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Investigation on running mortality syndrome (RMS) in *Penaeus vannamei* culture with control measures

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Abstract
The issue of Running Mortality Syndrome (RMS) in shrimp farming is a cause for concern as it can lead to significant economic losses. RMS is primarily caused by the *Vibrio parahaemolyticus* bacteria strain. RMS is characterized by reddening of body with reddish hepatopancreas and mortality started from 40 days of culture (DOC). In a recent study, advanced molecular techniques were used to isolate and identify the causative organism as *Vibrio parahaemolyticus*. Histopathological analysis revealed vacuolation of B cells, sloughing into the lumen of hepatopancreas, muscle necrosis, edema, and haemocytic infiltration in infected shrimp. *Ayapana triplinervis* was studied against *Vibrio parahaemolyticus* isolated from RMS infected shrimp and compared with Oxy-tetracycline (OTC) as a positive control by MIC test. Therefore, the use of *Ayapana triplinervis* as a potential herbal antimicrobial agent can be considered as a viable option for the control of the causative agent in the shrimp production sector, as part of eco-friendly and sustainable management practices.

Keywords: Running mortality syndrome, *Ayapana triplinervis*, vibrio parahaemolyticus, shrimp farming

1. Introduction
Shrimps are often referred to as the "pinkish gold of the sea" because of their high demand, delightful taste, and the considerable economic value they command in the global market (Nisar *et al.*, 2021) [23]. In 2009-2010, the Coastal Aquaculture Authority of India (CAAI) recommended the introduction of the exotic species *Penaeus vannamei*. Forward-thinking fish farmers enthusiastically adopted this recommendation for semi-intensive production in low saline water. Among the promising regions in India, the culture of *P. vannamei* is thriving in the coastal and brackish water areas of West Bengal, particularly in the Purba Medinipur District (Maiti *et al.*, 2019) [21]. Lightner's (1993) [19] research found that the growing demand for shrimp farming in India has led to significant advancements in shrimp hatchery production and controlled environment culture for export purposes. However, the high-volume production of both larvae and mature shrimp often resulted both infectious and non-infectious diseases. The accumulation of organic matter in the pond, resulting from excessive feeding (overfeeding), shrimp faecal matter, and dead algae, contributes to pond bottom pollution (Venkateswarlu *et al.*, 2023) [39]. Unfortunately, this industry has faced significant setbacks from bacterial pathogens, including *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio cholerae*, *Proteus penneri*, *Aeromonas schubertii*, and *Shewanella algae*, as reported by Aguirre-Guzmán (2004) [1]; Cao *et al.* (2015) [6]; Sudheesh *et al.* (2001) [33]; Zhou *et al.* (2012) [42]. According to Rao and Satyanarayana (2020) [28], *Penaeus vannamei* affected by Running Mortality Syndrome (RMS) displayed clinical signs such as antennae cutting, unusual reddening of the uropod, and a reddish-yellow tint in the hepatopancreas, along with ongoing internal mortality. However, the extensive and regular use of antibiotics in shrimp aquaculture has led to the development of antibiotic resistance in the pathogens affecting both cultured animals and humans, as highlighted by Holmström *et al.* (2003) [12]. The significant role of many herbal preparations in disease management is acknowledged due to their antioxidant and antimicrobial properties, as studied by Prasad and Padhyoy (1993) [25]. As highlighted by Sobrinho *et al.* (2017) [31], *Eupatorium* species are notably abundant in terpenes, phytosterols, and sesquiterpene lactones, possessing anticancer, antiplasmodial, and antibacterial properties. This characteristic makes them promising candidates for therapeutic development. *Eupatorium triplinerve* also recognized as *Ayapana triplinervis*, is a perennial herb predominantly found in Asia (Unnikrishnan *et al.*, 2014) [38].

The objective of the present research is to employ the Polymerase Chain Reaction (PCR) technique to identify the causative agent of Running Mortality Syndrome (RMS), examine the histopathological changes, and assess the potential properties of the eco-friendly antimicrobial agent *Ayapana triplinervis.*

2. Materials and Methods

2.1 Sample collection

Water and shrimp samples were collected from 48 farms located in Purba Medinipur district (Fig.1) of West Bengal, India. Samples were collected at three distinct stages; firstly, in the early stage (between 40 to 50 DOC when mortality starts); secondly, during the middle phase (during 60 to 70 DOC) and finally at the time of harvest (90 to 120 DOC). General information including stocking density, pond area, source water and health condition of shrimp was obtained from all the farms. Additional information regarding mortality, as well as patterns and progress in production were collected from the affected ponds.

