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Original Article

# Incidence of running mortality syndrome (RMS) in Pacific whiteleg shrimp, *Litopenaeus vannamei*, in an intensive biofloc grow-out pond

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**Abstract:** Bacterial disease is a major problem in Pacific whiteleg shrimp, *Litopenaeus vannamei* farming areas where farmers are facing a huge production loss due to epidemic diseases. The incidence of running mortality syndrome (RMS) was reported in *L. vannamei*, in an intensive biofloc culture system. Infected shrimps showed bacterial spots on the surface of the carapace, thick transparent mucous attached to the hepatopancreas, antennal cut, and cannibalism. Microscopical examination revealed a lichen-like structure with undulated margins varying about 22-650 µm size. Morphological characteristics of the colonies were smooth, circular, and opaque. Histopathological studies showed the sloughing of the tubule, multiplication of the bacterial plaque, and infiltration of the hemocytes in the infected hepatopancreas. Scanning electron microscopy of the infected shrimp revealed bacilli and cocci-shaped bacteria. Using transmission electron microscopy, bacterial populations were observed in the cytoplasm.

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## Introduction

In 2009, brackishwater aquaculture in India introduced the Pacific white shrimp, *Litopenaeus vannamei* as a prime species to be farmed using Biofloc technology (BFT). As a result, it doubled production in the last decade by contributing 53% (FAO, 2020). The biofloc system is a sustainable replacement in aquaculture for the control and reduction of various diseases where the media provides organic-rich matter comprising particulate biomass and colonization of bacteria with the addition of probiotic effect (Aguilera-Rivera et al., 2014). Infectious diseases are a common factor in shrimp culture, particularly in Andhra Pradesh and Tamil Nadu (Rao and Satyanarayana, 2020). Environmental factors trigger the expeditious multiplication of pathogens that are already in lower concentrations in the habitat (Johnson et al., 2010). Most of the outbreaks in culture waters are reported to be associated with *Vibrio* populations and have led to a loss of one billion USD $ in the Asia-Pacific region (Sung et al., 1999; FAO, 2013).

Bacterial infections occur in a weakened state and are a serious threat by causing mortality (Ganesh et al., 2010). It is caused by a gram-negative bacterium belonging to the family Vibrionaceae (Sizemore and Davis, 1985). The infected shrimp pond exhibits perpetuating mortality throughout the culture period and is not interdependent on water quality parameters. This condition is termed Running Mortality Syndrome (RMS) in shrimp (Alavandi et al., 2019). It is correlated with both mortality and morbidity, which eventually leads to lower productivity (Rao and Satyanarayana, 2020).

In RMS, low mortality starts during the 35-40 days of culture (DOC) and gradually increases, forcing the farmers to preharvest the crops (Poulos et al., 2006). Infected shrimps are characterized by pale or reddish carapace, lethargy, root and reddish telson and antenna, necrosis in the appendages and tissue, slow growth, opacity in the muscle, anorexia, and melanization (Karunasager et al., 1994; Lightner, 1996; Robertson et al., 1998; Smith, 2000). The present study reports the incidence of RMS in an

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intensive biofloc grow-out pond, *Litopenaeus vannamaei*, in Tamil Nadu. Microbiological, histopathology, and electron microscopic studies were performed to confirm the bacterial infection.

## Materials and Methods

**Description of the study area:** Samples were collected from a commercial shrimp farm (11˚ 21'33''N, 79˚ 48'45''E) in Tamil Nadu, India. The shrimps were maintained for 119 days in a biofloc rearing setup with no water exchange, constant aeration, and organic-rich media. Sampling was performed during the early hours of the day using a cast net. The collected samples were brought to the laboratory in a portable icebox.

**Gross observation and wet mount:** The moribund and normal shrimp were grossly observed and compared. The carapaces of the infected and normal shrimp were cut to a size of 4 $mm^2$ and placed on a clean glass slide with a drop of distilled water. It was observed under a light microscope (Magnus MLX).

**Water quality parameters:** Water samples were obtained from both biofloc ponds and subsequently transported to the laboratory for further analysis. Weekly assessments of water quality parameters such as temperature, pH levels, salinity, and dissolved oxygen content were conducted using a thermometer, pH pen, refractometer, and dissolved oxygen meter.

**Bacterial isolation and biochemical analysis:** The hepatopancreas was taken aseptically and crushed with pestle, and mortar in PBS solution. They were then inoculated into TCBS agar plates and maintained at 30±2℃ for 24 hrs. Subsequent subcultures were performed for the purification of the bacteria. Identification and characterization of the bacteria was performed based on their biochemical tests based on Buchanan and Gibbons (1974).

**Histopathology:** Normal and infected shrimps were dissected and the organs like gills and hepatopancreas were preserved in Davidson's fixative. The preserved samples were processed and embedded in paraffin. The embedded tissues were cut into thin sections. Furthermore, they are stained with haematoxylin and eosin (Bell and Lightner, 1988).

