AHPND disease: An investigation of the pVA1-type plasmid characteristics of pathogenic agents

Pazir M.Kh.[1*]; Ahmadi A.H.[2], Nazari M.A.[1]; Aein Jamshid Kh.[1], Sharifinia M.[1]; Jafari O.[3], Pourmozaffar, S[4].

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Abstract

Acute Hepatopancreatic Necrosis Disease (AHPND), caused by pathogenic strains of *Vibrio* parahaemolyticus and other *Vibrio* species carrying the pVA1-type plasmid, has emerged as a significant threat to global shrimp aquaculture since its first outbreak in 2009. Characterized by severe hepatopancreatic necrosis and high mortality rates, the disease has spread rapidly across Asia, the Americas, and other regions, resulting in catastrophic economic losses exceeding \$43 billion. The pVA1-type plasmid, which harbors the pirAB toxin genes, is central to the virulence of AHPND, enabling the production of binary toxins that disrupt shrimp cellular processes and lead to tissue damage. The plasmid's conjugative transfer capability facilitates its spread among *Vibrio* species and even to non-*Vibrio* bacteria, increasing the diversity of AHPND-causing pathogens. Environmental factors, such as poor water quality and high stocking densities, exacerbate disease outbreaks, while the shrimp's innate immune response often fails to combat the infection effectively. Despite advancements in understanding the pathogenesis of AHPND, including toxin mechanisms and plasmid dynamics, the disease remains a major challenge for the aquaculture industry. Effective control measures, including improved biosecurity, disease monitoring, and research into novel treatments, are urgently needed to mitigate its impact and ensure the sustainability of global shrimp production.

Keywords: AHPND, pVA1-type plasmid, *Vibrio parahaemolyticus*, pirAB toxins, shrimp aquaculture

1-Shrimp Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Bushehr, 75169-89177, Iran
2-Department of Biological Science and Technology, Faculty of Nano and Bio Science and Technology, Persian Gulf University, Bushehr, 75169, Iran
3-International Sturgeon Research Institute, Iranian Fisheries Science Research Institute, Agricultural Research (AREEO), Education and Extension Organization, Rasht, Iran
4-Persian Gulf Mollusks Research Station, Persian Gulf and Oman Sea Ecology Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research Education and Extension Organization (AREEO), Bandar-e- Lengeh, 75145, Iran
*Corresponding author's Email:dr.pazir@gmail.com

Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND) has significantly impacted the shrimp farming industry since its emergence. Primarily caused by pathogenic strains of the bacterium *Vibrio* parahaemolyticus, the disease has led to catastrophic economic consequences worldwide.

The first recorded AHPND outbreak occurred in southern China in 2009, marking the beginning of its global spread (Zorriehzahra and Banaederakhshan, 2015). By 2010, the disease was detected in Hainan Island, China, and by 2011, it had reached Vietnam and Malaysia (Kumar *et al*., 2021). By 2012, AHPND had spread to Thailand, severely impacting local shrimp production. The disease continued to spread, reaching Mexico by 2013, the Philippines in 2015, South America in 2016, and Bangladesh and the United States in 2017. By 2018, it had reached Taiwan Province of China, followed by South Korea in 2019 and Okinawa Prefecture, Japan, in 2020. These developments coincided with rising global shrimp demand, raising urgent concerns in the aquaculture industry (Ahmmed *et al*., 2019; Tang *et al*., 2020). According to reports from the Veterinary Organization of Iran, the disease was first detected in 2022 at a shrimp farm in Hormozgan Province, Iran (Figure 1).