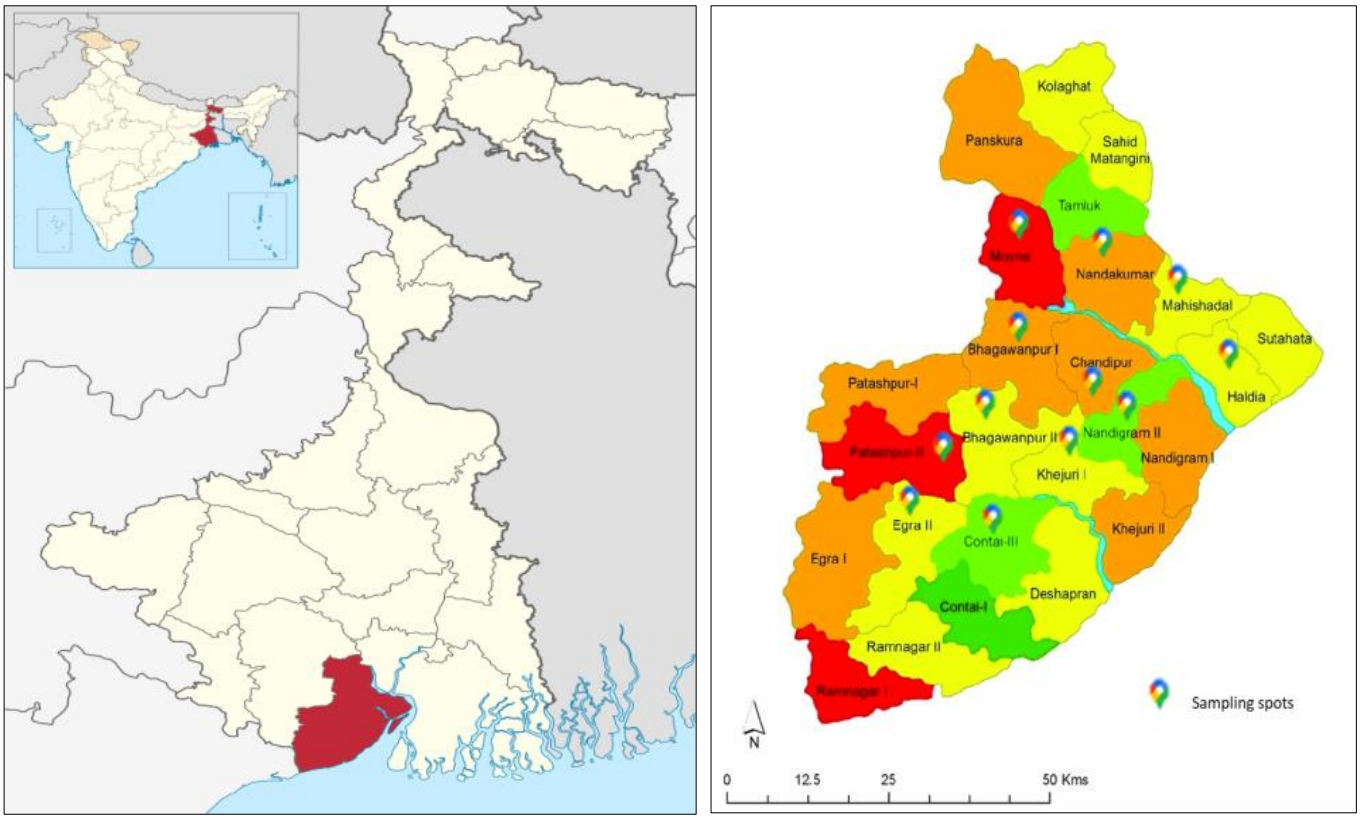

Fig 1: Sampling area

2.2 Bacteriological analysis

In the laboratory, inoculums were collected from affected external and internal parts of morbid shrimp, such as the hepatopancreas, gills, and intestine. This inoculum was then incubated in Alkaline Peptone Water (APW). Following this, the samples were streaked onto Thiosulfate–citrate–bile salt (TCBS) agar plates and further incubated. Single colony was picked up and purified by repeated streaking on TSA slants. A series of biochemical reactions as described by Bergey's manual, Holt *et al.* (1994) [13]; BAM method, Kaysner *et al.* (2004) [15] were performed to identify the target bacteria up to species level.

2.3 Bacterial DNA extraction and PCR amplification of 16S rDNA gene and sequencing

The genomic DNA of bacterial isolates was extracted by using genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. The 16S rDNA gene was amplified through PCR reaction that was performed in a Master cycler Pro S system (Eppendorf, Germany). The universal primers (forward primer 8F and reverse primer 1492R) were used. The PCR amplified products were sequenced at the Genomics Division, Barcode Biosciences Pvt. Ltd, Bangalore, India.

2.4 Histopathology

Live and moribund shrimps were collected and fixed immediately by injecting Davidson's fixative into the hepatopancreas and muscle region. The hepatopancreas and muscle region were dissected and processed for histology described by Roberts (2012) [29]. The haematoxyline and eosin stained tissue sections were observed under a microscope (Olympus, Japan, Model: BX51) and photographed.

2.5 Analysis of water parameters

The water samples were collected using 100 ml sterile polypropylene containers. The different water testing kits were used for measuring pH, salinity, total hardness, total alkalinity, total ammonia and nitrite.

2.6 Preparation of *Ayapana triplinervis* powder and extracts

Dried leaves of Ayapana were collected from the Aquatic Animal Health department, Faculty of Fishery Science, WB. The leaves were then dried in a hot air oven at 40 °C for 48 hours. The dried leaves were ground to fine powder and sieved (mesh size 0.9 mm). The sieved samples were then soaked in ethyl acetate with continuous shaking at 200 rpm at 30 °C for two days. The extracts were then filtered twice

using Whatman no. 1 filter paper and stored in -20 °C until further use.

2.7 Antimicrobial assay of *Ayapana triplinervis* leaf extract using Kirby-Bauer method

Vibrio parahaemolyticus was screened for its sensitivity to ethyl acetate extract of *Ayapana triplinervis* by agar disc diffusion technique using Kirby-Bauer method in triplicates (Bauer 1966; CLSI 2006) [4, 9]. A pure culture of the bacterium (20 h old) from TSA slant was inoculated into nutrient broth and incubated for 18 h at 37 °C. 0.1 ml from this 18 h grown culture was taken and spread using sterile cotton swab onto Mueller–Hinton agar plates. The sterile disc impregnated with 50 µl of 100 mg/ml, 50 mg/ml, 25 mg/ml of leaf extract, OTC (30 mg) disc and a control disc with 50 µl ethyl acetate were placed aseptically onto the inoculated agar plates at least 15 mm away from the edge, at equal distance from each other to avoid overlapping of the zone of inhibition. Following this, the plates were then incubated for 24 h at 37 °C and the diameters of the zone of inhibition (mm) were measured.

