

A surveillance study on motile *Aeromonas Septicemia* (MAS) affecting cultured *Litopenaeus vannamei* shrimp from Suez Governorate, Egypt

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ABSTRACT

This study aimed to investigate the prevalence of *Aeromonas* species among 150 cultured shrimp (*Litopenaeus vannamei*) collected from commercial farms in the Suez Governorate, Egypt. Shrimp samples were subjected to bacteriological, molecular and histopathological examinations. Our results revealed that isolated bacteria were identified as *Aeromonas hydrophila*, based on morphological and phenotypic characteristics using traditional biochemical tests and commercial API20E kits. The results were genetically confirmed by conventional PCR assay using a specific set of primers targeting the Hemolysin gene. PCR yielded amplicons with a size of 130 bp which were the characteristics of *A. hydrophila*. The total prevalence of *A. hydrophila* among infected shrimp was 36.67%, whereas the lowest seasonal prevalence of infection was recorded in spring (26%), followed by summer (40%) and autumn (44%). The gross lesions of infected shrimp revealed black cuticular lesions, broken rostrum, rotten gills, and hepatopancreas discoloration. The histopathological examination of the infected hepatopancreas revealed cellular degeneration, sloughing of cells and necrosis, while the infected gills showed sloughing and atrophy of the branchial lamellae along with cell proliferation. The muscle also showed broken muscle fibers and haemocyte infiltrations. It was concluded that these bacterial strains badly infected the cultured shrimp. Thus, serious steps should be followed to avoid the outbreaks by using immunostimulants (herbal additives, prebiotics and probiotics) from the onset of the farming period.

INTRODUCTION

Shrimp are highly-priced seafood typically caught in warm, tropical, and coastal seas. Shrimp support fisheries that are commercially profitable in various parts of the world (Ajani *et al.*, 2013). Shrimp forms one of the most popular and widely traded types of seafood in the world (Sun *et al.*, 2013). *Litopenaeus vannamei*, often known as the Pacific white-leg shrimp, is a crucial species in the aquaculture sector and is commonly raised in subtropical to tropical areas. This species can be raised inside (in both tanks or recirculating aquaculture systems) or outdoors (in ponds) and grows quickly; it has great survival rates even at high densities, a wide

tolerance range for salinity and temperature, and can be fed a variety of foods (Cuzon *et al.*, 2004). Disease outbreaks caused by harmful bacteria and viruses are causing significant commercial losses in the shrimp farming sector (Thitamadee *et al.*, 2016). Bacterial diseases have become a principle economic problem in the farming of the Pacific white shrimp, *L. vannamei*, affecting survival and growth (Toranzo *et al.*, 2005; Cao *et al.*, 2014). Bacterial diseases caused a annual estimate of 20% production loss in the shrimp industry (Hong *et al.*, 2016). Cuerrently, the world market needs healthy aquaculture products from farm to table (Chinabut & Puttinaowarat, 2005), which leads us to face such problems threatening this industry. Gram-negative, widespread *Aeromonas* species are frequently found in freshwater and estuarine habitats. These species cause a significant foodborne bacterial zoonotic disease in aquaculture and are native to aquatic settings all over the world (Janda & Abbott, 2010; Algammal *et al.*, 2020; Samayanpaulraj *et al.*, 2020). The three pathogens producing black disease are *Aeromonas hydrophila*, *A. sobria*, and *A. caviae*; when these bacteria infected thoracopods with the black disease, it resulted in generalized septicemia and irregular swimming. Death happened 24-48 h after the onset of black nodules (Saejung *et al.*, 2011) due to the production of a variety of virulence factors, especially toxins responsible for gastrointestinal infections including hemolysin (Castro-Escarpulli *et al.*, 2003). Thus, for the detection of virulence factors, hemolysin (*hlyA*) and aerolysin (*aerA*) genes are the most effective way for detecting *Aeromonas* genus (Yousr *et al.*, 2007). The *aerA* and *hlyA* genes in charge of producing the aerolysin and hemolysin toxins in this genus were assayed for using the polymerase chain reaction (PCR) technique in the genomes of the *Aeromonas* spp., isolated from ambient and seafood sources (Niamah, 2021). Consequently, this study aimed to isolate and identify *Aeromonas* species from cultured white leg shrimp (*L. vannamei*), using traditional standard ways of API 20E, which are not reliable tools for phenotypic characterization of *A. hydrophila* to the species level. Due to interspecies homogeneity and phenotypic similarity, researchers in the current study used a modern technology (PCR) for confirmation, recording the clinical and postmortem lesions on the affected shrimp and studying the histopathological pictures of infected shrimp organs.