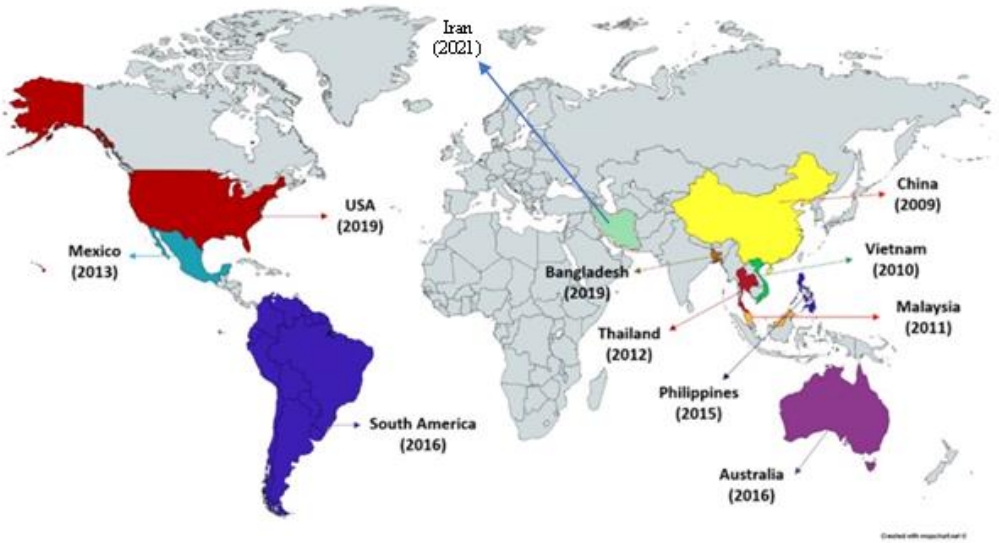

Figure 1: The global map showing the spread of AHPND.

Economic impact and control measures

AHPND has caused substantial economic losses, particularly in Asia, with initial estimates suggesting losses of around \$8 billion in Asia and \$4 billion in the Americas. More recent assessments indicate that global losses due to the disease have reached unprecedented levels, amounting to approximately \$43 billion. Shrimp production in affected regions dropped by about 60%, leading to increased unemployment due to the closure of farms and processing plants, which

reduced overall community revenue (Kumar *et al*., 2021). *Litopenaeus vannamei* is a key shrimp species in Iran, where culturing began in 1996. Production has been inconsistent due to disease outbreaks, and following the AHPND outbreak, annual shrimp production has steadily declined.

Host range and distribution

AHPND affects multiple species of shrimp, notably Penaeus monodon, *L. vannamei*, and Macrobrachium rosenbergii. The disease has been documented in various regions worldwide, including Asia and the Americas, leading to significant production losses and impacting the aquaculture industry considerably (Kumar *et al*., 2021).

Causative agents of AHPND

Vibrio species

Acute Hepatopancreatic Necrosis Disease (AHPND) is primarily caused by pathogenic strains of the bacterium *V. parahaemolyticus*, a Gram-negative, non-spore-forming bacterium known for its role in various aquatic diseases. In addition to *V. parahaemolyticus*, other *Vibrio* species, such as *V. harveyi, V. owensii, V. punensis, V. campbellii, V. alginolyticus,* and *Shewanella* sp., which contain the pVA1 plasmid, can also be responsible for outbreaks of AHPND. These species share similar virulence mechanisms and contribute to the overall threat posed by bacterial infections in shrimp (Han *et al*., 2015) (Figure 2).

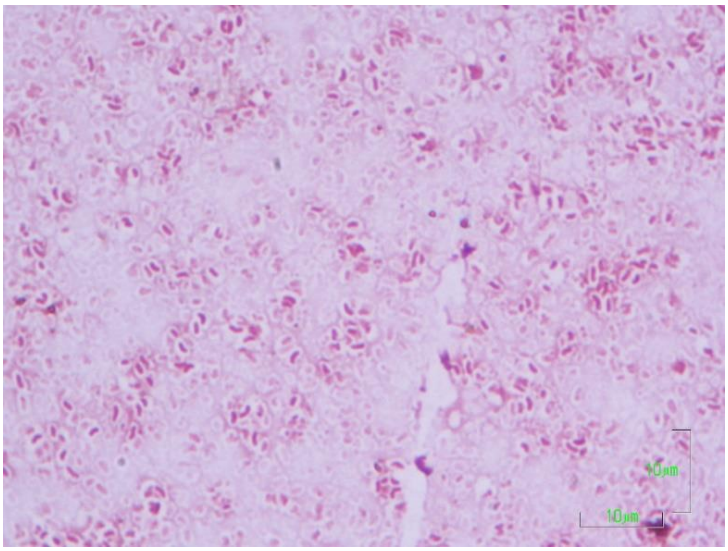

Figure 2:The pVA1-type plasmid of *V. parahaemolyticus* is isolated from *L. vannamei*.

Routes of AHPND entry

AHPND pathogens employ multiple entry routes to infect shrimp, leading to the onset of the disease. Understanding these routes is crucial for developing effective prevention and management strategies in shrimp aquaculture (Office International des Épizooties, 2003) (Figure 3).

