Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(6): 867 – 876 (2024)
www.ejabf.journals.ekb.eg

Histopathological Analysis of Acute Hepatopancreatic Necrosis Disease (AHPND) Impact on the Hepatopancreas of *Litopenaeus vannamei,* using Scanning Electron Microscopy

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ARTICLE INFO

Article History:
Received: Nov. 6, 2024
Accepted: Nov. 22, 2024
Online: Nov. 27, 2024

Keywords:
Acute hepatopancreatic necrosis disease (AHPND), Histopathology, *Litopenaeus vannamei*, Scanning electron microscopy (SEM), *Vibrio parahaemolyticus*

ABSTRACT

This study examined the histopathological impact of acute hepatopancreatic necrosis disease (AHPND) on the hepatopancreas of *Litopenaeus vannamei* using scanning electron microscopy (SEM). AHPND, caused by *Vibrio parahaemolyticus*, is known for its rapid and lethal effects on shrimp hepatopancreatic tissue, leading to substantial losses in the aquaculture industry. Through SEM, significant morphological changes were observed in infected hepatopancreatic samples, including necrosis, vacuolization, and mineralization. Necrosis was marked by cellular disintegration, vacuolization by hollow epithelial cells, and mineralization by dense white granules. Sample A5 displayed an intact hepatopancreatic structure, contrasting sharply with A1 and A6, which exhibited extensive tissue damage. These findings highlight the profound structural impact of AHPND on shrimp health, underscoring the need for targeted disease management practices in aquaculture.

INTRODUCTION

The shrimp species *Litopenaeus vannamei*, commonly referred to as the Pacific white shrimp, is recognized as one of the most economically significant species in global aquaculture. This species has gained prominence due to its rapid growth rate, high yield, and nutritional value, making it a preferred choice among aquaculturists worldwide **(Liu *et al.*, 2022; Zhang *et al.*, 2022; Andriani & Pratama, 2023)**. In 2018, the production of the Pacific white shrimp reached approximately 4.97 million tons, accounting for over half of the total output of crustacean aquaculture **(Liu *et al.*, 2022)**. However, the shrimp

farming industry faces substantial challenges, particularly from infectious diseases that can lead to significant production losses **(Amiin *et al.*, 2023)**.

Among the various diseases impacting shrimp populations, acute hepatopancreatic necrosis disease (AHPND) stands out as one of the most devastating. Initially identified in Asia, AHPND is caused by specific strains of *Vibrio parahaemolyticus* that produce the PirA and PirB toxins **(Dong *et al.*, 2017)**. These toxins lead to rapid deterioration of the hepatopancreas, resulting in high mortality rates within shrimp populations **(Restrepo *et al.*, 2018)**. The disease has been linked to outbreaks in several countries, including China, Vietnam, and Mexico, causing severe economic repercussions for shrimp producers **(Zeng *et al.*, 2013; Restrepo *et al.*, 2018)**. The pathogenicity of *Vibrio* species, particularly those harboring the pirAB genes, has been well-documented, indicating their role in the AHPND outbreaks **(Dong *et al.*, 2017; Durán-Avelar *et al.*, 2018)**.

Histopathological analysis is crucial for understanding the impact of acute hepatopancreatic necrosis disease (AHPND) on the cellular and tissue architecture of the hepatopancreas in *Litopenaeus vannamei*. The hepatopancreas is a vital organ responsible for digestion, nutrient absorption, and storage in shrimp, making it a key focus in studies of AHPND pathology **(Soto-Rodríguez *et al.*, 2015; Velázquez-Lizárraga *et al.*, 2019)**. Traditional microscopy techniques, while useful, often lack the resolution necessary to capture the intricate structural changes that occur in hepatopancreatic tissue during AHPND infection. In contrast, scanning electron microscopy (SEM) provides a higher-resolution imaging alternative that allows for detailed characterization of histological changes at the microstructural level **(Zeng *et al.*, 2013; Hong *et al.*, 2016)**.