**Scanning electron microscope:** The cultured bacteria were fixed in 1% glutaraldehyde (vol/vol) (Polysciences, Inc., Warrington, Pa.), 1.5% NaCl (wt/vol), and 0.1 M cacodylic acid (Sigma Chemical Co., St, Louis, Mo.), pH 7.60. After fixation, the plates were flooded with acid buffer, which was removed through vacuum aspiration, and stored overnight at 4˚C. Later the plate was processed through an ascending series of ethanol. The sample was dehydrated, and then coated with gold, and observed under a scanning electron microscope (JEOL JSM 5610LV at 15KV).

**Transmission electron microscope:** The hepatopancreas of the infected and the normal shrimp were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature. The samples were then washed with 0.1 M sodium cacodylate buffer and then dehydrated using an acetone series. Samples were embedded in 100 epoxy and incubated overnight at 60℃. Semithin sections were prepared and stained with toluidine blue. They were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynold's lead citrate (Reyonlds, 1963), and the samples were examined under a transmission electron microscope (Philips Tecnai TR spirit).

## Results

**Sample collection:** The area shrimp farm is approximately 0.55 hectare with a stocking density of 200/$m^2$. The shrimps weighed about 25-30g. Mortality was observed as early as 35 day of culture to 119 day of culture. Sampling was performed weekly. Based on the moribund and mortality recorded, the prevalence of the infection was 43-45%.

**Gross observation and the wet mount study:** The normal shrimp had long antennae with all the organs such as the hepatopancreas, eye, pleopods, and periopods intact, whereas the infected shrimps revealed anorexia, multifocal melanisation, and necrosis. The infected shrimps exhibited cannibalism, short antennae, loss of pleopods, bacterial spots and white patches on the carapace, partial loss, and redness on the uropods (Fig. 1a). The colour varied from pink

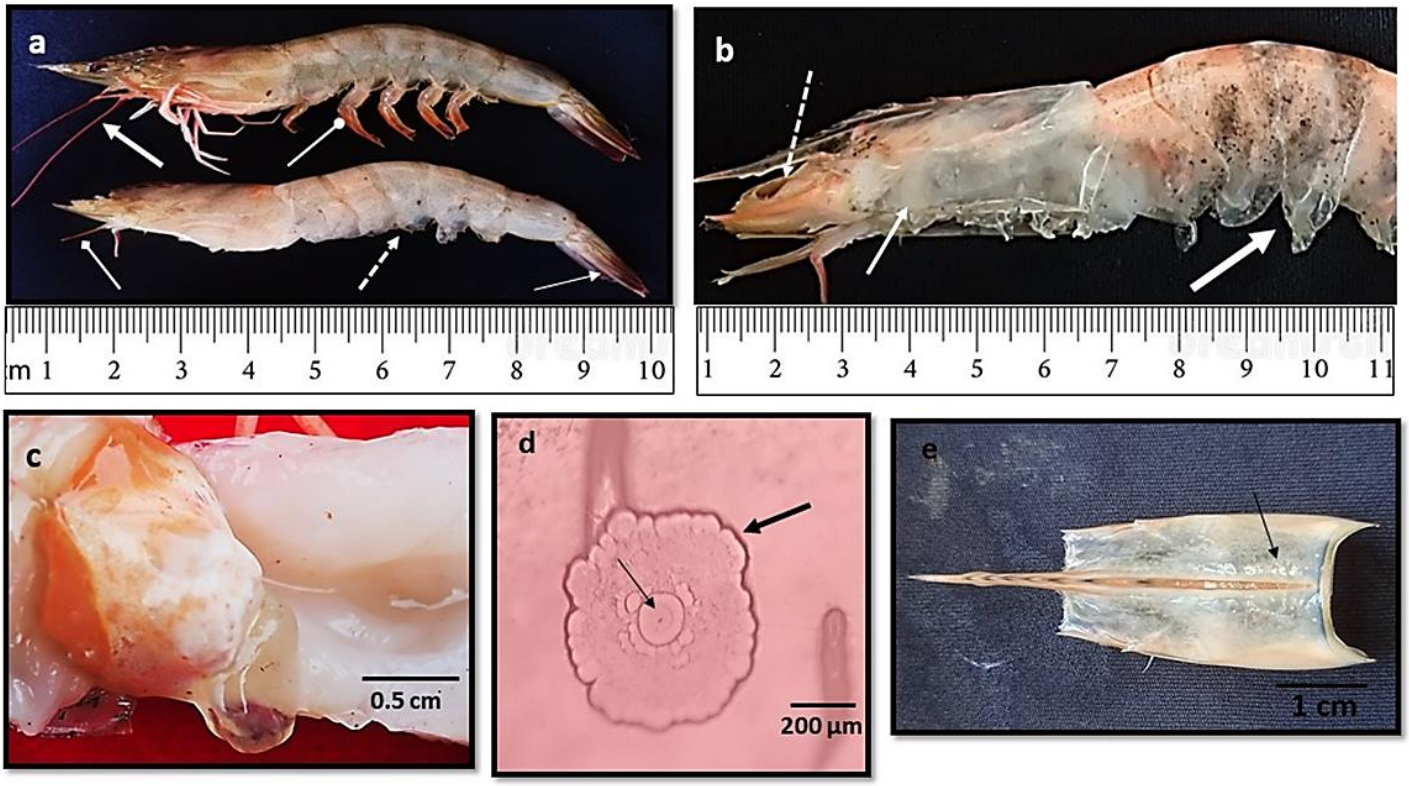


Figure 1. Comparison of the normal and the infected shrimp. **(a)** Lengthy antennae (thick arrow), pleopods (round head arrow), short antennae (diamond head arrow), loss of pleopods and pereiopods (dotted arrow), redness and loss in the uropods (thin arrow), **(b)** Loss of eyes (dotted arrow), soft shell, loss of gill (thin arrow), loss of pleopods (thick arrow), **(c)** Mucous accumulation in the infected hepatopancreas, **(d)** Single bacterial spot carrying the bacterial plaque in the centre (thin arrow), undulated margin (thick arrow), and **(e)** Multiple bacterial spots on the carapace (thin arrow)