- Horizontal transmission: The bacteria spread from infected shrimp to healthy ones through cannibalism.

- Vertical transmission: The pathogen can be passed from broodstock to offspring.
- Environmental transmission: Bacteria from infected farms can be disposed of in nature and spread to neighboring farms in the same area.

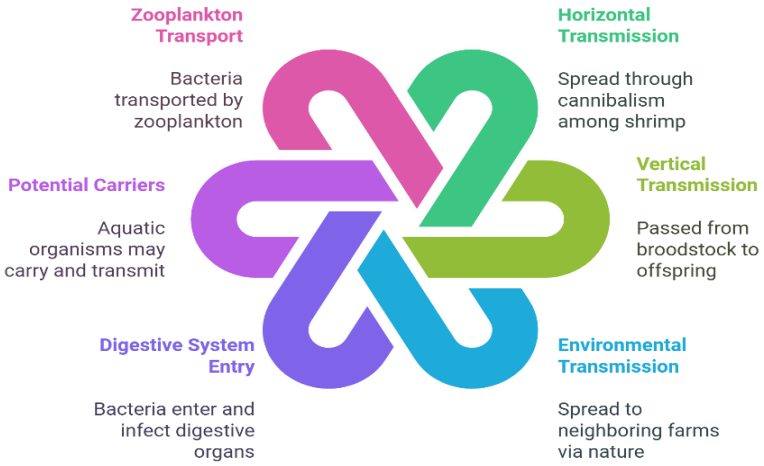

Figure 3:Routes for entering pVA1-type bacteria into shrimp rearing.

- Digestive system entry: Once inside the shrimp, the bacteria enter the digestive organs, first colonizing and infecting the stomach before moving to the hepatopancreas.
- Potential carriers: Aquatic organisms such as crabs, fish, plankton, and birds may potentially carry and transmit the disease, although this requires further confirmation.
- Zooplankton transport: The bacteria can be transported by zooplankton, potentially facilitating its spread.

Plasmid

Plasmids are extrachromosomal genetic elements—small, circular, double-stranded DNA molecules that can replicate independently of the bacterial chromosome. They exist separately from the bacterial chromosome and are found in many bacterial species, providing various benefits to their host cells. The number of plasmids per cell can vary greatly, from one to hundreds, depending on the plasmid type and bacterial species. Plasmids often carry genes that provide advantages to the host bacteria, such as antibiotic resistance or the ability to metabolize certain compounds. However, their presence is not essential for bacterial survival. All AHPND-causing *Vibrio* species harbor a highly homologous plasmid called the pVA1-type plasmid, which carries the pirAB toxin genes responsible for causing AHPND. Non-AHPND-causing strains lack this plasmid.

Plasmid types

Based on the research conducted, there are different plasmid types in *Vibrio* bacteria. While the exact number of different plasmid types in *Vibrio* bacteria is not provided, it is clear that

there are multiple types beyond just the pVA1-type. The presence and types of plasmids can vary depending on the specific *Vibrio* species and strain, as well as environmental factors and exposure to antibiotics.

- pAQU-type (Plasmid Aquatic-type) plasmids: These plasmids, found in *Vibrio* species, range from ~160 to 206 kb in size and harbor up to 111 core genes encoding conjugative, replication, and maintenance functions (Li *et al*., 2017).
- MDR (Multidrug-resistance) plasmids: The study identified pAQU-type plasmids as emerging multidrug-resistant (MDR) conjugative plasmids among important pathogens from different origins in Asia (Li *et al*., 2017) (Figure 4).

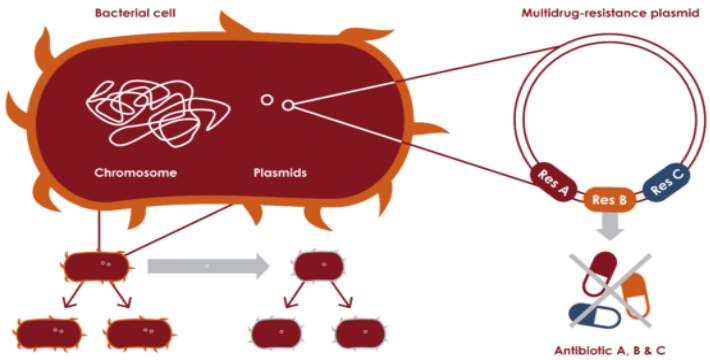

Figure 4:Multidrug-resistance (MDR) plasmids are a type of plasmid (a small, circular DNA molecule found in bacteria) that carry genes conferring resistance to multiple antibiotics or antimicrobial agents.

