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Clinical and histopathological evidence of *Hepatobacter penaei* in *Litopenaus vannamei* (Pacific white-shrimp) culture

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Abstract: With the appearance of high mortalities in the traditional cultivation of Pacific white shrimp (Litopenaus vannamei), the objective was to evaluate the health in semi-intensive nurseries in Mossoró, Rio Grande do Norte, Brazil. A random sample of 200 shrimp was collected, with 50 shrimp sampled per month during the dry season (August to November), was collected for presumptive clinical and histopathological examination of the hepatopancreas. Histologically, the hepatopancreatic fragments were evaluated for the presence of intracytoplasmic bacteria, and scores (1-4) were assigned according to the degree of severity of the lesions (DS). Morbidity percentages of 89.5% of shrimp infected with the bacterium Hepatobacter penaei were recorded. Hemolymph clotting time was not suitable for diagnosing individuals with necrotizing hepatopancreatitis. The affected individuals presented melanized lesions, such as focal to diffuse necrosis in the abdominal segments (45.5%), and the pleopods presented an expansion of chromatophores only in the most evolved cases of the infection, with 73% presenting uropods with expanded chromatophores. In the hepatopancreas, it was found that 57.5% had tubules with a significant reduction in lipid levels, 66.5% had DS 3, indicating the transition phase of the disease, and 23% had DS 4, considering the chronic phase. The absence of cytoplasm was observed in B cells with chromatin condensation. The findings suggest that abiotic factors, such as salinity (34.3‰ ± 2.78) and high temperatures (27°C ± 1.40), may have contributed to the pathogen's virulence in marine shrimp ponds, resulting in a high mortality rate.
Keywords: Bacterial Necrotizing Hepatopancreatitis, Rickettsia, Histopathology.

1. Introduction

The diseases of penaeid shrimp are caused by microorganisms that are part of their natural microbiota, which, when subjected to stress conditions, become pathogenic (Morales-Covarrubias, 2013a). In the cultivation of crustaceans, several viral and bacterial diseases are listed according to the list released by the World Organization for Animal Health (WOAH, 2023), as acute hepatopancreatic necrosis disease, infections with *Aphanomyces astaci* (crayfish plague), decapod iridescent virus 1, *Hepatobacter penaei* (necrotizing hepatopancreatitis), hypodermal and hematopoietic necrosis virus, myonecrosis virus, *Macrobrachium rosenbergii nodavirus* (white tail disease), Taura syndrome virus, white spot syndrome virus, and yellow head virus genotype 1.

NHP is a disease caused by an alpha proteobacterium (α Proteobacteria), an obligate intracellular of the Rickettsia type, under the proposed name of *Hepatobacter penaei*, which is a Gram-negative, pleomorphic bacterium, and its infectious form is mobile through flagella (Morales-CovarrubiaS, 2014). The leading causes of NHP transmission are cannibalism of infected shrimp and cohabitation of healthy shrimp and infected shrimp; however, it is also suggested that it occurs through vertical transmission (Morales et al., 2015).

The increase in the incidence of infectious outbreaks and their impact on production led to the development of different diagnostic techniques, which responded to the specific needs at the time of their implementation. Among these, there are techniques and procedures for clinical and fresh analysis, microbiological and histopathological tests, bioassay, use of antibodies, in situ hybridization, immunohistochemistry, and molecular PCR techniques (Varela and Choc-Martínez, 2020). The objective of this study was to identify the cause of high mortalities in marine shrimp ponds assessing the health of the shrimp in Rio Grande do Norte.

2. Materials e Methods

The study was carried out at the Aquatic Health Laboratory (LASA) at the Universidade Federal Rural do Semi-Árido (UFERSA), latitude -5.2065° S; longitude: -37.3193° W, using semi-intensive ponds with high mortality, from August to November 2022. 200 shrimp of the *Litopenaus vannamei* species were randomly collected, distributed in four net tanks, with a density of 30-35 shrimp/m2, with the aid of a cast net, and transported in plastic boxes with water and adequate aeration. The physicochemical parameters of the ponds, such as oxygen, pH, temperature, and salinity, were recorded.