to red based on melanisation along with loss of an eye, soft shell, partial loss of cephalothorax, and loss of pleopods (Fig. 1b). Infected shrimp secreted a thick transparent mucous attached to the hepatopancreas (Fig. 1c). The bacterial spot size varied between 22-650 µm and was a circular, round shape with an undulate margin (Fig. 1d). The bacterial spots on the carapace were white to patch and ellipsoidal in shape (Fig. 1e).

**Water quality parameters**

**Temperature:** The temperature of the normal pond ranged from 29.5-32.3℃ whereas in the infected pond, it was about 30-34.9℃. There was wide variation in temperature during the culture period. In comparison highest temperature in the infected pond was recorded on the 14$^{th}$ day of culture and the lowest on the 4$^{th}$ day of culture (Fig. 2).

**Salinity:** The salinity of the normal pond was about 29.8-32.4 PSU, whereas, in the infected pond, it was about 29.9-32.5 PSU (Fig. 3). On comparison, the lowest salinity was recorded on the 2$^{nd}$ day of culture and the highest during the 6$^{th}$ day of culture.

**pH:** On comparison, the normal pond ranged from 7.4 to 8.2 ppm, whereas the infected pond was approximately 7.4-8.2 (Fig. 4). There was a wide range of fluctuations in the pH throughout the culture.

**Dissolved oxygen:** The dissolved oxygen was about 6.9-10.3 mg/l in the normal pond, whereas in the infected pond, it ranged from about 4.6 to 6.7 mg/l. The highest DO in the infected pond was noted on the 13$^{th}$ day of culture and lowest on the 2$^{nd}$ day of culture, whereas in the normal pond, it was highest during the 11$^{th}$ and 12$^{th}$ days of culture (Fig. 5).

**Phenotypic observation and biochemical analysis:** The bacterial culture was plated by inoculating the infected hepatopancreas on TCBS agar. The morphological characteristics of the bacteria were smooth, motile, circular, and opaque colonies with an entire margin (Table 1).

**Histopathology**: The hepatopancreatic tubules were in the necrotic stage. Infiltration of hemocytes was evident in the cytoplasm of the hepatopancreas.

Table 1. Gram staining, preliminary biochemical analysis and sugar fermentation of the colonies.

| Gram Staining | Motality | Oxidase | Catalase | Indole | Methyl Red | Voges Proskaurer | Citrate | Urease | Nitrate reduction | Dextrose | Glucose | Lactose | Maltose | Sucrose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| – | Motile | – | + | + | + | + | + | + | – | + | + | + | + | + |

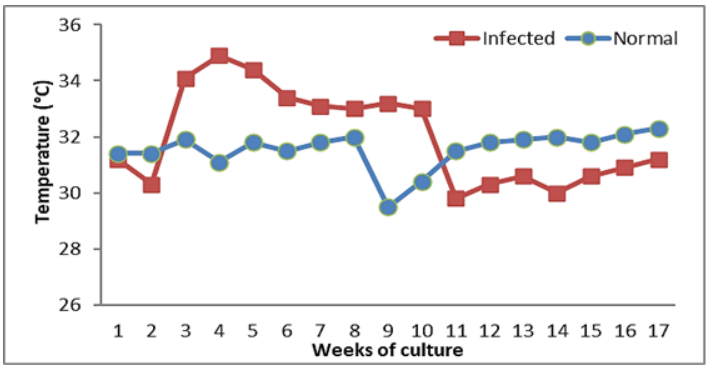


Figure 2. Temperature of normal and infected pond.

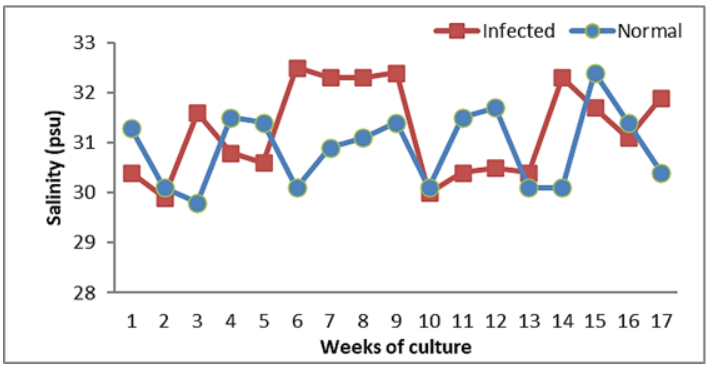


Figure 3. Salinity of normal and infected pond.