SEM's three-dimensional imaging capabilities enable researchers to visualize cellular and tissue surface alterations, which are essential for diagnosing the extent and progression of AHPND pathology **(Kumar *et al.*, 2019; Yu *et al.*, 2022)**. For instance, studies have shown that AHPND leads to significant histopathological changes in the hepatopancreas, including necrosis and cellular degeneration, which can be effectively observed using SEM **(Soto-Rodríguez *et al.*, 2015; Velázquez-Lizárraga *et al.*, 2019)**. By employing SEM, we can gain insights into the microstructural damage inflicted by *Vibrio parahaemolyticus*, the causative agent of AHPND, and its associated toxins, PirA and PirB **(Dong *et al.*, 2017; Kumar *et al.*, 2020)**. This understanding is critical for developing targeted disease management strategies and improving diagnostic accuracy, ultimately contributing to the sustainability of shrimp aquaculture **(Kumar *et al.*, 2020; Zermeño-Cervantes *et al.*, 2023)**.

In our study, we utilized SEM to examine the microstructural changes in the hepatopancreas of *L. vannamei* infected with AHPND. By characterizing these changes, we aimed to enhance the understanding of the disease's pathogenesis and its impact on hepatopancreatic tissue integrity. Insights gained from this research could inform future strategies for disease prevention and management in shrimp farming, helping to mitigate

the economic losses associated with AHPND outbreaks **(Soto-Rodríguez *et al.*, 2015; Fu *et al.*, 2017)**.

MATERIALS AND METHODS

Shrimp samples

Healthy juvenile *Litopenaeus vannamei* (average weight 5-10g) were sourced from a local aquaculture farm and acclimated under laboratory conditions for one week before experimentation. Shrimp were maintained in aerated tanks with controlled water temperature (28 ± 1°C), salinity (30ppt), and dissolved oxygen (6mg/ L) to ensure minimal stress and environmental stability prior to pathogen exposure.

AHPND challenge

Shrimp were divided into two groups: (1) an experimental group challenged with *Vibrio parahaemolyticus* strain known to induce AHPND and (2) a control group that received a sterile saline injection. The experimental group was injected with 10^7 CFU of *V. parahaemolyticus* per shrimp, targeting the hepatopancreas. Mortality and clinical signs of AHPND were daily monitored. Samples were collected from moribund and surviving shrimp over a 10-day observation period.

Hepatopancreas tissue collection

At specific time points post-injection (days 1, 3, and 7), shrimp from both groups were euthanized, and the hepatopancreas tissues were aseptically dissected. The samples were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4) to preserve tissue morphology for SEM analysis. Fixed tissues were stored at 4°C until processing.

Scanning electron microscopy (SEM) preparation

The fixed hepatopancreas tissues were washed in phosphate buffer (0.1 M, pH 7.4), and then subjected to dehydration through a graded ethanol series (30, 50, 70, 90, and 100%), with each step lasting 15 minutes. After dehydration, samples were dried using a critical-point drying method to prevent collapse of delicate tissue structures. Dried samples were mounted on SEM stubs and sputter-coated with gold-palladium to enhance conductivity.

SEM imaging and analysis

SEM imaging was performed using a scanning electron microscope (model: JEOL JSM-7500F) with an accelerating voltage of 5-10 kV. Images of hepatopancreatic tissues were captured at varying magnifications (500 to 5000x) to observe detailed morphological changes in the epithelial cells, tubules, and lumen. For comparative analysis, at least 10 fields per sample were examined to identify characteristic features associated with AHPND, including cellular detachment, tubule degradation, and vacuole formation. Images from the control group were also analyzed to serve as baseline data for healthy tissue structure.

Data analysis

SEM images were qualitatively analyzed to characterize distinct morphological changes in infected tissues compared to controls. Quantitative data, such as the percentage of affected tubules and cell detachment rates, were measured using image analysis software (ImageJ, NIH). Statistical comparisons between the experimental and control groups were conducted using Student's t-test, with significance set at $P< 0.05$.

RESULTS

Histopathological analysis is employed as evidence of disease infection, providing insights into health conditions through organ structural changes. Observations of the hepatopancreas histopathology in *vannamei* shrimp diagnosed with AHPND revealed alterations in tissue structure, examined under a magnification of 1500x (Fig. 1).

