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Challenges and strategies for detecting the AHPND pathogen in shrimp hatcheries

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Abstract

Acute Hepatopancreatic Necrosis Disease (AHPND) is a severe bacterial infection that threatens shrimp aquaculture, causing significant economic losses globally. The disease is attributed to specific strains of *Vibrio* spp. carrying the *pVA1* plasmid, which encodes *PirAB* toxins responsible for hepatopancreatic necrosis in shrimp. Early detection of AHPND is crucial for effective disease management, yet several challenges hinder accurate and timely diagnosis. This article examines key diagnostic challenges, including genetic variability in AHPND-causing bacteria, inconsistencies in toxin production, environmental influences, and limitations of current molecular detection methods. Additionally, alternative diagnostic approaches such as LAMP, CRISPR/Cas12a assays, and immunoassays are discussed for their potential to improve detection accuracy. Future research should focus on developing more robust and rapid diagnostic tools to overcome the limitations of current methods. Enhanced detection strategies, combined with biosecurity and preventive measures, will be essential in mitigating the impact of AHPND on global shrimp farming.

Keywords: AHPND, *Vibrio*, PirAB, Diagnosis, Aquaculture.

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Introduction

AHPND, or Acute Hepatopancreatic Necrosis Disease, is a bacterial disease affecting farmed penaeid shrimp, leading to substantial economic losses in the aquaculture industry (WOAH, 2023). The disease is caused by specific strains of *Vibrio* spp. bacteria that contain a plasmid encoding toxins that damage the shrimp's hepatopancreas (Figure 1). Since its emergence, AHPND has spread across multiple countries, causing significant drops in shrimp production and substantial financial losses (Kumar *et al*., 2021).

Figure 1: Clinical signs of *Litopenaeus vannamei* infected with Acute Hepatopancreatic Necrosis Disease (AHPND). Infected shrimp exhibit pale and atrophied hepatopancreas, empty digestive tract, lethargy, soft shell, and abnormal swimming behavior. Severe cases may result in mass mortality within the first 30–35 days of culture.

This disease was first reported in China around 2009, initially known as covert mortality disease (Estrada-Perez *et al*., 2020). The disease emerged as a significant threat to the shrimp farming industry, causing global losses. By 2010, it had spread to Vietnam, followed by Malaysia in 2011, Thailand in 2012, Mexico in 2013, the Philippines in 2015, and South America in 2016 (UNDRR, 2023). The disease's rapid spread highlights its contagious nature and the challenges in containing it. AHPND is caused by specific strains of bacteria, including *V. parahaemolyticus*, *V. punensis*, *V. harveyi*, *V. owensii*, *V. campbelli*, and *Shewanella* sp., which carry the pVA1 plasmid (Hong *et al*., 2016) (Figure 2). This plasmid contains genes that encode PirAVP and PirBVP toxins, which are homologous to insect-related toxins and are the primary virulence factors responsible for the disease (Shinn *et al*., 2018). These toxins damage the hepatopancreatic cells of shrimp, leading to the characteristic necrosis observed in AHPND-affected individuals. The presence of these toxins is crucial for the development of AHPND, distinguishing it from other vibriosis diseases (Aranguren Caro *et al*., 2020).

AHPND has had a profound economic impact on the shrimp aquaculture industry worldwide.

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Figure 2: Different *Vibrio* species isolated on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar. The colonies display varying morphologies and colors, with yellow colonies indicating sucrose-fermenting *Vibrio* spp. (e.g., *Vibrio alginolyticus* and *Vibrio harveyi*), while green colonies represent non-sucrose-fermenting species (e.g., *Vibrio parahaemolyticus* and *Vibrio vulnificus*). These bacterial species are commonly associated with shrimp diseases, including Acute Hepatopancreatic Necrosis Disease (AHPND).

The shrimp production in AHPND-affected regions has dropped to approximately 60%, leading to significant financial losses. Collective losses have exceeded an estimated USD 43 billion across Asia (China, Malaysia, Thailand, Vietnam) and in Mexico. In Vietnam alone, AHPND has caused a $2.56 billion loss since its emergence in 2011. From 2010 to 2016, combined losses from China, Malaysia, Mexico and Vietnam were significant. AHPND progresses through three phases: initial, acute, and terminal. In the initial phase, shrimp show signs of damage in the hepatopancreas and a partial or total absence of food in the gut. The hepatopancreatic tubular epithelial cells become modified, elongated, and cellular desquamation occurs. During the acute phase, shrimp exhibit anorexia, lethargy, and an empty digestive tract, with the hepatopancreas becoming atrophied and whitish. Microscopically, massive shedding of hepatopancreatic tubular epithelial cells is observed, along with cell necrosis and accumulation of haemocytes in the lumen. The terminal phase is characterized by expanded chromatophores, atrophied and whitish hepatopancreas, and fibrous appearance when the hepatopancreas is squashed (Kumar *et al*., 2021).