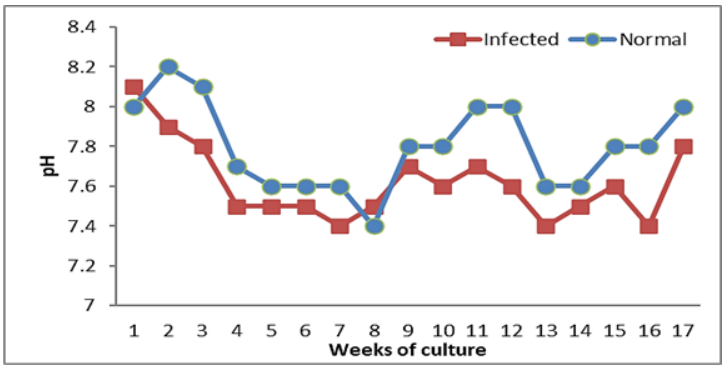


Figure 4. pH of normal and infected pond.

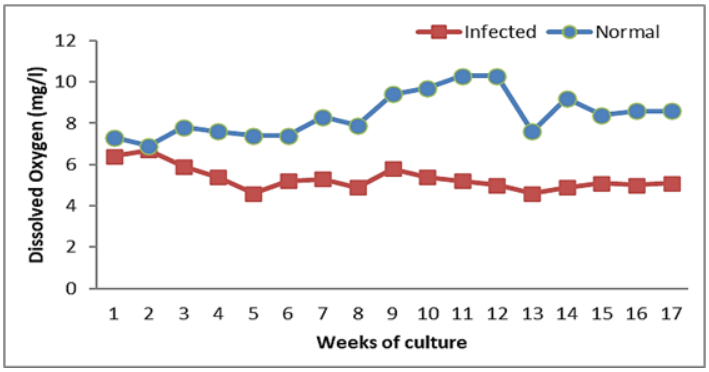


Figure 5. Dissolved oxygen of normal and infected pond.

Sloughing of the hepatopancreatic tubule was observed in the medial region (Fig. 6A). Hepatopancreatic tubules were atrophied. The lumen size is greatly varied. Multifocal granulomas with bacterial colonies were distributed. Hemolytic infiltration in the interstitial space is moderately diffused. Lipid droplets are released from the lumen of the hepatopancreatic cell. Bacterial plaque in the lumen of the hepatopancreas (Fig. 6B). Histopathology of the infection gills showed pcynotic cells diffused in the lamellae, degradation of the gill structure (Fig. 6C), necronization of the connective tissue, vacuolization, basophilic inclusion diffused in the cytoplasm, and disintegration of the gill structure (Fig. 6D).

**Scanning electron microscope:** Scanning electron microscope studies revealed the presence of bacteria in the infected shrimp. Bacilli-shaped bacteria were evident in the infected animal. The size of the bacillus bacteria was about 2 µm (Fig. 7A). The size of the cocci-shaped bacteria was about 2 µm (Fig. 7B).

**Transmission electron microscope:** The transmission electron microscope confirmed the presence of bacteria in the cytoplasm of the hepatopancreas. The bacteria were densely populated in some cells, and cell organelles such as endoplasmic reticulum were pushed to the corner of the cell. The chromatin was marginated (Fig. 8A). The size of the bacillus is about 2 µm whereas the endoplasmic reticulum is spread out in the cytoplasm (Fig. 8B). The cocci-shaped bacteria ranged about 0.5 µm. Bacterial plaque is diffused in the connective tissue of the hepatopancreas (Fig. 8C). Various bacterial cells are spread out in the cytoplasm of the cells, and the hemolytic membrane is electron-dense. Lipid rafts were also in the hepatopancreatic tissue. The bacterial