- Antibiotic resistance plasmids: Many *Vibrio* species harbor plasmids carrying antibiotic resistance genes. These plasmids can vary in size and gene content (Li *et al*., 2017).
- Other unspecified plasmids: Plasmid presence can vary significantly even within the same *Vibrio* species. Some strains may carry plasmids while others do not (Li *et al*., 2017).
- Non-pVH plasmids: The results mention 13 related plasmids (pVH-r family) without the pirAB genes isolated from a variety of species within the *Vibrio* harveyi clade (Xiao *et al*., 2017).

Plasmid integration

There are two types of plasmid integration into a host bacterium. Single crossover integration, also known as Campbell-like integration, occurs when the plasmid integrates into the bacterial chromosome through a single homologous recombination event. Double crossover integration involves two separate recombination events between the plasmid and the bacterial chromosome (Heap *et al*., 2012) (Figure 5).

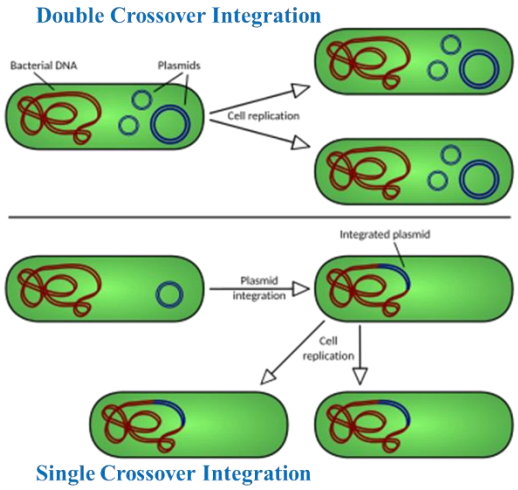

Figure 5: Plasmid integration into a host bacterium occurs via single or double crossover.

pVA1-type plasmid

The pVA1-type plasmid is highly homologous, which refers to the similarity in structure or sequence between genes, proteins, or other biological molecules, indicating that they share a common evolutionary origin. The pVA1-type plasmids are highly homologous across different AHPND-causing *Vibrio* species. The plasmid size can range from approximately 69 kb to 73 kb (Dong *et al*., 2019). This plasmid (pVA1) contains about 92 other open reading frames (ORFs) that include the pirAB toxin genes (Wang *et al*., 2020) (Figure 6).