The incidence of AHPND has varied across different regions and years. In infected farms, the prevalence of AHPND at the pond level was reported to be 45.64%, with 49.27% of the area infected (Tang and Bondad-Reantaso, 2019). In Malaysia, annual prevalence rates of AHPND were reported as 50% in 2011, 26% in 2012, and 73% in 2013, with the disease persisting but at lower rates in subsequent years. AHPND can lead to mortality rates of up to 100% in infected shrimp within 30–35 days of stocking, posing a significant threat to shrimp farms (Yen *et al*., 2021).

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Acute Hepatopancreatic Necrosis Disease (AHPND) poses a significant threat to shrimp aquaculture, particularly in hatchery centers and farms, where early detection is crucial. While several detection methods exist, the unique characteristics of AHPND-causing pathogens present considerable diagnostic challenges (Nunan *et al*., 2014a). This article addresses challenges such as the genetic variability of AHPND-causing bacteria, inconsistencies in toxin production, environmental influences, factors affecting molecular diagnosis, and the introduction of rapid and reliable detection methods along with their associated problems.

Genetic variability of AHPND-causing bacteria

The genetic diversity of AHPND-causing bacteria complicates accurate and consistent detection (Kumar *et al*., 2021). *Vibrio parahaemolyticus* (VpAHPND) strains exhibit significant genetic variations, particularly in the pVA1 plasmid, which harbors the PirAVP and PirBVP toxin genes. This plasmid can mobilize to other Vibrio strains and even non-Vibrio species, leading to the conversion of non-pathogenic strains into pathogenic ones. The 70-kb AHPND plasmid in V. parahaemolyticus strains is genetically diverse, suggesting that the virulent plasmid has been acquired from several genotypes through lateral gene transfer (Wang *et al*., 2022), therefore, this genetic variability might lead to mismatches with the primers used in PCR assays, resulting in non-detection (Li *et al*., 2017) (Figure 3).

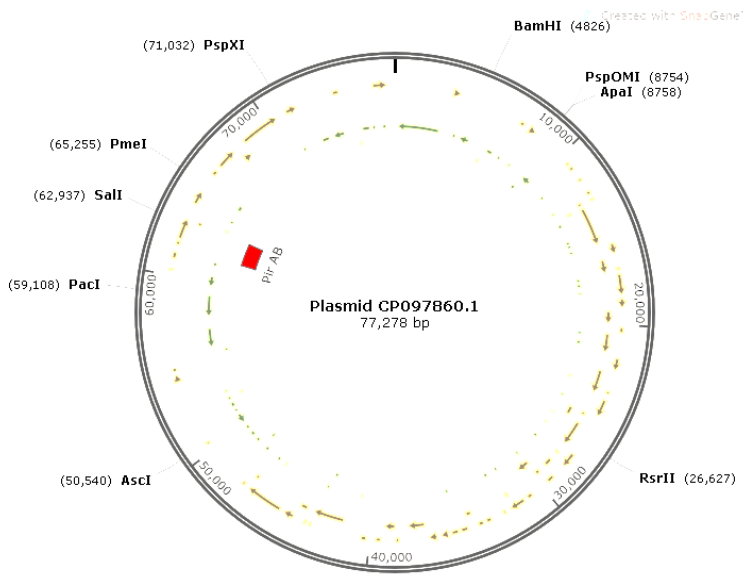

Figure 3: Circular map of the AHPND-associated plasmid *pVA1* (Plasmid CP097860.1, 77,278 bp). The plasmid contains multiple coding sequences, including virulence-related genes. The *PirAB* toxin genes, essential for Acute Hepatopancreatic Necrosis Disease (AHPND) pathogenesis, are highlighted in red. Various restriction enzyme sites and primer binding regions are indicated. The plasmid plays a crucial role in the virulence of *Vibrio parahaemolyticus* strains responsible for AHPND outbreaks in shrimp.