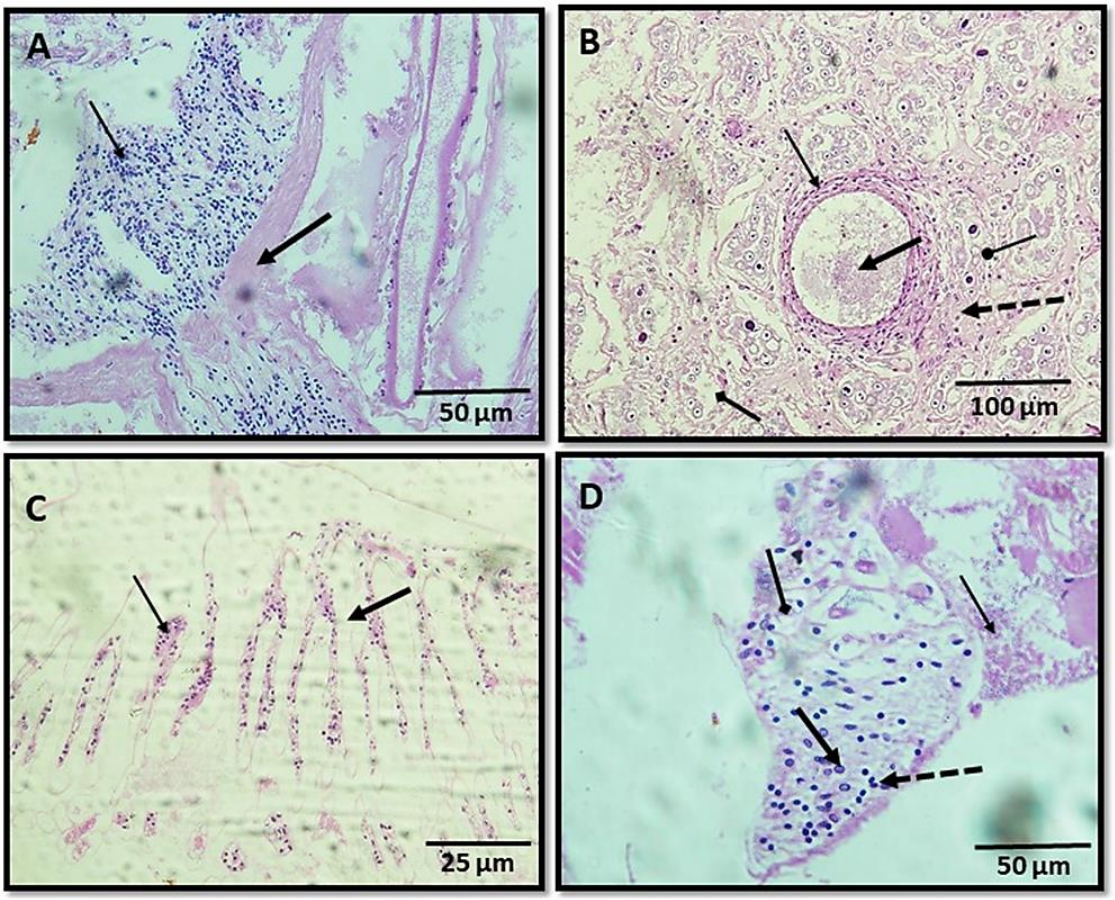


Figure 6. Histopathology of the running mortality syndrome infected hepatopancreas. Infilterated hemocytes in the cytoplasm of the hepatopancreatic cells (thin arrow), sloughing of the hepatopancreatic tubule epithelial cells in the medial region of the hepatopancreas (thick arrow) (A); multifocal granulomas (thin arrow), bacterial colonies (thick arrow), moderately diffused hemocytic infilteration in the interstitial space (dotted arrow) (B); Histopathology of the running mortality syndrome of infected gills. Pcynotic cells in the gill lamellae (thin arrow), disintegration on the structure of the gills (thick arrow) (C), marginated chromatin (thick arrow), basophilic inclusion spread out in the cytoplasm (dotted arrow), disintegration of the gill structure (thin arrow) (D).

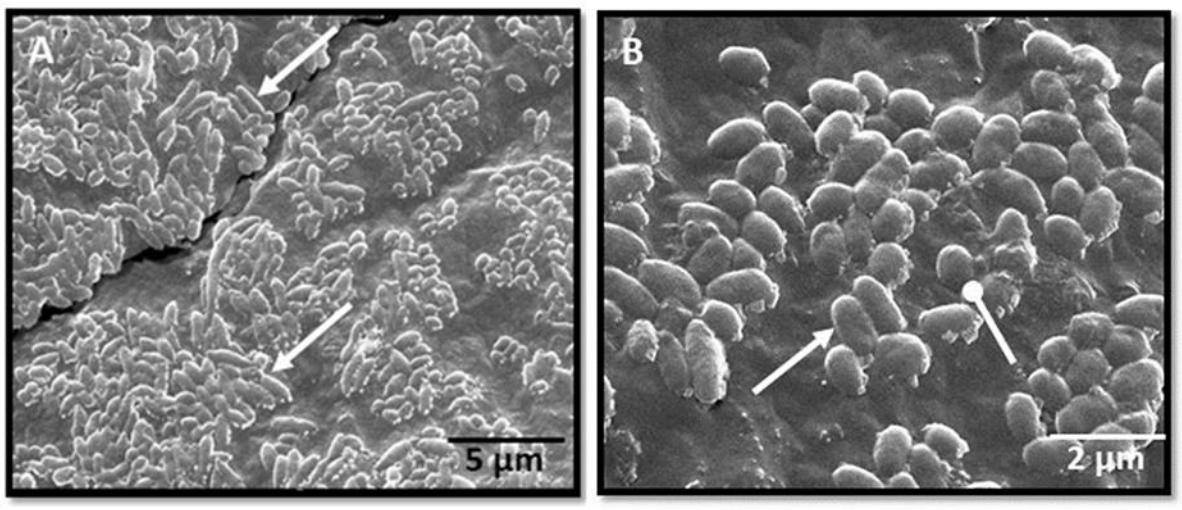


Figure 7. SEM image of the cultured bacteria. Bacillus bacteria in clusters or in groups (bold arrow). Cocci shaped bacteria (round head arrow), bacillus shaped bacteria (bold arrow).