Shrimp health monitoring was evaluated through presumptive clinical examination and histopathological analysis of the hepatopancreas. In the presumptive examination, the hemolymph clotting time according to Aguirre-Guszmán and Sánchez

Martinez (2005) was observed, as well as the macroscopic appearance of the shrimp and microscopic analysis of the hepatopancreas according to the method proposed by Morales and Cuéllar-Anjel (2014).

The macroscopic aspects were characterized according to the degree of severity of the lesions, ranging from 0 to 3 (Lightner 1996), for the degree of melanization of the lesions in the cuticle, presence of muscular opacity, and expansion of chromatophores in the appendages (Table 1).

DEGREE OF SEVERITY	CLINICAL SIGNS
0	Without macroscopic alterations suggestive of disease.
1	Few macroscopic alterations suggestive of disease.
2	Moderate macroscopic changes suggestive of disease.
3	Numerous macroscopic changes suggestive of disease.

Source: Modified from Lightner (1996)

Table 1 – Quantitative degree of severity for melanization of cuticle lesions, presence of muscle opacity, and expansion of chromatophores in the appendages.

For microscopic analysis, the animals were stunned and sacrificed on ice through thermal shock. The hepatopancreas was analyzed under an optical microscope with 10×, 20×, 40×, and 100× objectives, subsequently characterized in terms of the number of lipids (Table 2), tubular atrophy, and tubule deformities (Table 3) according to the classification by Morales-Covarrubias (2008). With the aid of tweezers, the hepatopancreas membrane was removed with a scalpel, and it was sectioned in half (longitudinally) to observe under the microscope the color of the liquid, texture, melanization, and tubular necrosis.

DEGREE OF SEVERITY	CLINICAL SIGNS
1	Hepatopancreas with very little lipid content (below 25%).
2	Hepatopancreas with low lipid content (25 - 50%).
3	Hepatopancreas with moderate lipid content (50 - 75%).
4	Hepatopancreas with a high level of lipids (above 75%).

Table 2 – Quantitative degree of severity for the amount of lipids in the hepatopancreas.

DEGREE OF SEVERITY	CLINICAL SIGNS
1	Hepatopancreas of normal size with normal tubules absent of atrophy.
2	Hepatopancreas atrophied, and in some regions, the presence of atrophied tubules surrounded by some layers of hemocytes.
3	Atrophied hepatopancreas characterized by atrophied tubules, melanization, necrosis, and formation of hemocytic nodules.
4	Atrophied hepatopancreas characterized by the presence of fibrosis, melanized, necrotic tubules, and the presence of granulomas.

Source: Modified from Morales-Covarrubias (2008)

Table 3 – Quantitative degree of severity for atrophy and deformities in the hepatopancreatic tubules.

Hepatopancreas fragments were immersed for fixation in 250 ml flasks with Davidson's solution for 48 hours, then rinsed with running water and preserved in 70% ethyl alcohol until histological analysis (Bell and Lightner 1988). To read the slides, a light microscope with 5×, 10×, 40×, and 100× objectives was used. Images were recorded with an Infinity 5.0–Olympus-Japan digital camera adapted to the microscope. The percentage of shrimp affected by diseases was recorded based on the equation: Percentage = Number of individuals affected by the disease with clinical symptoms ÷ Total number of analyzed individuals × 100. The data from the presumptive test were subjected to descriptive statistical analysis, and the Tukey Test was used at the 5% probability level to compare the means between the environments. The statistical program used for the statistical analysis was the SISVAR software. For quantitative time data, regression analysis was performed.