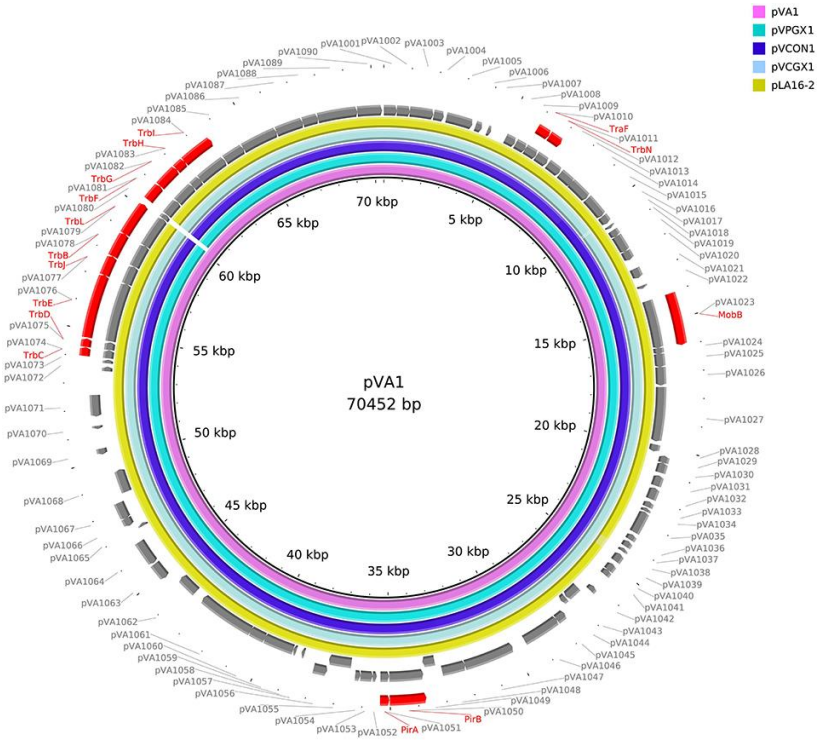

Figure 6: Structure of pVA1-type isolated from AHPND-infected shrimp

These ORFs are involved in various functions such as horizontal gene transfer, plasmid transmission and maintenance, toxin secretion, and protection against host defense mechanisms. The presence of these genes contributes to the spread and persistence of the pVA1 plasmid among different *Vibrio* species (Dong *et al*., 2019) (**Error! Reference source not found.**).

Table 1: The functional of various ORFs in the pVA1-type.

Toxin genes:	• pirAvp (ORF51) and pirBvp (ORF50) - The main virulence factors causing AHPND in shrimp
Transposases:	• ORF15, ORF48, ORF55, ORF57, and ORF68 - Involved in gene transposition and mobility
Conjugation factors:	• ORF10, ORF11, ORF75, ORF76, ORF78, ORF79, ORF81, ORF82, ORF83, and ORF84 - Involved in plasmid conjugative transfer
Antirestriction proteins:	• ORF32 and ORF35 - Likely involved in protecting the plasmid from host restriction systems
Secretion systems:	• ORF3, ORF86, ORF89, and ORF90 - Components of type II and III secretion systems that may facilitate toxin release
Plasmid maintenance:	• ORF7 (pndA) - Part of a post-segregational killing (PSK) system
DNA methyltransferases:	• ORF47 and ORF56 - Likely involved in DNA modification
Type IV Secretion System (T4SS):	• A cluster of 14 genes including traF, traG, and a trb cluster (trbB1, -B2, -C, -D, -E, -F, -G, -H, -I, -J, -L, and -N) - Mediates conjugative transfer of the plasmid

pVA1-type plasmid transfer

The pVA1-type plasmids can be transferred between bacterial cells through conjugation. This process allows the plasmid to move from one bacterial species to another without being fully integrated into the chromosome. This conjugative ability helps explain how the plasmid has spread to multiple *Vibrio* species, increasing the diversity of AHPND-causing bacteria (Dong *et al*., 2019).

The pVA1-type plasmid contains a post-segregational killing (PSK) system, ensuring that only bacteria retaining the plasmid survive after cell division. These conjugation factors collectively enable the pVA1 plasmid to efficiently transfer between bacterial cells, spreading the virulence genes (like pirAB) to new hosts and contributing to the increased diversity of AHPND-causing *Vibrio* species (Han *et al*., 2015) (Figure 7).

Different strains of the pVA1-type in the world

There are multiple strains from different geographical locations. This strain has been reported extensively in Asia, particularly in countries like Vietnam and Malaysia, as well as in the Americas, especially in Mexico since 2013 (Dong *et al*., 2019).

- Southeast Asia: The plasmid was first identified in *Vibrio* parahaemolyticus strains causing AHPND in this region.
- Mexico: There are references to "Mexican-like" or "Mexican-type"

plasmids, suggesting distinct strains from this country.

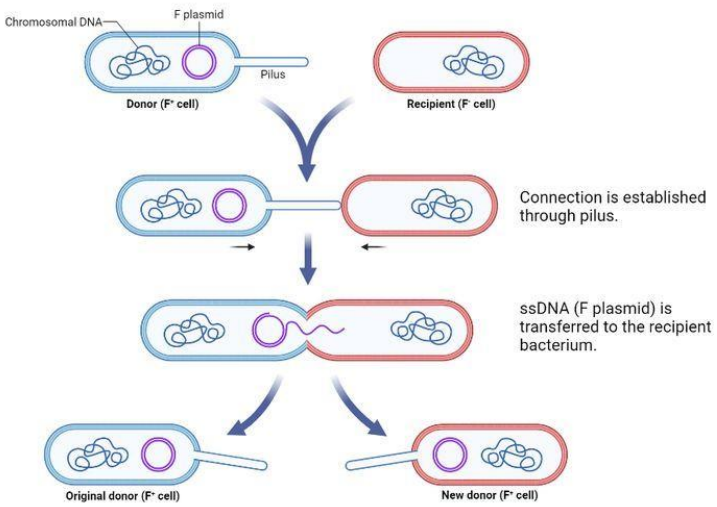

Figure 7: The dissemination of pVA1-type plasmids among Vibrio species via conjugation.