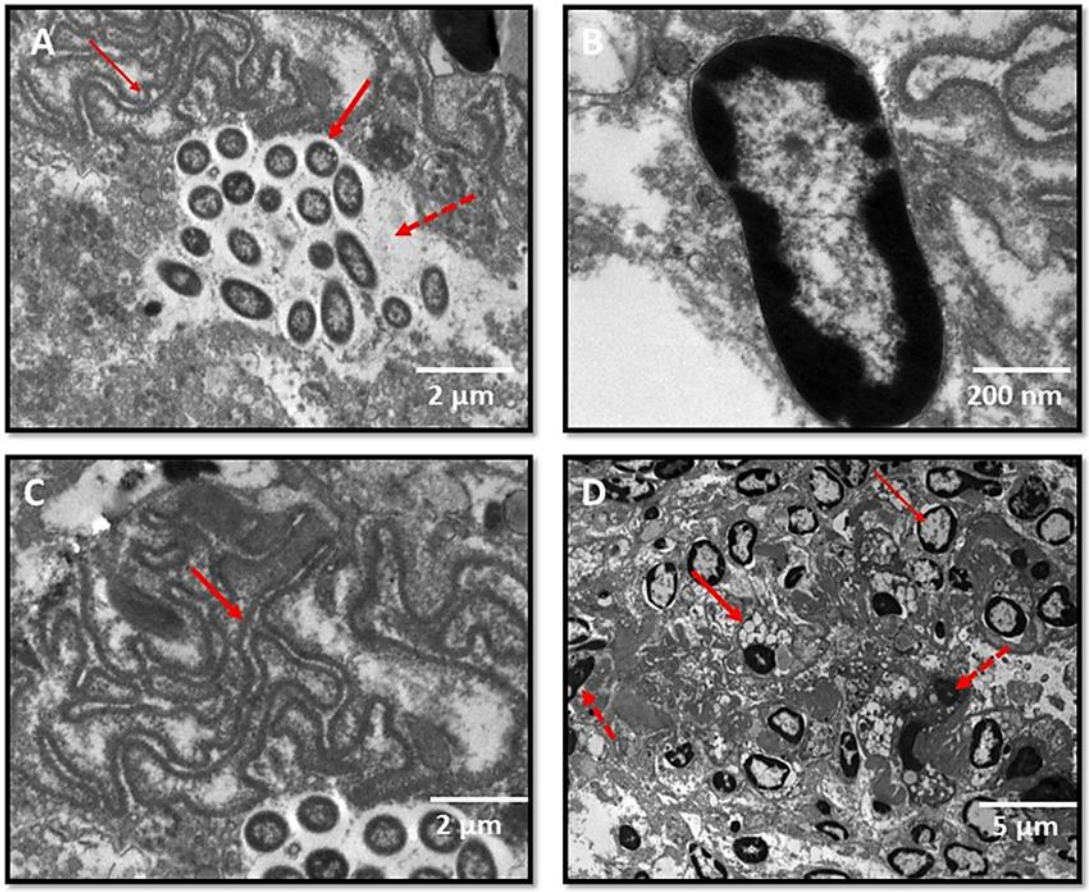


Figure 8. Ultrastructural studies of the infected hepatopancreas. (A) Bacterial population in the infected hepatopancreas (dotted arrow), number of bacteria in the cell cytoplasm of the marginated nucleus (thick arrow), and endoplasmic reticulum are also seen (thin arrow), (B) Single bacillus bacteria with endoplasmic reticulum on one side, (C) Coccus bacteria accumulate on one side with endoplasmic reticulum on one side, and (D) Lipid raft surrounded by haemocytes (thick arrow), marginated chromatin (thin arrow), bacteria present in the cytoplasm of the dense chromatin cell (dotted arrow).

cells were spread out in large numbers and both cocci and bacilli-shaped were observed. The lipid droplets accumulated are increased in size in the cytoplasm (Fig. 8D).

## Discussions

We report an outbreak of the bacterial infection, Running Mortality Syndrome (RMS) in the Pacific whiteleg shrimp, *L. vannamei* in an intensive biofloc grow-out pond, Tamil Nadu. A similar outbreak of vibriosis was reported in a biofloc culture by Aguilera-Rivera et al. (2019**).** The environmental factors that trigger the multiplication of bacterial infection include crowding, increased water temperature, and decreased dissolved oxygen (Brock and Lightner, 1990; Kannapiran et al., 2009). Vaseeharan et al. (2007) in *Penaeus monodon* reported mass mortality and wounds on the surface of the body.

In moribund and dead shrimps, bacterial spots that measured about 22-650 µm were noticed on the surface of the carapace. Microscopic examination revealed that they were lichen-like with an undulated margin and varied in shape and size. Similar observations were recorded by Wang et al. (2000) and Dewangan et al. (2022). The characterization of the infected shrimp was similar to other reports, which included redness, lethargy, anorexia (Chen, 1992), a whitish opaque body (Longyant et al., 2008), redness on the uropods, cannibalism, and antennal cut (Wu et al., 2001; Rao and Satyanarayana, 2020). Infected shrimp showed signs such as white necrotic areas in the muscles, and necrotic and reddened areas, which aligns with the results of Melena et al. (2012) and Alavandi et al. (2019).

In shrimp culture, poor water quality parameters coupled with viral and bacterial diseases are major

mortality factors (Chamberlain, 1997). In our study, there is a variation in salinity that is similar to that of Tedengren et al. (1988). Similar to our studies, overstocking can cause a decrease in water quality and increase disease transmission (Tendencia et al., 2010). Alavandi et al. (2019) stated that water parameters with high temperature and stocking density combined with low feed intake can result in mortality.