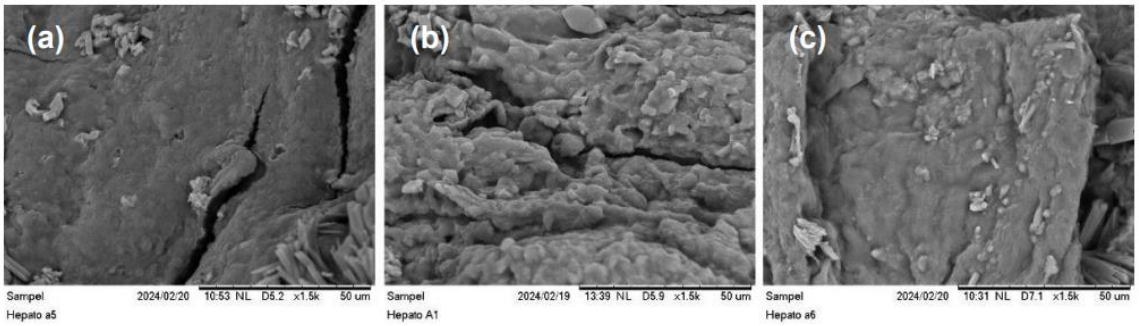


Fig. 1. Hepatopancreas histopathology (1500x): **(a)** Histopathology of sample A5; **(b)** Histopathology of sample A1; **(c)** Histopathology of sample A6

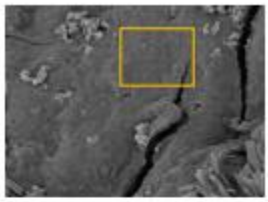
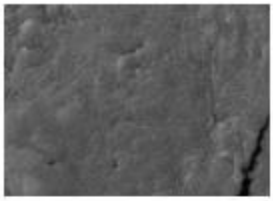
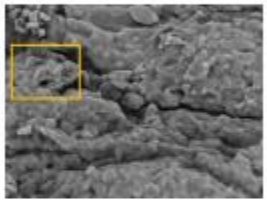
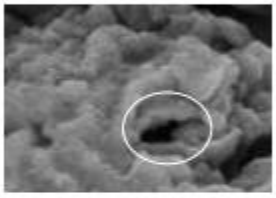
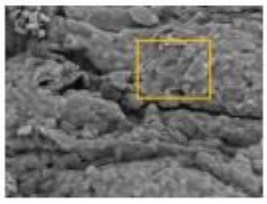
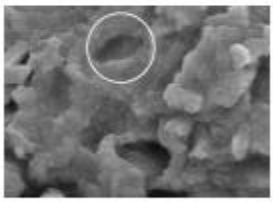
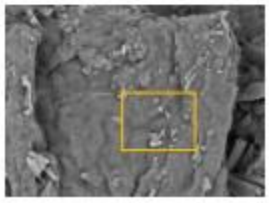
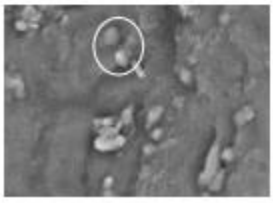
In Fig. (1), the hepatopancreas of sample A5 appears to have intact tissue structure. In contrast, the hepatopancreas in samples A1 and A6 displays irregular tissue compared to the histopathology of sample A5. The hepatopancreas in sample A5 is the most intact, attributed to immunostimulants administered by farmers to mitigate the impact of AHPND on *vannamei* shrimp. Meanwhile, the irregular forms observed in samples A1 and A6 suggest lysis within the hepatopancreatic tissue **(Anjaini *et al.*, 2018)**.

Criteria of *vannamei* shrimp hepatopancreas organs

The histopathology of *vannamei* shrimp hepatopancreas examined with SEM shows that sample A5 maintains intact tissue structure without any protrusions or surface damage. However, the histology of hepatopancreas in A1 and A6, both diagnosed with AHPND, indicates necrosis, vacuolization, and mineralization. Necrosis is characterized by cell or tissue death, leading to a loss of normal tissue integrity, often caused by biological agents like viruses, bacteria, fungi, and parasites. Vacuolization, observed in hepatopancreas, involves cavity formation within cells due to lipid degeneration, where tubular epithelial cells appear hollow **(Marbun *et al.*, 2019)**. Bacterial infection, particularly from *Vibrio*

spp., causes significant necrosis, structural tissue damage, tubular epithelial cell shrinkage, and the formation of vacuoles alongside cell release into the lumen **(Dharmawan *et al.*, 2020)**. Mineralization within the hepatopancreas is marked by numerous white spots, typically resulting from nitrogenous waste accumulation in the culture environment (feed residue, waste, and excretion) **(Putri & Prayitno, 2015)**. Details of tissue damage resulting from AHPND are shown in Table (1).