3. Results

The nurseries in the region of Mossoró in Rio Grande do Norte had physicochemical water parameters with minimum and maximum variations (mean and standard deviation) for oxygen, pH, temperature, and salinity of 2.9mg/L – 9.7mg/L (5.6mg/L ± 2.46), 8 - 9 (8.5 ± 0.5), 25°C – 29°C (27°C ± 1.40), and 30.9‰ – 37.0‰ (34.3‰ ± 2.78), respectively. Between the study months, a mortality rate of 70% was observed in the nurseries studied, in which temperature and salinity variations were moderately high. A presumptive clinical examination showed a morbidity rate of 89.5% in the shrimp sample. In the presumptive clinical examination of the sick shrimp, it was verified that 86% had hemolymph clotting time ≤ 30s, with a mean and standard deviation (13 ± 5.21); 1.5% had coagulation > 30s and < 60s (33 ± 1.52), and 2% with coagulation ≥ 60s. In 10.5% of the healthy animals, a clotting time ≤ 30s (15 ± 4.56) was observed.

Among the sick individuals, which represent 89.5% of the evaluated animals, 66.5% did not present melanized lesions in the cuticle (DS 0), 14.5% had a single lesion in the exoskeleton (DS 1), 6% had two to three lesions in different regions of the exoskeleton, and 2.5% had multiple lesions throughout the entire length of the shrimp exoskeleton. It was found that 45.5% of the shrimp had focal to diffuse necrosis in the abdominal segments. The abdominal musculature of the diseased shrimps presented 44% translucent musculature without necrosis (DS 0), 20% only a focus of muscular necrosis reaching a single abdominal segment (DS 1), 13.5% two abdominal segments affected with muscular necrosis (DS 2), and 12% diffuse necrosis affecting the entire length of the animal's body (DS 3).

In 64.5% of the pleopods, they were normal (DS 0), 20% had a slight expansion of the chromatophores (DS 1), 3.5% had a moderate expansion of the chromatophores (DS 2), and 1.5% had numerous expanded chromatophores (DS 3). However, 73% of sick shrimp had uropods with expanded chromatophores. 16.5% had normal uropods absent of expanded chromatophores (DS 0), 19% had a slight expansion of chromatophores (DS 1), 32.5% had a moderate expansion of chromatophores (DS 2), and 21.5% had numerous expanded chromatophores (DS 3).

In the histological analysis regarding the level of lipids in the hepatopancreas, a significant reduction in lipids in the tubules was observed in 57.5% of the sick shrimp. 5% of the shrimp had hepatopancreatic tubules with very few lipids (DS 1), 52.5% had tubules with a low level of lipids (DS 2), 31% had tubules with a moderate level of lipids (DS 3), and 1% had tubules with a high level of lipids—a lipid level considered adequate (DS 4) (Table 2).

As for the deformity of the tubules, it was found that 66.5% of the shrimp had DS 3, considering the transition phase of bacterial necrotizing hepatopancreatitis (NHP-B), showing pale coloration inside the tubules, atrophy, necrosis, and tubular strangulation. However, in 23% of the shrimp, DS 4 was observed, presenting the chronic phase of NHP-B characterized by tubular atrophy with an increase in cells with necrosis, as well as melanized nodules around the tubules (Figure 1).

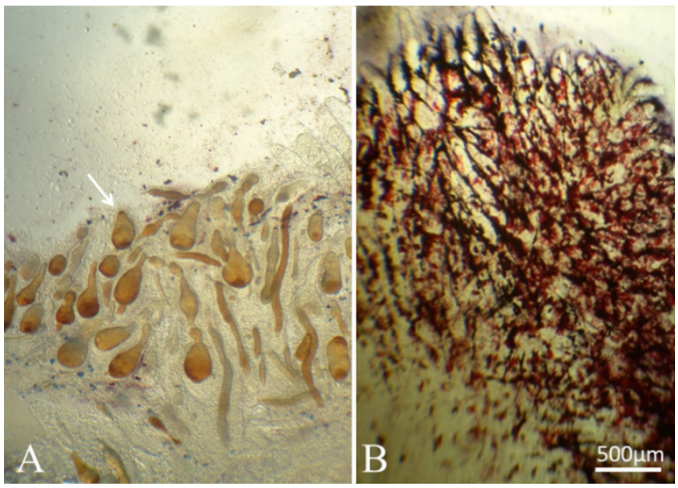

Figure 1 – Longitudinal view of diseased shrimp (*Litopenaus vannamei*). (A) Severity grade 3 showing atrophy, deformity, necrosis, and tubular strangulation. (B) Severity grade 4 characterized by increased necrosis in the cells of the hepatopancreatic tubules, as well as a greater number of melanized nodules around the tubules.