MATERIALS AND METHODS

1. Samples collection

A total of freshly 150 cultured white-leg shrimp (*L. vannamei*) individuals, with an average body weight of (7.39 ± 2.27) g and length of (10.7 ± 3.2) cm were randomly collected from commercial shrimp farms in the Suez Governorate from March 2020 to November 2020. Alive samples were then transferred in tanks supplied with an air-blower to the Fish Diseases Laboratory, the National Institute of Oceanography and Fisheries, Suez and Aqaba Gulfs' branch for further examinations. Clinical and postmortem investigations for detecting abnormalities were performed according to the method of Austin and Austin (2007).

2. Bacteriological examination

Isolation of *Aeromonas* was aseptically achieved from shrimp samples, which were thoroughly mixed with sterile tryptone soy broth (Hi media) for it was so difficult to separate each organ individually; then, the homogenates were incubated at 27°C for 24h. A loopful from each enriched homogenate was streaked onto the dried surfaces of duplicate plates of *Aeromonas* selective medium base supplemented with ampicillin (Oxoid) for selective isolation of *A. hydrophila*. The plates were then incubated at 27°C for 24h. Presumptive *A. hydrophila* colonies (opaque green colonies with dark centers) were picked up, purified onto nutrient agar (Hi media) and subjected to a series of confirmatory biochemical tests according to **Palumbo et al. (2001)**. Each single presumptive *A. hydrophila* colony was subjected to microscopic, biochemical analysis using API 20E strips (BioMerieux, France) and molecular identification.

3. Molecular identification of *Aeromonas* species

Genomic DNA was extracted according to **Devi et al. (2009)** using the boiling method. PCR amplification for specific virulence gene (Hemolysin gene) using specific primers AH-F 5'GCCGAGCGCCCAGAAGGTGAGTT'3 as forwarding primer and AH-R 5'GAGCGGCTGGATGCGGTTGT'3as reverse primer as discussed in the study of **Olewi (2013)**. The PCR reaction was run in a PTC-100 Peltier thermal cycler (Techne, England). A typical mixture of 5µl of the extracted DNA was prepared, with the addition of 12.5µl of PCR master mix, and 1µl of forwarding and reverse primers in a volume of 25µl. The cycle conditions were as follows: pre-denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 30sec, annealing at 72 °C for 45sec, and extension at 72°C for 7min. To confirm the targeted PCR amplification, (8-10µl) of the amplicons was separated by 1% of agarose gel at a constant of 80 V for 30min. The amplified product was visualized as a single compact band of expected size under UV light (Edvotek series).

4. Histopathological examination

Specimens were freshly taken from affected organs and tissues of naturally infected shrimps for the histopathological test. Specimens were trimmed and fixed in 10% of phosphate-buffered formalin and then washed with running tap water for 24h. The samples were dehydrated in gradients of ascending alcohol concentrations, and cleared with xylol and embedded in paraffin wax to support the tissue for thin sectioning (5-micron thickness). Sections were stained with hematoxylin and eosin (H&E) stains, and then microscopically examined by Leica Icc50 HD microscope according to **Roberts (2001)**.

RESULTS

1. Clinical and postmortem examination

Clinical examination of infected white leg shrimp, *L. vannamei* recorded blackish patches and broken rostrum (Fig. 1A), rotten gills and hepatopaneas discoloration (Fig. 1B), paleness of hepatopaneas and whitish musculature (Fig. 1C), reddish tail, whitish hepatopaneas (Fig.

1D), whitish hepatopancreas, white patches on the body (Fig. 1E), blackish coloration of the whole body (Fig. 1F).