- South America: The results mention South American isolates with plasmids similar to the Mexican-like origin.
- Korea: There is mention of a Korean mutant *V. parahaemolyticus* strain carrying a pVA1-like plasmid.
- China: Some of the studies referenced were conducted on isolates from China.

These strains have exhibited significant virulence, leading to high mortality rates in affected shrimp populations and causing economic losses in the aquaculture industry (Castellanos *et al*., 2023).

Bacterial species containing pVA1-type

Acute Hepatopancreatic Necrosis Disease (AHPND) is primarily caused by specific strains of *Vibrio* species that harbor a plasmid containing the pirAB toxin genes. The key *Vibrio* species known to cause AHPND are: *V. parahaemolyticus, V. harveyi, V. owensii, V. campbellii, V. punensis*, and *V. alginolyticus*. These *Vibrio* species become pathogenic and capable of causing AHPND when they acquire the pVA1-type plasmid, which carries the pirAB toxin genes responsible for the disease's characteristic symptoms in shrimp (Wang *et al*., 2020).

Research has identified two non-*Vibrio* bacterial species, *Micrococcus luteus* and *Algoriphagus* sp., that may cause AHPND and possess associated genes. A study showed that the pirA and pirB genes could transfer from the AHPND-causing *V. parahaemolyticus* to the non-pathogenic *Algoriphagus* sp., enabling it to induce AHPND-like symptoms in shrimp after acquiring these genes (Durán-Avelar *et al*., 2018; Muthukrishnan *et al*., 2019).

Mechanism of pathogenesis

The pathogenesis of AHPND involves several critical steps, including adherence, colonization, and toxin production.

Adherence and colonization

Upon entering the shrimp's gastrointestinal system, *Vibrio* species adhere to the epithelial cells of the gut.

- Biofilm Formation: Bacteria typically form biofilms, enhancing their stability and increasing resistance to the host immune response, thereby facilitating colonization.
- Gastrointestinal Interaction: The bacteria may interact with the hepatopancreas, the essential organ for digestion and metabolism in shrimp, leading to subsequent pathological changes.

Toxin production

Once colonized, the bacteria commence the production of binary toxins, which are central to their pathogenic mechanisms.

- PirAB Toxins: The PirA and PirB proteins disrupt cellular processes, leading to apoptosis in hepatopancreatic cells. This disruption is primarily due to the activation of specific apoptotic pathways, which are crucial for cell signaling during host responses.
- Cytotoxic Effects: The toxins induce cytotoxic effects on the hepatopancreas, resulting in necrosis of the tissues vital for digestion and nutrient absorption. The degeneration of these tissues leads to impaired growth and increased mortality rates in infected shrimp.

Mechanism of toxin action

1. Toxin Binding: The PirAVP toxin binds to specific receptors on the epithelial cell membranes of the shrimp's hepatopancreas. This binding initiates a damaging cascade within the cell, leading to cellular dysfunction and death. The binding is facilitated by the toxin's lectin-like activity, which recognizes mucin-like O-glycosidic structures on the cell surface (Kumar *et al*., 2019; Soto-Rodriguez *et al*., 2022).
2. Pore Formation: The PirBVP component facilitates the formation of pores in the cell membrane. This pore-forming activity is crucial for the observed necrosis in affected cells, as it disrupts cellular integrity, leading to cell lysis and death. The interaction between PirAVP and PirBVP forms a heterotetrameric complex that activates the pore-forming domain, mimicking the action of insecticidal Cry toxins (Lee *et al*., 2015; Lin *et al*., 2019).

These mechanisms collectively contribute to the severe histopathological changes and high mortality rates associated with AHPND in shrimp.

Immune response

The innate immune response of shrimp plays a role in combating AHPND; however, the efficiency is often compromised.

- Immune Evasion: The rapidity of the infection and the highly virulent nature of the strains significantly circumvent the shrimp's innate immune defenses, such as phagocytosis and the production of antimicrobial peptides.
- Systemic Effects: The widespread tissue damage can provoke systemic

inflammatory responses, further exacerbating the pathological outcomes.

Clinical manifestations

Infected shrimp display characteristic clinical signs indicative of AHPND.