2.8 Statistical analysis

The data on season wise prevalence of different *Vibrio sp.* were analysed by two-way ANOVA. They confirmed the significance of difference by Tukey post-hoc test for comparison of means. One way ANOVA analysis was done with the concentration of *Ayapana triplinervis* extraction (mg/disc) and inhibition zone. The water quality parameter were analysed by correlation test. All the statistical analyses were done using Statistical Package Tools for Social Sciences (IBM-SPSS), version: 22.0.

3. Results and Discussion

3.1 Seasonal occurrence of RMS Disease

The study area included Purba Medinipur district in West Bengal (Fig.1), a major shrimp farming hubs and that reports frequent occurrence of RMS. Out of the 48 shrimp farms, 18 were healthy while the remaining farms were affected with running mortality syndrome (RMS) from October 2022 to September 2023. In farms affected by running mortality syndrome (RMS), shrimp mortalities started as early as 40 DOC and continued up to 120 DOC. The stocking density in ponds that were in a healthy condition ranged between 45 ± 5 m², while in ponds affected by running mortality syndrome (RMS), the stocking density ranged from 66 ± 4 m². The mortality rates steadily increase with the progression of the culture period (Fig.2.) and increase in stocking density. These findings are quite similar with the findings of Kumar and Ramulu (2013) [17], who observed that environmental stress factors like elevated water temperatures, pH fluctuations, low dissolved oxygen levels, overcrowding, heavy parasite infestation, excessive organic matter, spawning activity, rough handling, and transportation led to disease outbreaks.

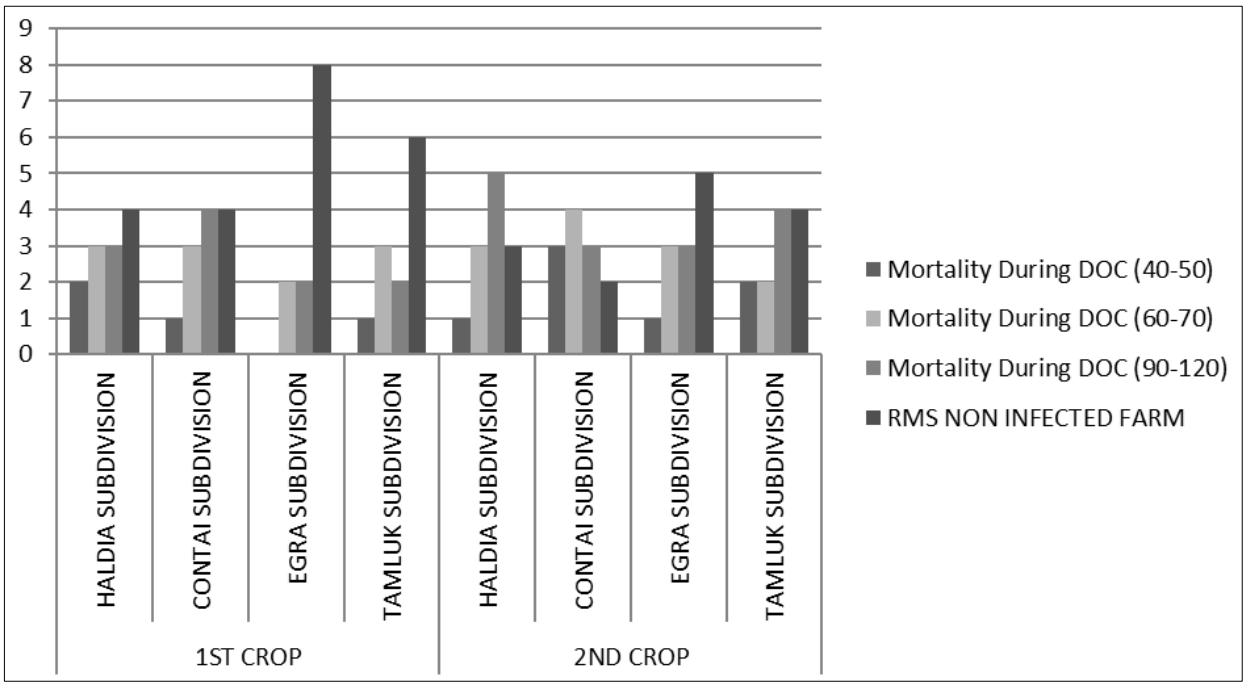

Fig 2: Mortality levels during different DOC of RMS infected *Penaeus vannamei.*

3.2 Clinical signs

Shrimp affected by RMS commonly display indications of total loss of appetite, leading them to refuse feed consumption. Additionally, they often exhibit difficulties in maintaining their usual balance. Infected shrimp typically manifest a reddish coloration in the hepatopancreas and a distinctive red discoloration of the body (Fig.3) before discontinuing feeding, ultimately leading to mortality, which are consistent with the findings of Rao and Satyanarayana (2020) [28].