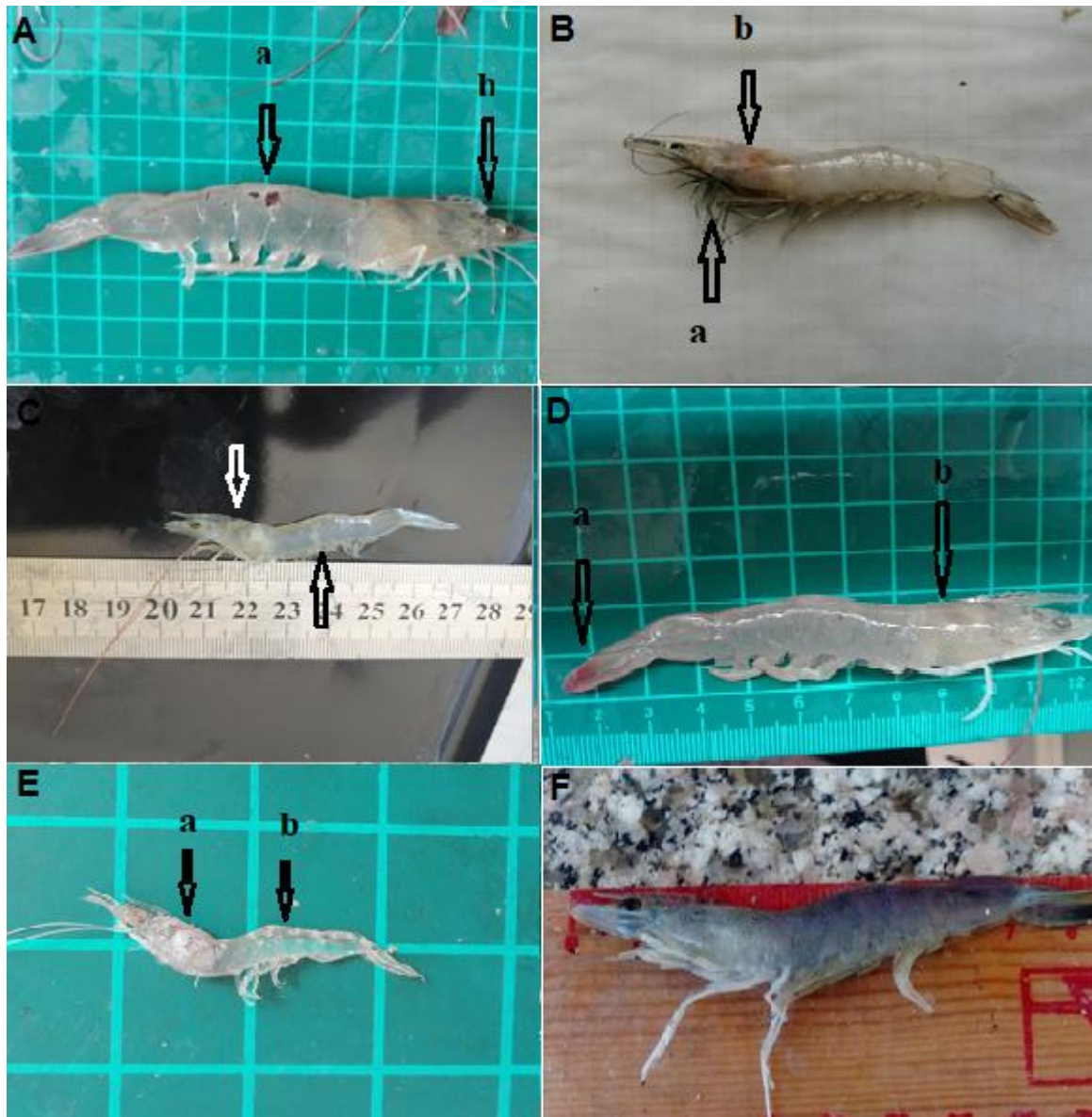


Fig. 1. Infected *L. vannamei* showing (A) Blackish patches (a) and broken rostrum (b); (B) Rotten gills (a) and hepatopancreas discoloration (b); (C) Paleness of hepatopancreas and whitish musculature; (D) Reddish tail (a), whitish hepatopancreas (b); (E) Whitish hepatopancreas (a), white patches on the body (b), and (F) Blackish coloration of the whole body.