The histopathological analysis of the fragments of the hepatopancreas of the sick individuals verified atrophy of the hepatopancreatic tubules, hemocyte nodules, a smaller amount of lipid vacuoles (R cells), and secretory vacuoles (B cells) showing cytoplasm condensation. In these vacuoles, intracytoplasmic bacteria of the rickettsia type with pyknotic nuclei and basophils were also observed (Figure 2).

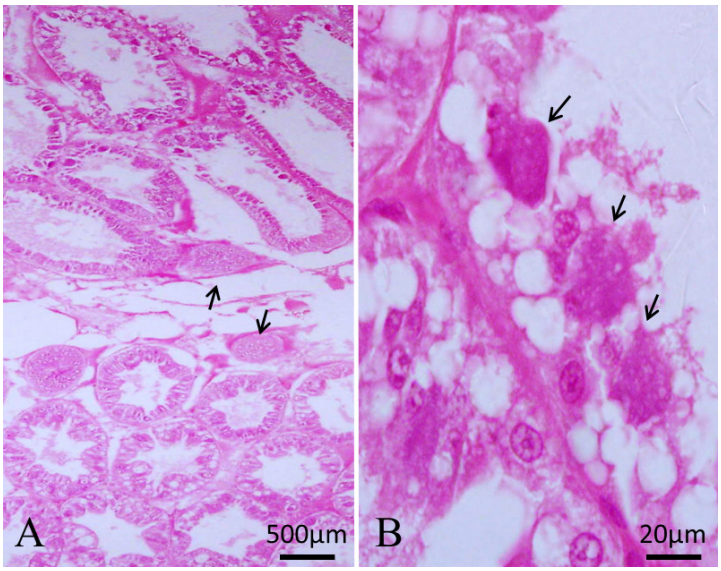

Figure 2 – Cross-section of diseased shrimp (*Litopenaus vannamei*). (A) View of the medial region of the hepatopancreas tubules showing some atrophied tubules and hemocytic nodules around the tubules (arrows). (B) View of the proximal region of the hepatopancreas tubule showing an atrophied tubule with cell detachment and the appearance of rickettsia within the B cells (arrows).

4. Discussion

Abiotic factors such as prolonged periods of high temperatures above 29°C and mean salinity of 37.0‰ are in line with variables reported by WOAH (2023b) and Morales-Covarrubias et al. (2011), favoring the replication of the etiological agent of NHP-B. Morales-Covarrubias (2010) reported similar results to the present study, where between September and October, there were mortalities above 20% in marine shrimp farms. These mortalities occur in these months due to high temperatures during the day and low temperatures at night, which favor the replication and growth of NHP-B, as reported by the WOAH (2023b). Therefore, the pathogenicity of the etiological agent will depend on the degree of virulence, the nutritional status of the individual, and its ability to cross the host's immune system (Lightner 1996).

In the presumptive examination, it was verified that the hemolymph coagulation time did not prove to be a suitable parameter for diagnosing sick individuals since they presented a coagulation time of less than 30 seconds, which is considered normal coagulation according to Aguirre-Guszmán and Sánchez-Martínez (2005). However, clotting time may be associated with other factors related to physical, chemical, or biological stress. Jussila et al. (2001) and Lightner (1996) reported in culture that the increase in hemolymph clotting time is associated with physiological stress or various infections. Stressors addressed by Maggioni et al. (2004), using the technique of unilateral ablation of the ocular stalk in species of *L. vannamei*, verified a decrease in the concentration of agglutinins, consequently increasing the time of plasma coagulation.