Fig 3: Distinctive red discoloration of the body of RMS infected *Penaeus vannamei*

3.3 Bacteriological analysis

Vibrio parahaemolyticus, *Vibrio alginolyticus* and *Vibrio proteolyticus* were isolated from hepatopancreas of infected shrimp samples by biochemical test. Predominant *Vibrio sp.* isolates phenotypically confirmed as *Vibrio parahaemolyticus* (OR941081) (Fig.5.), was further confirmed by the 16s rRNA gene sequencing. The present study showed that shrimps in the wet season were more contaminated with different *Vibrio sp.* than those of the dry season. The statistical analysis conducted through two-way ANOVA (Table 1) revealed that there were no significant differences (p>0.05, df=3) concerning various seasons. However, there were significant differences (p<0.05, df=2) observed in the prevalence of different *Vibrio sp.* According to Alavandi *et al.* (2019) [2], the bacterial study of haemolymph and hepatopancreas from shrimp affected by RMS showed that particularly *Vibrio parahaemolyticus* and *Vibrio azureus* were predominant. The study also revealed that the mortality percentage gradually increases as the days of culture progresses. Notably, *Vibrio parahaemolyticus* was found to be dominant in RMS-infected cultured *P. vannamei* with higher mortality rate during 90-120 DOC. The overall seasonal variation pattern of *Vibrio parahaemolyticus* (OR941081), as observed by Bughe *et al.* (2016) [5], closely relates with the findings of the current study, highlighting increased contamination in shrimps during the wet season compared to the dry season(Fig.4.). This result contradicts with the findings of Xu *et al.* (2016) [40], where higher occurrence percentage of *Vibrio parahaemolyticus* was recorded in aquatic products during the summer (50%) then winter (22.7%). Zarei *et al.* (2012) [41] investigated the seasonal prevalence of Vibrio species in shrimps. They emphasized the highest occurrence of *Vibrio parahaemolyticus* during the summer season, attributing the rapid multiplication of its cells in ambient temperature. Sabir *et al.* (2011) [30] recorded seasonal variations and spatial distribution of *Vibrio alginolyticus* in the marine environment, noting the highest concentration during the warm season and minimal levels during the cold season which also support present study (Fig.4).

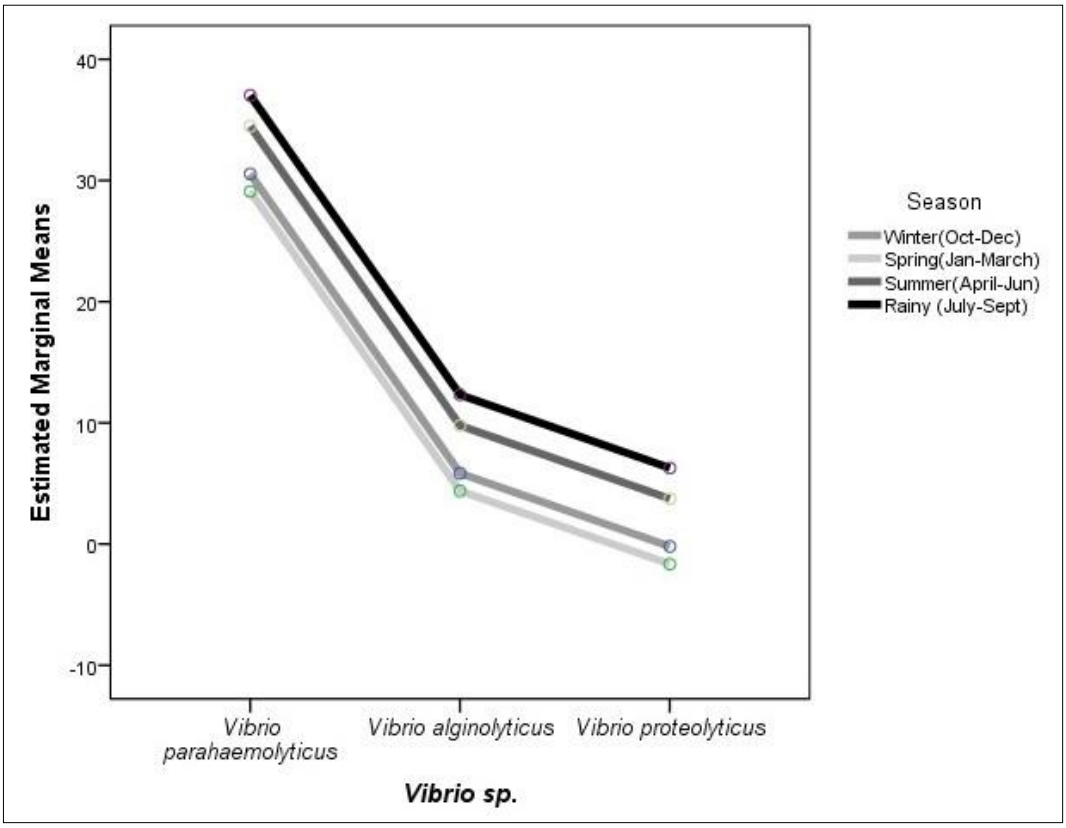

Fig 4: Estimated Marginal Means of prevalence of *Vibrio sp.* across four seasons

Table 1: Two way ANOVA to assess the prevalence of Vibrio sp. in relation with different season.