Table 1. Hepatopancreas organ criteria at 1500X magnification

Kategori	Perbesaran 1500X	Detail Kerusakan
Normal		
Nekrosis		
Vakuolisasi		
Mineralisasi		

The hepatopancreatic tissues in samples A1 and A6, both affected by acute hepatopancreatic necrosis disease (AHPND), exhibit significant pathological changes, including necrosis, vacuolization, and mineralization. Necrosis is a critical process characterized by cell and tissue death, leading to a loss of cellular cohesion and integrity. This degradation manifests as disintegration in tissue structure, often triggered by pathogens such as *Vibrio parahaemolyticus*, which disrupt cellular functions and induce cell death **(Han *et al.* 2015; Santos *et al.*, 2019)**. The localized tissue degradation

associated with necrosis can contribute to broader dysfunction within the hepatopancreas, impairing its essential roles in digestion and nutrient absorption **(Santos *et al.*, 2019)**.

Vacuolization, another prominent feature observed in the hepatopancreatic tissues of A1 and A6, refers to the formation of vacuoles or empty spaces within cells. This phenomenon is often a result of lipid degeneration or cellular distress, indicating a cellular response to metabolic imbalance. In the context of the hepatopancreas, vacuolization reflects the loss of essential cellular contents, leading to hollow or "empty" appearances in tubular epithelial cells **(Santos *et al.*, 2019)**. Such cellular degeneration can severely impact the organ's functional efficiency, ultimately weakening the shrimp's digestive capabilities **(Santos *et al.*, 2019)**.

Additionally, mineralization is evident within the hepatopancreatic tissues of samples A1 and A6, appearing as dense white spots or granules. This process involves the deposition of mineral compounds, often calcium-based, within the tissue, which can result from the accumulation of nitrogenous wastes in the aquaculture environment, including feed residues, metabolic waste, and fecal matter **(Qiu *et al.*, 2017; Surawut *et al.*, 2023)**. Over time, mineralization can impede normal cell function, leading to rigidity in tissue and further structural compromise. The mineralized tissue loses flexibility, which likely contributes to the organ's reduced capacity to process nutrients efficiently **(Qiu *et al.*, 2017; Surawut *et al.*, 2023)**.

Collectively, these pathological features—necrosis, vacuolization, and mineralization—underscore the detrimental impact of AHPND on hepatopancreatic tissue integrity and functionality in *vannamei* shrimp. Understanding these changes is crucial for developing effective management strategies to mitigate the effects of AHPND and enhance the sustainability of shrimp aquaculture **(Santos *et al.*, 2019)**.