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Variability in pathogen load

Studies have shown that virulence plasmid copy numbers can vary widely in infected shrimp, ranging from 4 to 105-106 copies per mg of tissue. This variability can make it challenging to establish reliable detection thresholds (Han *et al*., 2015).

Inconsistent toxin production

Not all V. parahaemolyticus strains harboring the pVA1 plasmid and PirABVP genes produce the AHPND-causing PirABVP toxins. Some strains that test positive for these genes via PCR fail to exhibit characteristic AHPND histopathological lesions and mortality in shrimp (Li *et al*., 2017). The virulence of AHPND-causing V. parahaemolyticus depends on the production of secreted proteins, specifically the PirABVP toxins, rather than solely on the copy number of the PirAVP/PirBVP genes. This inconsistency makes relying solely on gene detection methods unreliable for diagnosing AHPND (Castellanos *et al*., 2023). Additionally, other virulence factors, such as the Type VI secretion system, may also play significant roles in the pathogenicity of these strains, further complicating the diagnosis and understanding of AHPND (Li *et al*., 2017).

Environmental factors and bacterial load

The detection of AHPND pathogens can be influenced by environmental factors and the overall bacterial load in hatchery environments (Wang *et al*., 2022). V. parahaemolyticus is part of the autochthonous microflora of estuarine and coastal environments, and its levels can vary depending on environmental and geographical factors. The bacteria thrive in high sodium chloride concentrations (0.5 to 10%) and moderate temperatures (5 to 37°C). High bacterial loads and the presence of other Vibrio species can interfere with the accurate detection of AHPND-causing strains (Wang *et al*., 2020; Cavanagh *et al*., 2024).

Environmental factors significantly influence the detection of AHPND (Acute Hepatopancreatic Necrosis Disease) pathogens in shrimp farming environments. These factors affect not only the presence and proliferation of the causative bacteria but also the efficacy and reliability of diagnostic methods. Key environmental parameters such as temperature, salinity, pH, and water quality play a crucial role in AHPND outbreaks and pathogen detection (Boyd and Phu, 2018).

Temperature

Temperature is a critical factor affecting the growth and virulence of Vibrio parahaemolyticus, the primary causative agent of AHPND. Optimal temperatures for V. parahaemolyticus proliferation generally range from 25°C to 37°C. Higher temperatures can promote bacterial growth, increasing the likelihood of AHPND outbreaks and facilitating easier detection due to higher pathogen loads (Zhang *et al*., 2021; Cavanagh *et al*., 2024). Conversely, lower temperatures may reduce bacterial

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activity and abundance, making detection more challenging. (López-León *et al*., 2016)

Salinity

Salinity levels also significantly impact the occurrence and detection of AHPND pathogens. V. parahaemolyticus thrives in brackish and marine waters with moderate to high salinity. Studies indicate that salinity ranges between 27.87±3 ppt are optimal for the prevalence of Vibrio parahaemolyticus (Peña-Navarro *et al*., 2025). Lower salinity levels (below 20 ppt) have been observed to reduce the incidence of AHPND. Therefore, salinity influences the distribution and concentration of the pathogen, affecting the sensitivity of detection methods (Yuhana and Afiff, 2023) .

pH

The pH of the water influences the physiological state and activity of V. parahaemolyticus. The optimum pH range for V. parahaemolyticus is approximately 7.96±0.1 (López-León *et al*., 2016). Deviations from this range can affect bacterial growth and toxin production, which in turn impacts the ability to detect the pathogen effectively. Extreme pH levels can inhibit bacterial proliferation, leading to lower pathogen concentrations and potentially false-negative results in diagnostic tests (Li *et al*., 2017).

Water quality

Overall water quality, including parameters such as dissolved oxygen, nutrient levels, and organic matter content, affects the microbial community and the proliferation of Vibrio species. Poor water quality, characterized by low dissolved oxygen and high organic matter, can promote the growth of pathogenic bacteria, including V. parahaemolyticus, increasing the risk of AHPND outbreaks (Nguyen *et al*., 2021). High levels of suspended solids and organic matter can also interfere with certain detection methods, such as PCR, by inhibiting enzymatic reactions or reducing DNA extraction efficiency (Kumar *et al*., 2022).