Source	Type II Sum of Squares	df	Mean Square	F	Sig.
Vibrio sp.	2121.905	2	1060.952	48.060	.000
Season	118.374	3	39.458	1.787	.249
Error	132.452	6	22.075		
Corrected Total	2372.731	11			

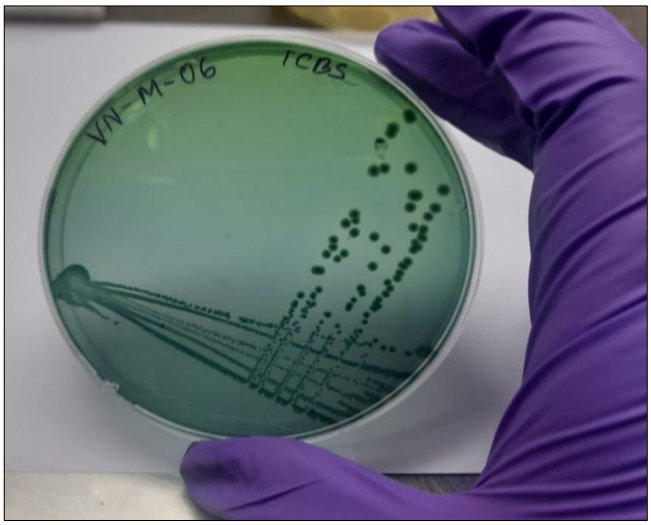

Fig 5: *Vibrio parahaemolyticus* green colony in TCBS Agar plate

3.4 Water quality of healthy and RMS affected farms

Among the pond water quality parameters examined, the RMS-affected ponds displayed notable elevations in salinity, nitrite and Ammonia levels. A correlation analysis was performed between the different water quality parameters of RMS infected pond in various seasons viz., Winter (Oct-Dec), Spring (Jan-March), Summer (April-Jun) and Rainy (July-Sept). A significant positive correlation was obtained in Nitrite and Ammonia (Correlation is significant at the 0.01 level) during different seasons (Table 2). Other water parameters are non-significant during different seasons. As demonstrated by Mastan and Ahmed (2013) [22], the outbreak of bacterial diseases in aquatic animals is predisposed by factors such as water quality, elevated organic load, contaminated feed, and unhygienic conditions. Furthermore, Chamberlain (1997) [7] highlighted that mortality in shrimp aquaculture is significantly influenced by viral and bacterial diseases, coupled with unfavourable physicochemical conditions. Li and Chen (2008) [18], examined the impact of pH stress on diminishing the resistance of white shrimp (*P. vannamei*) to *Vibrio sp*. and impeding their immune response. Tedengren *et al.* (1988) [37] noted that variations in salinity lead to physiological stress, diminishing the tolerance to environmental stressors. Takashashi *et al.* (1995) [35], reported that variations in water quality parameters like pH, salinity, temperature, and hardness may increase the vulnerability of shrimp to pathogens. In the present investigation, ponds affected by RMS exhibited increased nitrite and ammonia levels, a pattern that closely aligns with the findings reported by Alavandi *et al.* (2019) [2]. Cheng and Chen (2000) [8], thoroughly documented the influence of ammonia on the physiological response and immune resistance of shrimp and other decapods. It was widely recognized that unionized ammonia, even at low concentrations, has the potential to damage cells by permeating membranes, especially at elevated pH levels, as emphasized by Lo and Kou (1998) [20].

Table 2: Correlation of RMS Infection of *P. vannamei* and different water quality parameters in various seasons during October 2022 to September 2023

	RMS Infection	pH	Salinity	Nitrite	Ammonia	Total hardness	Total Alkalinity
RMS Infection	1						
pH	-.351	1					
Salinity	-.143	.447*	1				
Nitrite	-.919**	.487*	.398	1			
Ammonia	-.785**	.647**	.379	.891**	1		
Total hardness	-.219	.412*	.373	.240	.130	1	
Total Alkalinity	.050	.113	-.776**	-.191	-.081	-.315	1

**. Correlation is significant at the 0.01 level (2-tailed), *. Correlation is significant at the 0.05 level (2-tailed).

3.5 Histopathological studies

Pathology of hepatopancreas of RMS affected shrimp from most farms showing vacuolation with sloughing of B cell (Fig.6), karyomegaly, increased inter hepatopancreatic tubular space (Fig.7), were recorded in 69% cases. Necrosis associated with haemocytic infiltration (Fig.8) and oedemas (Fig.9) of muscles were identified in samples from 57% of the farms surveyed. The hepatopancreas plays a vital role related to the immune response and to the heat stress (Sun *et al.*, 2014) [34]. Infection with *V. parahaemolyticus* can lead to damage in the hepatopancreas organ (Khimmakthong and Sukkarun, 2017) [16]. The pathology of the hepatopancreas in cases of oral/enteric vibriosis showed severe necrosis, structural loss, tubule epithelial cell atrophy, vacuolation, and the rounding and sloughing of cells into the lumen (Ambipillai *et al.*, 2003) [3]. Raja *et al.* (2017) [27] observed that *Vibrio parahaemolyticus*-infected shrimp displayed histopathological alterations, including disrupted hepatopancreatic tubules with diffuse interstitial oedema, karyomegaly featuring prominent nucleoli, rounding and shedding of hepatopancreatic tubular epithelium, bacterial colonies, and the presence of apoptotic bodies. The findings also encompassed tubule regeneration, cystic, dilated, and vacuolated appearances of hepatopancreas tubules, hypoplastic changes in the tubules with the absence of B, R, and F cells, granuloma formation, concretions in tubules, calcification, and necrosis. Alavandi *et al.* (2019) [2], RMS infected shrimps showed haemocytic infiltration in abdominal muscle. Soto -Rodriguez *et al.* (2006) [32] noted that experimental infection with *V. harveyi* in *P. vannamei* resulted in observable haemocytic infiltration, inflammation and melanisation in the skeletal muscles. As studied by The muscle necrosis observed in *V. harveyi* infected shrimp were similar with the muscle effected by viral infections, such as Penaeus vannamei nodavirus (PvNV) (Tang *et al.*, 2007) [36], and Infectious Myonecrosis Virus (IMNV) (Poulos *et al.*, 2006) [24]. In the current study, the histopathological changes in both the hepatopancreas and muscle were consensus with findings from previous studies.