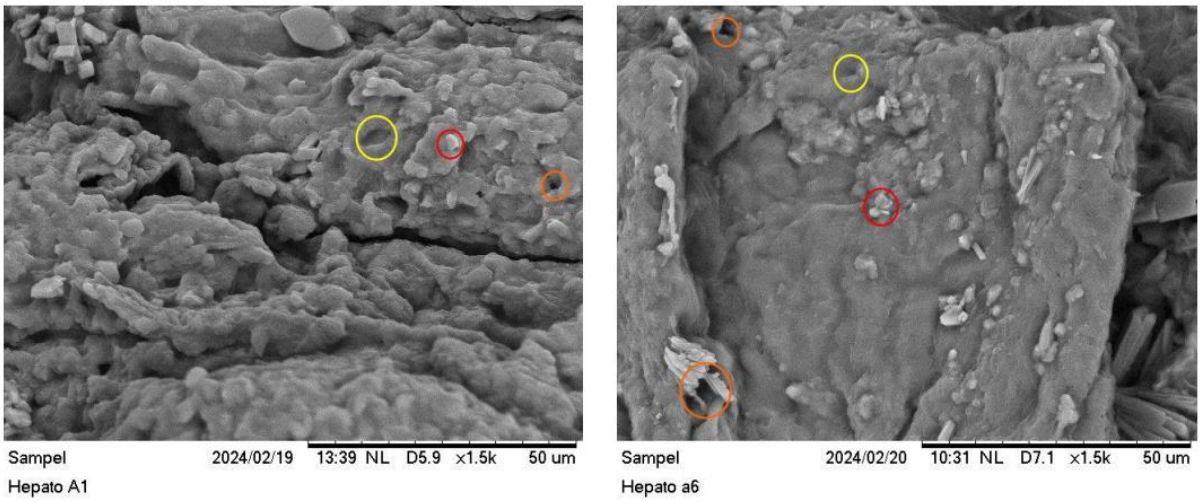


Fig. 2. Hepatopancreas damage analysis: Necrosis (orange circle); Vacuolization (yellow circle); Mineralization (red circle)

Based on Fig. (2), histopathological examination using scanning electron microscopy (SEM) reveals significant changes in the hepatopancreas of *vannamei* shrimp infected by acute hepatopancreatic necrosis disease (AHPND). In samples A1 and A6, notable cavities indicative of vacuolization damage are present, alongside the holes on the tissue surface that mark necrosis. Additionally, the presence of white granules suggests mineralization within the affected tissues. The damage observed is primarily attributed to bacterial infections from *Vibrio parahaemolyticus*, which specifically target hepatopancreatic cells in shrimp, leading to cell release and tissue damage. This ultimately results in hepatopancreatic dysfunction and cell death, contributing to necrosis **(Kongrueng *et al.*, 2014; Hong *et al.*, 2016; Jintasataporn *et al.*, 2021)**.

The histopathological changes, particularly in sample A1, demonstrate more extensive damage compared to sample A6. This observation aligns with quantitative PCR (qPCR) results, which indicate a higher level of AHPND infection in sample A1 than in sample A6. The necrosis observed in the hepatopancreas is characterized by the sloughing of epithelial cells and the infiltration of hemocytes, which are indicative of an immune response to the bacterial infection **(Padilah *et al.*, 2022; Loo, 2023)**. The vacuolization noted in the hepatopancreatic tissues reflects a cellular response to metabolic imbalance, where cells lose essential contents, resulting in hollow or "empty" spaces within the tubular epithelial cells **(Velázquez-Lizárraga *et al.*, 2019; Miao *et al.*, 2023)**.

Furthermore, mineralization, marked by the deposition of calcium-based granules, is observed in the hepatopancreas of both samples. This process is often a consequence of accumulated nitrogenous wastes in the aquaculture environment, which can arise from feed residues and metabolic waste **(Boonchuen *et al.*, 2020; Hernández-Cabanyero *et al.*, 2023)**. Over time, mineralization can lead to rigidity in the tissue, further compromising the organ's ability to function effectively in nutrient processing **(Dabu *et al.*, 2015; Jintasataporn *et al.*, 2021)**.

In summary, the pathological features of necrosis, vacuolization, and mineralization collectively reflect the severe impact of AHPND on the integrity and functionality of the hepatopancreas in *vannamei* shrimp. Understanding these changes is crucial for developing effective management strategies to mitigate the effects of AHPND in shrimp aquaculture **(Dabu *et al.*, 2015; Miao *et al.*, 2023)**.

CONCLUSION

The SEM-based histopathological analysis revealed extensive tissue damage in *Litopenaeus vannamei* hepatopancreas infected by AHPND. Samples A1 and A6 exhibited distinct signs of necrosis, vacuolization, and mineralization, in contrast to the intact tissue observed in sample A5. Necrosis in infected tissues manifested as disorganized and degraded cells, vacuolization appeared as hollow spaces within cells, and mineralization presented as dense, white granules within the tissue. These pathological changes underscore the virulent nature of *Vibrio parahaemolyticus* infection, which severely

compromises hepatopancreatic integrity and function. The insights gained from this study are essential for the development of more effective strategies for early detection, prevention, and management of AHPND in shrimp aquaculture, aiming to mitigate economic losses and enhance shrimp health.

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