Biofilm formation

V. parahaemolyticus can form biofilms on surfaces in aquaculture systems, which can serve as reservoirs for the pathogen. Biofilms protect bacteria from disinfectants and other control measures, making it difficult to eradicate the pathogen completely (Mizan *et al*., 2016). The presence of biofilms can lead to false-negative results if water samples are tested without properly dislodging and dispersing the biofilm (Nguyen *et al*., 2024). Therefore, effective detection strategies must account for the potential presence of V. parahaemolyticus within biofilms.

Influence of other microorganisms

The presence of other microorganisms in the water can also affect the detection of AHPND pathogens. Some bacteria may inhibit the growth of V. parahaemolyticus through competition or the production of inhibitory substances, reducing its abundance and

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detectability. Conversely, other bacteria may promote the growth of V. parahaemolyticus or enhance its virulence, increasing the risk of AHPND outbreaks and potentially improving detection rates. The complex interactions within the microbial community can thus influence the accuracy and reliability of AHPND detection methods (Avery *et al.,* 2020; Bacian *et al*., 2021).

Cross-reactivity

PCR assays for other pathogens can sometimes cross-react with non-target organisms. This could potentially lead to false positives if primers are not highly specific to the AHPND-causing Vibrio strains (Roux *et al*., 2019).

Competition for PCR reagents

In samples with multiple pathogens, there could be competition for PCR reagents, potentially reducing the efficiency of amplification for the AHPND-specific targets (Xu *et al*., 2022).

Masking effects

High levels of other pathogens or microorganisms in a sample could potentially mask the presence of low levels of AHPND-causing Vibrio, especially if using methods that rely on general bacterial detection before specific testing (Cruz-Flores *et al*., 2019).

Implications for diagnostic testing

Given the significant influence of environmental factors on AHPND pathogen detection, it is crucial to consider these factors when designing and implementing diagnostic testing programs. Regular monitoring of temperature, salinity, pH, and other water quality parameters can help predict periods of increased AHPND risk (Karunasagar and Karunasagar, 2018). Sample collection strategies should account for potential variations in pathogen distribution due to environmental gradients and biofilm formation (Weller *et al*., 2020). Additionally, quality control measures should be implemented to minimize the impact of interfering substances on diagnostic test results. Integrating environmental monitoring with advanced molecular and immunological detection methods can improve the accuracy and effectiveness of AHPND surveillance and control efforts (Li *et al*., 2017).

Need for rapid and sensitive detection methods

Early detection of AHPND in hatchery centers is essential to prevent widespread outbreaks and minimize economic losses (Arunrut *et al*., 2016). Traditional methods, such as histological examination, are time-consuming and require specialized expertise. While PCR-based methods offer faster detection, their reliability is compromised by genetic variability and inconsistent toxin production. There is a pressing need for rapid, sensitive, and specific detection methods that can accurately identify AHPND-causing pathogens, even in the presence of low

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bacterial loads and genetic variations (Wang *et al*., 2023; Dong *et al*., 2024). Notably, the failure to detect the agent of AHPND in shrimp post-larvae at hatchery centers using real-time PCR and PCR methods can be attributed to several factors:

Low bacterial load

The bacterial load of Vibrio parahaemolyticus might be below the detection limit of these methods in early stages, making it difficult to identify the pathogen (Tinwongger *et al*., 2014). Therefore, for environmental or low-level samples, an enrichment step may be necessary before PCR to increase bacterial numbers to detectable levels (Hong *et al*., 2016).

Uneven distribution

The pathogen may not be uniformly distributed throughout all post-larvae in a batch, leading to false negatives if only a small sample is tested (Cruz-Flores *et al*., 2019).

Strain variations

Some AHPND-causing strains may have genetic variations that are not detected by existing PCR primers and probes (Sirikharin *et al*., 2015).

Sample Quality

Poor sample collection (Improper sampling techniques), testing too few individuals or handling can degrade DNA, affecting the sensitivity and accuracy of PCR detection and may result in missing infected post-larvae (De Schryver *et al*., 2014; Lee *et al*., 2015; Yu *et al*., 2020).

Sample type considerations

While hepatopancreas tissue is typically sampled, other sample types like water may also be used but could have different detection sensitivities (Han *et al*., 2015).

PCR inhibitors

Substances in the shrimp tissue or environment may inhibit the PCR reaction, leading to false negative results (Sirikharin *et al*., 2015).