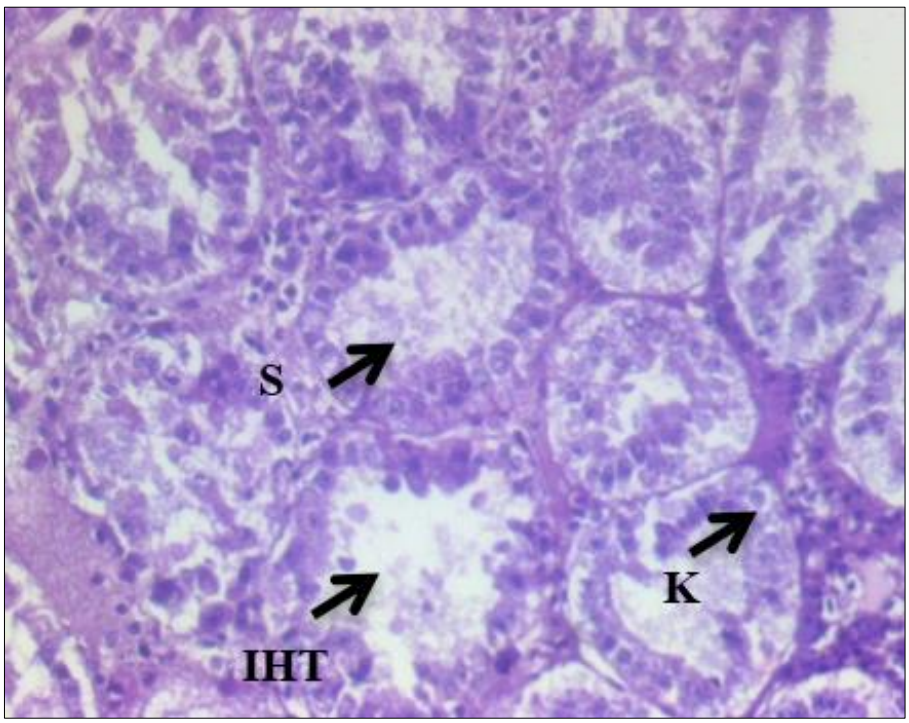

Fig 7: Hepatopancreaus of RMS infected *Penaeus vannamei* showed karyomegaly (K) and increased inter hepatopancreatic tubular space (IHT).

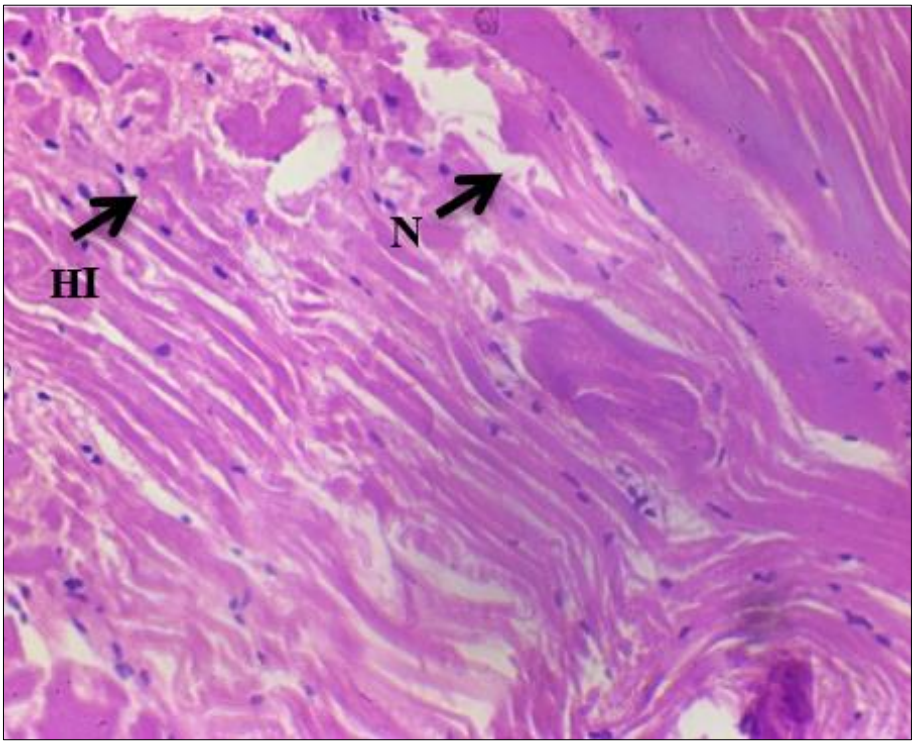

Fig 8: Muscle of RMS infected *Penaeus vannamei* showing necrosis (N) and haemocytic infiltration (HI)