2. Bacteriological examination

As shown in Table (1), all suspected isolates of *A. hydrophila* are Gram -ve, short rod, scattered and actively motile. They gave dark green, opaque with dark centers on *Aeromonas* selective medium base. The results of the conventional biochemical tests and commercial

API20E system revealed that all isolates were positive for ONPG, ADH, H₂S, TDA, IND, GEL, GLU, MAN, SAC, oxidase and catalase tests, while they were negative for INO, SOR and MEL tests. Some isolates showed variable reaction (+/-) to LDC, ODC, CIT, URE, VP, RHA, AMY, and ARA tests. The code numbers on API20E strips were **7477135** & **3766126**, which confirmed that the isolates belonged to *A. hydrophila*.

Table 1. Phenotypic and biochemical characteristics of *A. hydrophila* recovered from infected *L. vannamei*

Test	<i>A. hydrophila</i>
Gram stain reaction	-ve
Shape	Short rod, Scattered
Motility	Actively motile
Growth on <i>Aeromonas</i> isolation medium.	Dark-green, opaque with dark centers
Growth at:	
-25-27°C	+
-37°C	+
-40°C	-
Growth on tryptone soy broth with:	
-0% NaCl	+
-3% NaCl	+
-8% NaCl	+
B-Galactosidase production (ONPG)	+
Arginine dihydrolase production (ADH) +	
Lysine decarboxylase production (LDC)	+/-
Ornithine decarboxylase production (ODC)	+/-
Citrate utilization(CIT)	+/-
H ₂ S production (H ₂ S)	+
Urease production (URE)	+/-
Tryptophane deaminase production (TDA)	+
Indole production (IND)	+
Acetoin production (VP)	+/-
Gelatinase production (GEL)	+
acid from:	
glucose (GLU)	+
mannitol(MAN)	+
insitol(INO)	-
Sorbitol (SOR)	-
Rhaminase (RHA)	+/-
sucrose (SAC)	+
melibiose (MEL)	-
amygdalin (AMY)	+/-
arabinase (ARA)	+/-
Cytochrome oxidase (OX)	+
Catalase	+

3. Molecular identification of *A. hydrophila*

Based on specific primers and the target sequence of AH primer for hemolysin gene, molecular identification of some isolated species revealed *A. hydrophila*. Gel electrophoresis revealed visible bands at 130 bp (Fig. 2).

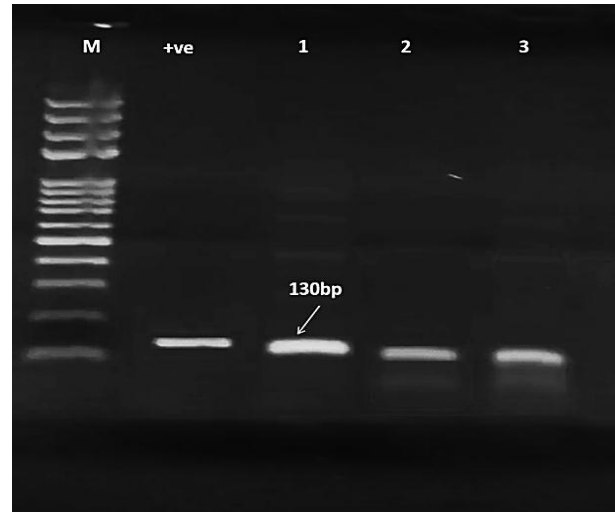


Fig. 2. Agarose gel showing the results of PCR for detection of *A. hydrophila* strain.

Lanes: M, ladder. Lane 1-3 is positive for the hemolysin gene with 130 bp PCR amplicon. +ve: positive control.

Total and seasonal prevalence of *A. hydrophila* among cultured *L. vannamei*

The total prevalence of *A. hydrophila* among examined cultured *L. vannamei* was 36.67 %. The seasonal prevalence was 26 % in spring and increased in summer to record 40 %, while it reached its highest in autumn with 44 %.