- Clinical Symptoms: Signs include lethargy, reduced feed intake, and distinctive visual symptoms such as white, opaque hepatopancreas and swelling (Figure 8).

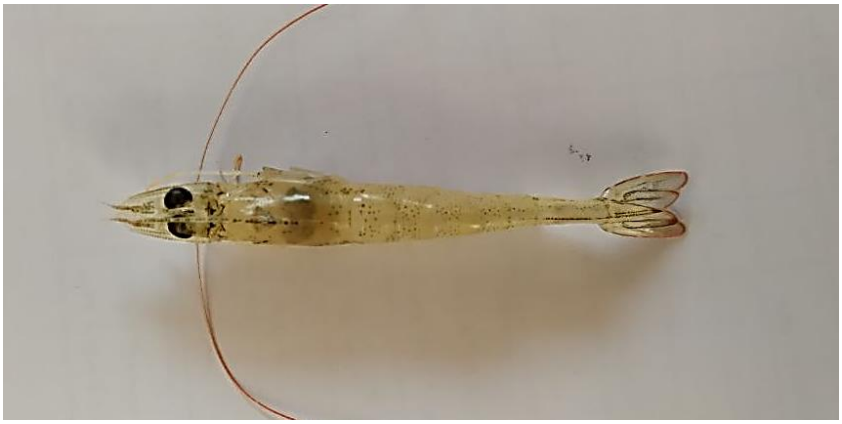

Figure 8: AHPND-infected *L. vannamei* shrimp.

Clinical pathology of hepatopancreas

The hepatopancreas serves essential functions in nutrient absorption, digestion, and enzyme production in crustaceans. Pathological changes due to AHPND impact the overall health and viability of shrimp populations, leading to high mortality rates (Figure 9).

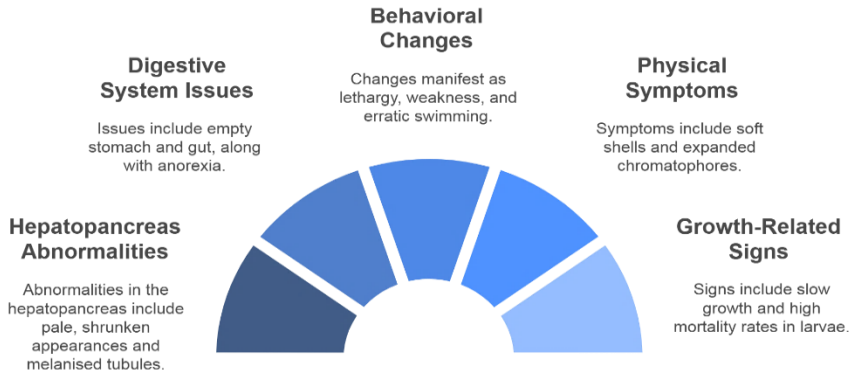

Figure 9: Clinical signs of AHPND disease in the shrimp.

Gross pathology

The hepatopancreas exhibits distinct gross pathological changes during AHPND.

- Color Changes: The infected hepatopancreas often appears pale or white, losing its usual reddish-brown coloration. This change results from necrotic processes affecting the tissue structure.
- Swelling and Fluid Accumulation: Affected shrimp may display swelling of the hepatopancreas, often accompanied by an accumulation of clear to yellow fluid. This is indicative of tissue damage and

inflammation, leading to an enlargement of the organ.
- Transparency: Observations may reveal an increased transparency of the hepatopancreatic tissue, hinting at the loss of cellular integrity due to necrosis.

Histopathological changes

Histopathological examinations reveal specific cellular alterations indicative of AHPND.
- Cellular Death: Extensive necrosis of the hepatopancreatic tissues is a hallmark of AHPND. Histological studies indicate severe degeneration of epithelial cells lining the tubules of the hepatopancreas. The presence of apoptotic cells is significant; the binary toxins (PirA and PirB) produced by *Vibrio* species directly induce apoptosis in hepatopancreatic cells.
- Inflammation: There is an observable infiltration of hemocytes (the immune cells in shrimp) around the tissue areas undergoing necrosis. This infiltration represents an immune response attempting to combat the infection. Accumulations of granulocytes suggest active immune engagement but are often insufficient in controlling the progression of the disease due to the virulence of the pathogenic strains.