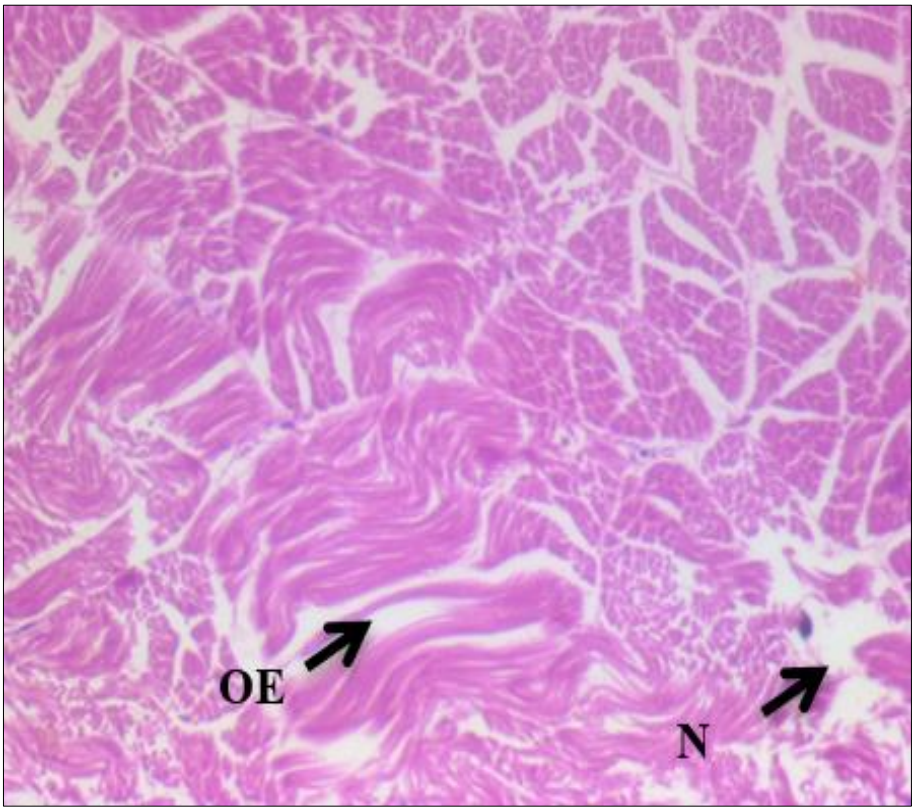

Fig 9: Muscle of RMS infected *Penaeus vannamei* showing oedema (OE) and necrosis (N)

3.6 Antimicrobial affectivity test of *Ayapana triplinervis* by disc diffusion test

Vibrio parahaemoliticus were screened for their sensitivity to *Ayapana triplinervis* ethyl acetate extract (ALE). Statistical analysis (Table 3) by one way ANOVA revealed that there were significant differences ($p<0.05$, df=3) in the concentration of the active component (mg/disc) and inhibition zone. A cellulose disc (6 mm) containing 50 mg/ml ayapana leaf extract (ALE) gave 8 mm inhibition zone. The disc containing 100 mg/ml ayapana leaf extract (ALE) gave 14.75 ± 0.95 mm inhibition zone in comparison to a 30 mg OTC disc which gave 27.25 ± 0.95 mm inhibition zone (Fig.11). Disc containing 25mg/ml ayapana leaf extract (ALE) and the control disc soaked in only ethyl acetate extract gave no inhibition zone (Fig. 11). Present study (Fig.10) is supported by Sobrinho *et al.* (2017) [31], who demonstrated the antibacterial properties of *Ayapana triplinervi* against a range of Gram-positive and Gram-

negative bacterial strains. These included *Bacillus megaterium, B. subtilis, B. cereus, S. aureus, E. coli, S. dysenteriae, S. sonnei, S. typhi, S. paratyphi,* and *P. aeruginosa*. Significantly, Rahman and Junaid (2008) [26] noted that 22 mm inhibitory zone was observed against *Vibrio sp*. when employing a 1000 µg/disc chloroform extract of *Ayapana triplinervis*. Ether extract of the *Ayapana triplinervis* demonstrated the inhibition of bacterial growth, affecting strains such as *B. subtilis, Staphylococcus epidermidis, S. aureus, M. luteus, E. coli, P. aeruginosa, Salmonella typhi, Shigella species, Vibrio cholerae,* and *Vibrio parahaemolyticus*, as reported by Gupta *et al.* (2002) [11].

Table 3: One way ANOVA with in Concentration of the active component (mg/disc) and inhibition zone

Inhibition Zone	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1300.700	4	325.175	591.227	.000
Within Groups	8.250	15	.550		
Total	1308.950	19			