4. Histopathological examination

The histopathological examination for hepatopancreas of infected *L. vannamei* shrimp revealed cellular degeneration with connective tissue detachment, necrosis, distended abnormal Lumen, vacuoles and flattened hyperchromatic cells with sloughing of hepatopancreas cells (Fig. 3), while others showed sloughing hepatopancreas cells, melanisation of cells, and loss of tubules (Fig. 4). Whereas, the gills revealed sloughed off, atrophy of the haemolymphatic lacuna of branchial lamellae and cell proliferation (Fig. 5). The musculature of infected shrimp revealed broken muscle with haemocytes infiltration (**Fig. 6**). Finally, the subcuticular gut epithelium showed haemocyte plug, eosinophilic inclusion bodies, necrotic inflammation and haemocytic infiltration (Fig. 7).

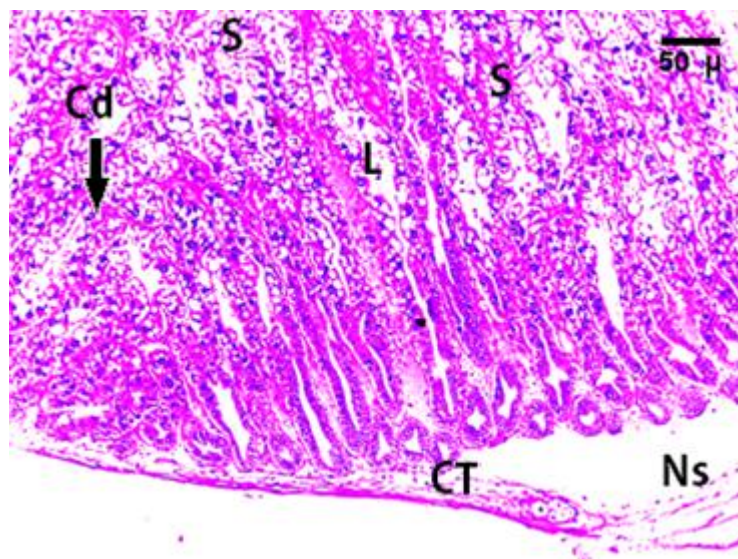


Fig. 3. Infected *L. vannamei* hepatopancreas showing cellular degenerative globules (Cd); connective tissue detachment (CT), distended lumen and flattened hyperchromatic cells (L), sloughing hepatopancreas cells (S), and necrosis (Ns)

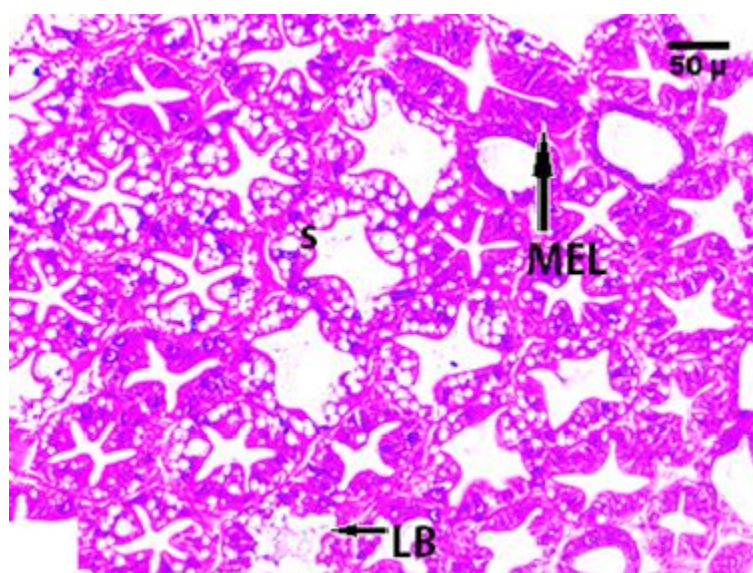


Fig. 4. Infected *L. vannamei* hepatopancreas showing sloughed cells (S), melanisation of cells (MEL), and loss of tubules (LB)