The hepatopancreas is a target organ in most shrimps for the entry of opportunistic pathogens (Chen et al., 1992). In our study, the morphology of the bacterial colonies was isolated from the hepatopancreas cultured in thiosulphate citrate bile salt sucrose (TCBS agar), which coincides with the report of Longyant et al. (2008) and Alavandi et al. (2018). Histological studies revealed the presence of haemocytic infiltration in the tubular or intestinal space, necrosis, sloughing, and basophilic inclusion which is in agreement with the results of Alavandi et al. (2018). Bacterial plaque was noticed in the lumen of the hepatopancreas (Dewangan et al., 2022). Rao and Satyanarayana (2020) reported sloughing of the hepatopancreatic tubules. Conditions such as these can be an obstacle to the digestion and absorption of nutrients in shrimps (Vogt et al., 1989; Vogt, 1994). Similar conditions were reported by Soto-Rodriguez et al. (2015) in *Litopenaeus vannamei* infected by *Vibrio parahemolyticus*. Multifocal granulomas, pycnotic cells, degeneration, and bacterial colonies in the hepatopancreas were also reported by Aguirre-Guzman et al. (2010).

Scanning electron microscopical studies showed the presence of bacteria on the surface of the bacteria in the infected shrimp. Similar white spots on the carapace revealed evidence of bacterial infections in *Penaeus monodon* (Wang et al., 2000). The infected hepatopancreas revealed the manifestation of bacterial infections through transmission electron microscopy. The bacterial plaque was spread out in the connective tissue, cytoplasm, and hepatopancreatic tubules, and the endoplasmic reticulum spread out in the hepatopancreatic cells. The proliferation of the bacteria resulted in the transfer of the nucleus in the margin of the cell. Bacterial infection triggers the endoplasmic reticulum stress sensor, which leads to unfolded protein response (UPR) which results in autophagy and stress granule formation.

## Conclusion

The proliferation of aquaculture has led to the frequent occurrence of outbreaks, posing a significant threat to the sustainability of shrimp production. Better management practices (BMP) along with biosecurity measures such as pond preparation, water filtration, disinfection of water, fencing, and procuring of seeds from Coastal Aquaculture Authority (CAA) registered hatcheries should be followed. Bacterial infections can be managed by continuous water supply, reducing stress, probiotics, and sanitation. There must be a proper mode of communication with aquaculture farmers on disease prevention and management of shrimp health. These are some of the factors that are important for managing emerging and prevailing pathogens.

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## References

Aguilera-Rivera D., Prieto-Davó A., Escalante K., Chávez C., Cuzon G., Gaxiola G. (2014). Probiotic effect of floc

on Vibrios in the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture, 424-425: 215-219.

Aguilera-Rivera D., Prieto-Davó A., Rodríguez-Fuentes G., Escalante-Herrera K.S., Gaxiola G. (2019). A vibriosis outbreak in the Pacific white shrimp, *Litopenaeus vannamei* reared in biofloc and clear seawater. Journal of Invertebrate Pathology, 167: 107246.

Aguirre-Guzmán G., Sánchez-Martínez J.G., Pérez-Castañeda R., Palacios-Monzón A., Trujillo-Rodríguez T., dela Cruz-Hernández N.I. (2010). Pathogenicity and infection route of *Vibrio parahaemolyticus* in American white shrimp, *Litopenaeus vannamei*. Journal of the World Aquaculture Society, 41(3): 464.

Alavandi S.V., Muralidhar M., Dayal J.S., Rajan J.S., Praveena P.E., Bhuvaneswari T., Otta S.K. (2019). Investigation on the infectious nature of Running Mortality Syndrome (RMS) of farmed Pacific white leg shrimp, *Penaeus vannamei* in shrimp farms of India. Aquaculture, 500: 278-289.

Bell T.A., Lightner D.V. (1988). A handbook of normal penaeid shrimp histology. Journal of the World Aquaculture Society. 30 p.

Brock J.A., Lightner D.V. (1990). Diseases of crustacea. Diseases caused by microorganisms. Diseases of Marine Animals, 3: 245-349.

Buchanan R.E., Gibbons N.E., Cowan S.T. (1974). Bergey's manual of determinative bacteriology. 8th Edition, Williams and Wilkins, Baltimore, 1268 p.

Chamberlain G.W. (1997). Sustainability of world shrimp farming. Global Trends: Fisheries Management, 20: 195-209.

Chen D. (1992). An overview of the disease situation, diagnostic techniques, treatments and preventatives used on shrimp farms in China. In: Fuls, K.L. Main, (Eds.), Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute Hawaii. pp. 47-55.

Dewangan N.K., Gopalakrishnan A., Shankar A., Ramakrishna R.S. (2022). Incidence of multiple bacterial infections in Pacific whiteleg shrimp, *Litopenaeus vannamei*. Aquaculture Research, 53(11): 3890-3897.

FAO (2013). Report of the FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp (under TCP/VIE/3304). Hanoi, Viet Nam, 25-27 June 2013. FAO Fisheries and Aquaculture Report No. 1053, Rome. 54 p.