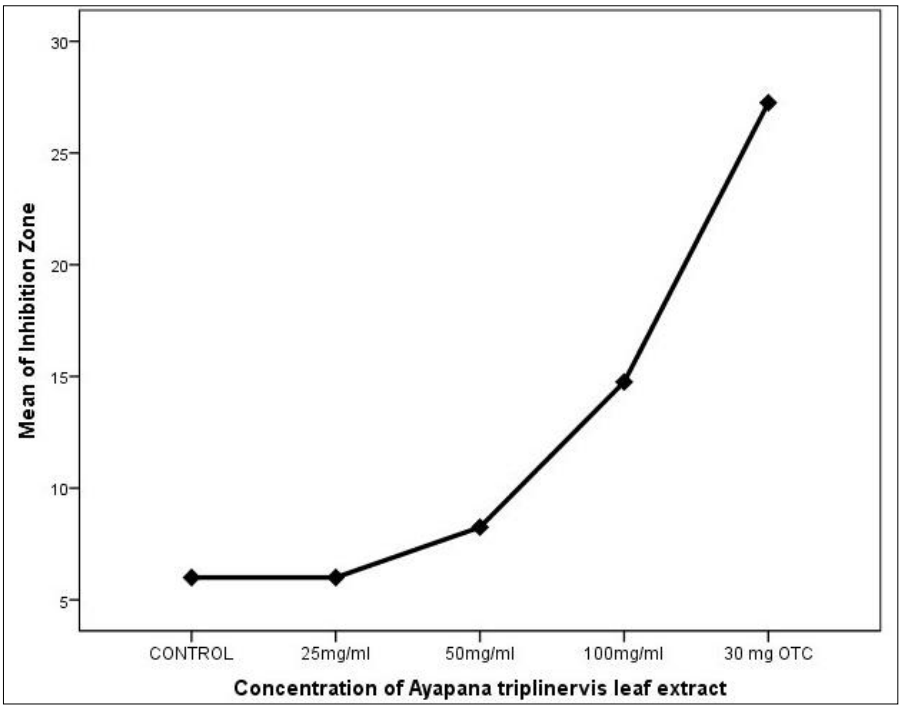

Fig 10: Graphical representation of mean zone of inhibition of *Vibrio parahaemolyticus* using *A. triplinervis* leaf extract and OTC

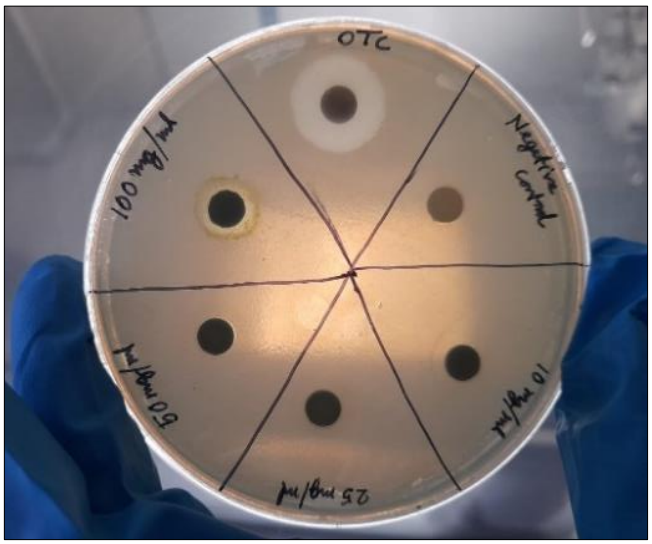

Fig 11: Zone of inhibition of *Ayapana triplinervis* ethyl acetate extract against *Vibrio parahaemolyticus* by disc diffusion assay

3.7 Management of Disease

The disease can be managed by reducing stocking density with partial harvesting process, as evident from the farm. Reduction in feed quantity also helps to reduce water pollution which is supportive for recovery from the RMS disease. Approximately, 8% of farmers are able to recover their shrimp production by maintaining good management practice and partial harvest. According to the shrimp farmers, turmeric powder, garlic powder, Potassium mono per sulphate (53%), Iodine and Benzalkonium chloride (BKC) are mostly used to control RMS disease. Currently, commercially available probiotic products in the market, containing *Lactobacillus* sp., *Bacillus* sp., *Carnobacterium* sp., *Enterococcus* sp., and yeast, specifically *Saccharomyces cerevisiae* have a positive response in controlling bacterial disease in shrimp culture. Specific pathogen free (SPF) juveniles of *P. vannamei* with limited water exchange under biosecure conditions will minimize the disease occurrence probability. The improvement guidelines for limited water exchange production systems employing phytoplankton for assimilation of dissolved nitrogen to maintain a suitable C: N ratio to facilitate nitrification. Rao and Satyanarayana (2020) [28] reported elevated B cells in the hepatopancreas in the early stage of during the initial disease stages, indicate suggesting over feeding. Overfeeding leads to water quality deterioration, inducing stress in the cultured organisms with poor immune response. Frequently, inadequate pond management practices can lead to beyond the optimal levels irrespective of stocking density. According to Joseph *et al.* (2001) [14] the accumulation of organic matter, including unused feed and faecal material, contributes to increase in levels of nutrients and metabolites (such as ammonia, nitrate, nitrite, and sulfide) in high stocking shrimp ponds. Cohen *et al.* (2005) [10] recommended that the use of viral-pathogen-free PL along

with effective water preparation and management practices as a suitable approach to prevent outbreaks of viral and bacterial pathogen diseases.

4. Conclusion

In conclusion, this study aimed to investigate the underlying factors contributing to running mortality syndrome (RMS) in shrimp. Data collected from various locations in the Purba Medinipur District of West Bengal between October 2022 and September 2023 revealed that the persistent mortality of shrimp due to RMS is influenced by a combination of crucial environmental factors and stocking density, leading to pathogenic attacks, particularly by *Vibrio parahaemolyticus*. Our findings suggest that RMS may be a syndrome associated with aquaculture systems, where shrimp mortality occurs when key system factors deviate from their optimal levels. This deviation is likely attributed to sub-optimal management practices, such as inadequate pond preparation and insufficient intervals for pond drying between crops. To address the issue without resorting to antibiotic use, we explored the use of *Ayapana triplinervis* to control *Vibrio parahaemolyticus*, the presumed causative agent of RMS, with promising results observed in in-vitro studies. Despite these positive outcomes, further investigation is necessary to gain a comprehensive understanding of this phenomenon and to validate our findings.

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