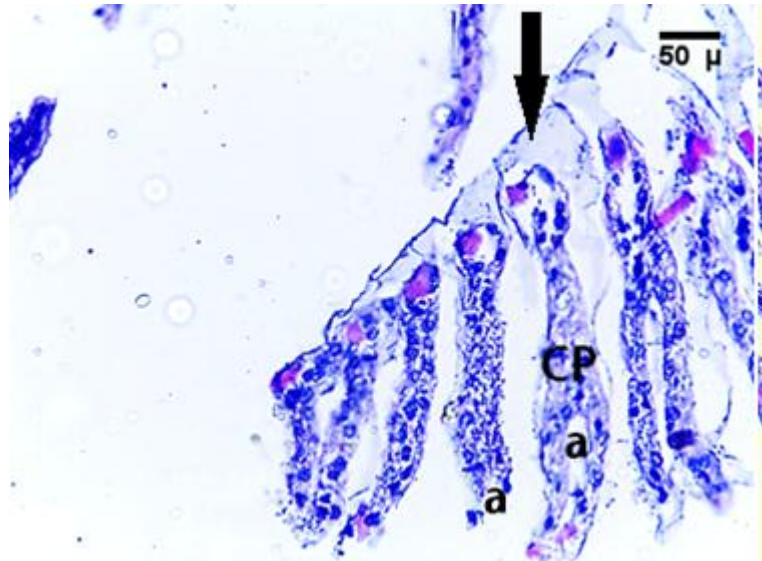


Fig. 5. Infected *L. vannamei* gills showing sloughed off (arrows), atrophy of the haemolymphatic lacuna of branchial lamellae (**a**), and cell proliferation (**CP**)

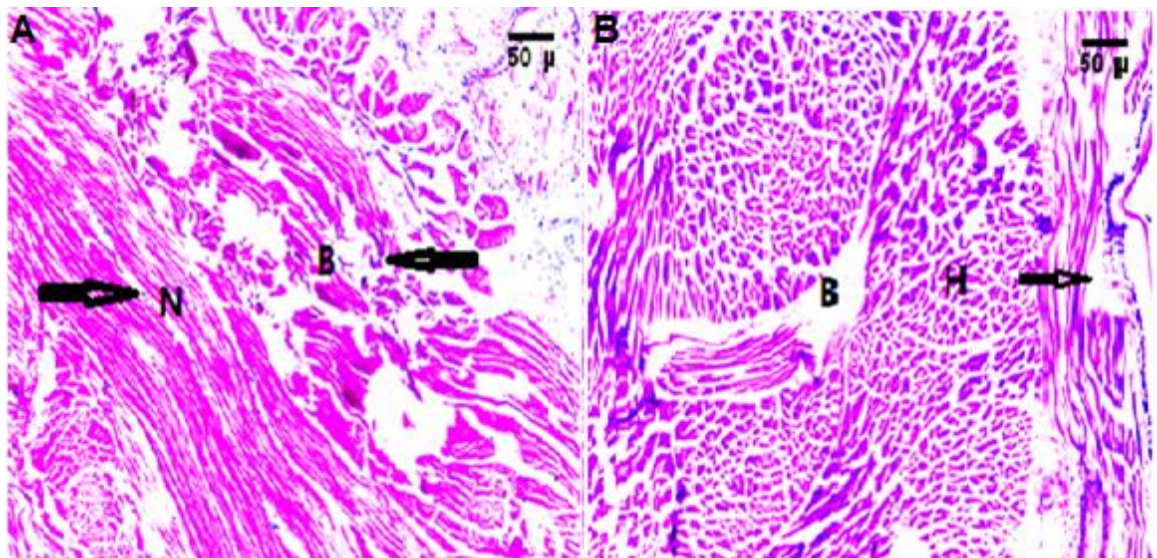


Fig. 6. Infected *L. vannamei* muscle showing broken muscle fibres (**B**), haemocytes infiltration (**H**) compared to normal tissue (N) in sub Fig. (A).