FAO. (2020). The state of world fisheries and aquaculture 2020. Sustainability in Action. Rome: FAO. doi: 10.4060/ca9229en.

Ganesh E.A., Das S., Chandrasekar K., Arun G., Balamurugan S. (2010). Monitoring of total heterotrophic bacteria and *Vibrio* spp. in an aquaculture pond. Current Research Journal of Biological Sciences, 2(1): 48-52.

Johnson C.N., Flowers A.R., Noriea N.F., Zimmerman A.M., Bowers J.C., DePaola A., Grimes D.J. (2010). Relationships between environmental factors and pathogenic vibrios in the Northern Gulf of Mexico. Applied and Environmental Microbiology, 76: 7076-7084.

Karunasagar I., Pai R., Malathi G.R., Karunasagar I. (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture, 128: 203-209.

Kannapiran E., Ravindran J., Chandrasekar R., Kalaiarasi A. (2009). Studies on luminous, *Vibrio harveyi* associated with shrimp culture system rearing *Penaeus monodon*. Journal of Environmental Biology, 30(5): 791.

Lightner D.V. (1996). A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, Louisiana. 305 p.

Longyant S., Rukpratanporn S., Chaivisuthangkura P., Suksawad P., Srisuk C., Sithigorngul W., Sithigorngul, P. (2008). Identification of *Vibrio* spp. in vibriosis *Penaeus vannamei* using developed monoclonal antibodies. Journal of Invertebrate Pathology, 98(+1): 63-68.

Melena J., Tomalá J., Panchana F., Betancourt I., Gonzabay C., Sonnenholzner S., Amano Y., Bonami R.J. (2012). Infectious muscle necrosis etiology in the Pacific White Shrimp (*Penaeus vannamei*) cultured in Ecuador. Brazilian Journal of Veterinary Pathology, 5: 31-36.

Poulos B.T., Tang K.F.J., Pantoja R.C., Bonami J.R., Lightner D.V. (2006). Purification and characterization of infectious myonecrosis virus of penaeid shrimp. Journal of General Virology, 87: 987-96.

Rao N.V., Satyanarayana A. (2020). Running Mortality Syndrome (RMS)-A New Disease that Struck the Indian Shrimp Industry. International Journal of Current Microbiology Application Science, 9(1): 1928-1934.

Reynolds E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, 17(1): 208–212.

Robertson P.A.W., Calderon J., Carrera L., Stark J.R., Zherdmant M., Austin B. (1998) Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. Diseases of Aquatic Organisms, 32: 151-155.

Sizemore R.K., Davis J.W. (1985). Source of *Vibrio* spp. found in the hemolymph of the blue crab, *Callinectes sapidus*. Journal of Invertebrate Pathology, 46(1): 109-110.

Soto-Rodriguez S.A., Gomez-Gil B., Lozano-Olvera R., Betancourt-Lozano M., Morales-Covarrubias M.S. (2015). Field and experimentally evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Applied and Environmental Microbiology, 81(5): 1689-1699.

Smith P.T. (2000) Diseases of the eye of farmed shrimp *Penaeus monodon*. Diseases of Aquatic Organisms, 43: 159-173.

Sung, H.H., Li, H.C., Tsai, F.M., Ting, Y.Y., Chao, W.L., (1999). Changes in the composition of Vibrio communities in pond water during tiger shrimp (*Penaeus monodon*) cultivation and in the hepatopancreas of healthy and diseased shrimpJournal of Experimental Marine Biology and Ecology, 236: 261-271.

Tedengren M., Arner M., Kautsky N. (1988). Ecophysiology and stress response of marine and brackish water Gammarus species (Crustacea, Amphipoda) to changes in salinity and exposure to cadmium and diesel-oil. Marine Ecology Progress Series, 47: 107-116.

Tendencia E.A., Bosma R.H., Verreth J.A.J. (2010). WSSV risk factors related to water physico-chemical properties and microflora in semi-intensive *Penaeus monodon* culture ponds in the Philippines. Aquaculture, 302: 164-168.

Vaseeharan B., Sundararaj S., Murugan T., Chen J.C. (2007). *Photobacterium damselae* ssp. Damselae associated with diseased black tiger shrimp *Penaeus monodon* Fabricius in India. Letters in Applied Microbiology, 45(1): 82-86.

Vogt G. (1994). Life-cycle and functional cytology of the hepatopancreatic cells of *Astacus astacus* (Crustacea, Decapoda). Zoomorphology, 114: 83-101.

Vogt G., Stocker W., Zwilling R. (1989). Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. Histochemistry, 91: 373-381.

Wang X.H., Leung K.Y. (2000). Biochemical characterization of different types of adherence of Vibrio species to fish epithelial cells. Microbiology, 146: 989-998.

Wang Y.G., Lee K.L., Najiah M., Shariff M., Hassan M. D. (2000). A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* and its comparison with white spot syndrome (WSS) caused by virus. Diseases of Aquatic Organisms, 41(1): 9-18.

Wu J.L., Namikoshi A., Nishizawa T., Mushiake K., Teruya K., Muroga K. (2001). Effects of shrimp density on transmission of penaeid acute viremia in *Penaeus japonicus* by cannibalism and the waterborne route. Diseases of Aquatic Organisms, 47(2): 129-135.