Degenerative changes

The tissue damage can lead to several degenerative changes seen histologically.
- Loss of Cellular Architecture: Histological evaluations often reveal a disruption of the normal architecture of the hepatopancreas, with profound vacuolization of hepatopancreatic cells attributable to the necrotic process. This vacuolation can severely impair the organ's functionality.
- Atrophy: Atrophic changes in the hepatopancreatic cells, particularly in the digestive tubules, occur as the cells undergo necrosis and fail to regenerate adequately, affecting the overall digestive efficiency (Figure 10).

Effects on functionality

The pathological conditions of the hepatopancreas critically affect the shrimp's overall biological functions.
- Digestive Impairment: The degeneration of the hepatopancreas severely compromises its ability to synthesize digestive enzymes, leading to poor digestion and nutrient absorption among infected shrimp.
- Growth Reduction: Due to impaired digestive functions, infected shrimp exhibit reduced growth rates, impacting the aquaculture yield and profitability significantly.

Mortality

The severe clinical pathology observed in AHPND can swiftly lead to increased mortality in affected shrimp populations.
- Mortality Rate: The disease can lead to rapid mortality, with some reported losses exceeding 80% within a short span, usually occurring within a few days of infection.

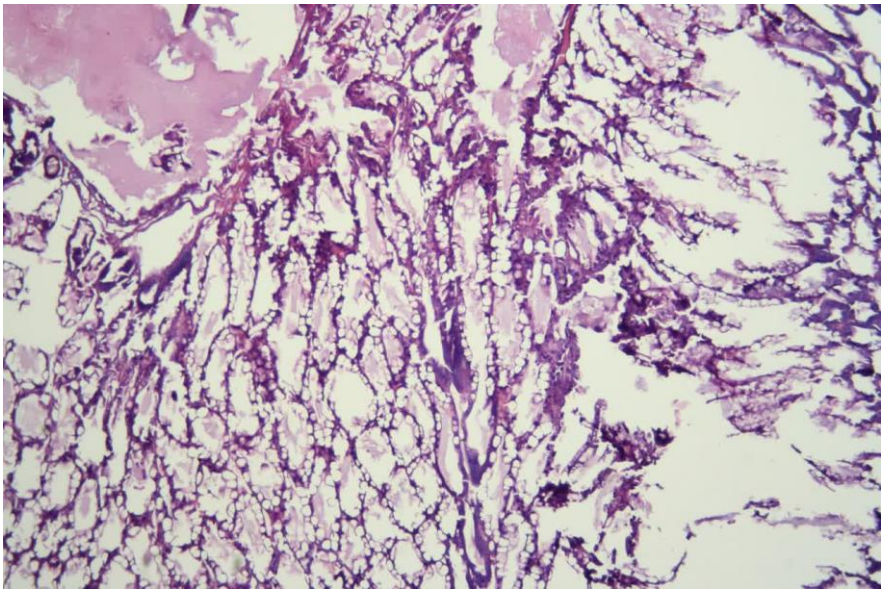

Figure 10: H & E-stained section of the hepatopancreas of *L. vannamei* revealing sludgy lesions in the epithelial lumen associated with acute hepatopancreatic necrosis disease (AHPND).

Environmental factors

Environmental conditions play a crucial role in the pathogenesis of AHPND.

- Stressors: Factors such as high stocking densities, fluctuating water quality parameters, increased temperatures, and the presence of external stressors can exacerbate the progression of AHPND.
- Outbreak Risks: The interaction of these stressors with pathogenic strains leads to conditions conducive to the outbreak of AHPND within shrimp farming operations.

Conclusion

Acute Hepatopancreatic Necrosis Disease (AHPND), caused by pathogenic *Vibrio* species carrying the pVA1-type plasmid, has had a devastating global impact on shrimp farming since its emergence in 2009. The disease, characterized by severe hepatopancreatic necrosis and high mortality rates, has spread across Asia, the Americas, and other regions, causing significant economic losses exceeding \$43 billion. The pVA1-type plasmid, which harbors the pirAB toxin genes, plays a central role in the virulence and spread of AHPND, facilitated by its conjugative transfer among *Vibrio* species and other bacteria. Despite efforts to understand its pathogenesis, including toxin mechanisms and immune evasion strategies, AHPND remains a major challenge for the aquaculture industry. Effective control measures, including improved biosecurity, disease monitoring, and research into novel treatments, are essential to mitigate its impact and safeguard global shrimp production.

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