Potential for false negatives

Surviving shrimp or those in early infection stages may carry low quantities of pathogenic bacteria that are difficult to detect, but could still act as disease vectors (Han *et al*., 2015).

Need for specific primers/probes

PCR methods require carefully designed primers and probes to detect the specific AHPND toxin genes. Genetic variations in some strains could potentially lead to false negatives if not accounted for in assay design (Cruz-Flores *et al*., 2019).

Biosecurity and prevention strategies

In addition to improving detection methods, implementing robust biosecurity practices in hatchery facilities is crucial for preventing AHPND. Maintaining good sanitary conditions, screening broodstock, nauplii, and post-larvae, and avoiding rapid changes in water conditions can help minimize the risk of AHPND outbreak (Zhang *et al*., 2023).

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Prophylaxis measures, such as pond management and disinfection before stocking shrimp post-larvae, are also essential. Focusing on producing clean, healthy, stress-free, and genetically improved animals can enhance their resistance to AHPND (Zorriehzahra and Banaederakhshan, 2015).

AHPND detection and associated issues

Detection of Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp, particularly in post-larvae (PL12), involves several advanced molecular techniques (Nunan *et al*., 2014)

PCR methods

While PCR is widely used, it requires expensive and sophisticated equipment, which may not be readily available in farm settings. Additionally, some PCR methods may produce false positives, as seen with design some primers (Tinwongger *et al*., 2014).

LAMP with gold nanoparticles

Although this method is simpler and cost-effective, it still requires careful handling and specific conditions to avoid false results. The sensitivity, while high, may not be appropriateness for all field conditions (Arunrut *et al*., 2016).

CRISPR/Cas12a assays

These assays are rapid and specific but may still require some level of technical expertise and equipment for fluorescence detection, which can be a barrier in less-equipped settings (Li *et al*., 2022).

Immunoassays

These methods, such as ELISA, can detect toxins at the protein level but may not detect all strains, especially if the strains do not produce the toxins as proteins. They also require specific antibodies, which can be costly to produce (Jeon *et al*., 2023).

Overall, while these methods are effective, their application can be limited by cost, equipment availability, and the need for technical expertise.

Alternative detection approaches

Acute hepatopancreatic necrosis disease (AHPND) is one of the most devastating diseases in aquaculture, causing significant economic losses in seafood supplies worldwide. To overcome the limitations of current detection methods, alternative approaches are being explored. Loop-mediated isothermal amplification (LAMP) combined with visual detection offers a simpler yet equally sensitive approach for detecting VP AHPND (Arunrut *et al*., 2016). Novel sandwich immunoassays are being developed to detect AHPND-causing V. parahaemolyticus strains, addressing the issue of strains that do not produce toxins as proteins (Jeon *et al*., 2023). Next-generation sequencing (NGS) technologies are also contributing to genomic studies of AHPND, providing insights into the genetic diversity and virulence mechanisms of the pathogen (Fu *et al*., 2020).

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Future directions

Future research should focus on developing more reliable and comprehensive diagnostic tools that account for the genetic variability and toxin production inconsistencies of AHPND-causing bacteria (Wang *et al*., 2023). These tools should be rapid, sensitive, and capable of detecting the pathogen in complex hatchery environments. Further investigation into the virulence mechanisms of AHPND and the factors influencing toxin production is also needed. Combining improved detection methods with stringent biosecurity and prevention strategies will be essential for mitigating the impact of AHPND on shrimp aquaculture (Li *et al*., 2017).

Conclusion

AHPND remains a significant challenge in shrimp aquaculture, primarily due to the genetic diversity of its causative bacteria and the limitations of existing diagnostic methods. The variability in *pVA1* plasmid structure, toxin production inconsistencies, and environmental factors contribute to diagnostic difficulties, leading to false negatives and unreliable results. Current molecular techniques, such as PCR, face limitations due to genetic mutations and inhibitor interference. Alternative methods, including LAMP, CRISPR/Cas12a assays, and immunoassays, offer promising solutions but still require further validation for field application. Future advancements in diagnostic technology should aim to improve sensitivity, specificity, and adaptability to diverse aquaculture environments. Additionally, implementing stringent biosecurity measures, regular pathogen monitoring, and preventive management strategies will be crucial in reducing the spread and economic burden of AHPND. Addressing these challenges through integrated detection and prevention strategies will be essential for sustainable shrimp farming.

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