Macroscopically, it was observed that sick shrimp had at least one melanized lesion in the exoskeleton, focal to diffuse necrosis in the abdominal segments, and expanded chromatophores, mainly in the uropods. Melanized lesions confer a greater predisposition to bacteriosis, corroborating one of the clinical signs of NHP-B reported by the WOAH (2023b). The generalized paleness of the body affecting all abdominal segments, adding an expansion of the chromatophores at the edges of the uropods observed in the present study, is the primary clinical sign of NHP-B referred to by Morales-Covarrubias (2008); Lightner (1996); Lightner; Redman (1998), and the WOAH (2023b). Microscopically, there was a significant reduction in the levels of lipids in the hepatopancreatic tubules, also correlated by Aranguren et al. (2006) and Cahu et al. (1995) in *L. vannamei nauplii*. These authors found a considerable decrease in triglyceride levels in females infected with NHP-B.

Concerning the deformity of the hepatopancreatic tubules, they are similar to those described by WOAH (2023b) and Morales-Covarrubias (2008). In the transition phase of NHP-B (DS 3) defined by Morales-Covarrubias (2008), atrophy and tubular deformity, necrosis of hepatopancreatic tubule cells, as well as strangulation of the tubules with cell detachment, are observed, similar to those identified in this study (Figure 4a). According to the same author, referring to the chronic phase of NHP-B (DS 4), atrophy with increased necrosis is reported in the cells of the hepatopancreatic tubules, as well as a more significant number of melanized nodules around the tubules, similar to the characteristics determined here (Figure 4b).

As the transition phase to the chronic one observed in this study progressed, the number of hepatopancreatic cells containing NHP-B increased during evolution, suggesting bacterial multiplication associated with increased tissue injury. These characteristics align with those found by Vincent and Lotz (2005) in *L. vannamei* in the chronic phase of NHP-B. The phases of NHP-B reported in the study align with those described by Frelier et al. (1992). The same author considered DS 3 the second stage of the disease, characterized by numerous wrinkled, hypertrophic, necrotic tubules and presenting tubular ectasia. DS 4 was considered by the same author the third stage of the disease, characterized by multiple scattered necrotic tubules, lack of intact tubular epithelium, melanization, and fibrosis.

According to the WOAH (2023b), histopathology is considered a standard and low-cost diagnostic method with good sensitivity and specificity for NHP-B. Still, other confirmatory methods, such as In-situ DNA, real-time PCR, quantitative PCR, and Sequencing, are also available. Histologically, the presence of tubular atrophy, hemocytic nodules, a decrease in R cells, and condensation of the cytoplasm of B cells, showing bacteria of the rickettsia type with pyknotic and basophilic nuclei, are by those cited by Lightner and Redman (1994), Lightner (1996), and Morales-Covarrubias (2008) in the confirmatory diagnosis of NHP-B. The histological characteristics reported in the present study are equivalent to those of Morales-Covarrubias et al. (2011) in analyses of hepatopancreas of *L. vannamei* in eight regions of Latin America showing tubular atrophy, hemocytic nodules, and bacterial granulomas. According to Morales-Covarrubias et al. (2006), fresh examination is a vital health monitoring method in shrimp farming; however, it should not be used for the principal diagnosis of NHP-B. However, the interpretation of the observed lesions and the judgment of the degrees of severity of the samples can be variable according to the observations of the person performing the examination.

5. Conclusion

The diseased shrimps showed at least one melanized lesion in the exoskeleton, focal to diffuse necrosis in the abdominal segments, expanded chromatophores in the uropods, a significant reduction in the levels of lipids in the hepatopancreatic tubules, presenting a higher percentage of the transition phase. Given these presumptive clinical signs, bacterial infection by NHP-B is suggested. Adding the diagnosis of the disease, the histopathology confirmed that the high mortality in the nurseries occurred due to intracytoplasmic infection by Bacterial Necrotizing Hepatopancreatitis (NHP-B). It is concluded that abiotic factors such as salinity and high temperatures during the study may favor the pathogen's virulence in marine shrimp ponds, triggering a high mortality rate.

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