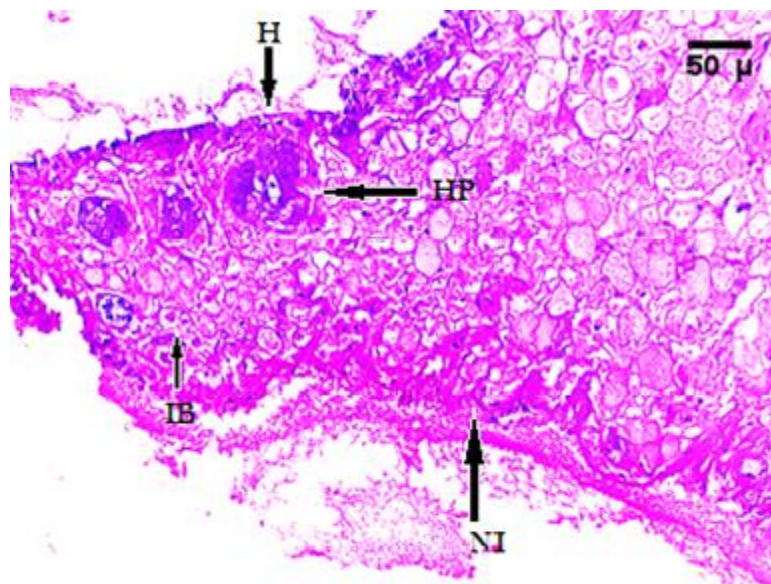


Fig. 7. Infected *L. vannamei* subcuticular gut epithelium showing haemocyte plug (**HP**), eosinophilic inclusion bodies (**IB**), necrotic inflammation (**NI**), and haemocytes infiltration (**H**)

DISCUSSION

The losses in shrimp aquaculture have been determined at approximately 60% of disease losses caused by viral pathogens and 20% by bacterial pathogens (**Flegel, 2012**). In the present study, the clinical signs and postmortem examination of infected *L. vannamei* showed black or brown cuticular lesions; this result is similar to that of **Abdolnabi et al. (2015)** who reported that, the basic clinical signs of *A. hydrophila* in *M. rosenbergii* were the presence of one to several focal melanin lesions on the outer body surface. Others showed deformity of the rostrum, melanization and darkening of appendages tips (telson), heavily darkened fouled gills, whitish opaque abdominal musculature and black colored shrimp, which agree with the findings of **Alavandi et al. (1995)**. These results may be attributed to the distribution of melanophores in the affected area, and that makes a black ring separating the healthy tissue from the affected one (**El-bouhy et al., 2006**). Additionally, other shrimps showed atrophied hepatopancreas, a finding which concurs with that of **Zhou et al. (2019)** who detected the gross signs of affected *L. vannamei* with *A. hydrophila* in the disease outbreak region in diseased shrimp showing the atrophic hepatopancreas. In other infected cases, the reddish coloration of appendages (telson) and tail may attribute to the expansion of chromatophores (**Alavandi et al., 1995**), opaque discolored hepatopancreas, the enlarged and blackish coloration of hepatopancreas, brown colored patches on cuticle and white empty gastrointestinal tract and black patches with white deposits on the carapace up to 3mm in diameter which linked to each other's to form larger plates. These different signs agree with those recorded in the work of **El Far et al. (2015)** and **Hong et al. (2016)**. They may be associated with bacterial toxins and

extracellular products (**Turska-Szewczuk *et al.*, 2012**), which may lead to opaque and whitish musculature (**Cheng *et al.*, 2016**).

Results of bacteriological examination confirmed the presence of *A. hydrophila*. Furthermore, the results of microbiological and biochemical characteristics using both traditional tests and API20E kits coincide with those of **Long *et al.* (2016)**, **Niamah (2021)** and **Zhou *et al.* (2019)**. Concerning the results of PCR, our results revealed that the virulent strains of *A. hydrophila* yielded amplification products of expected molecular size at 130 bp. These results are similar to those of **Wang *et al.* (2003)**.

For the total and seasonal prevalence among infected shrimps, the total prevalence of *Aeromonas hydrophila* was 36.67%. The seasonal prevalence of *Aeromonas hydrophila* was 26 % in spring, followed by summer (40 %) and autumn (44%). This result is comparable to **Khamesipour *et al.* (2014)** who revealed that, the total prevalence of *A. hydrophila* was 13.88% in the examined shrimp collected from shrimp farms in one province (Bushehr) along the Persian Gulf on the south coast of Iran. Moreover, findings are matched with those of **Seethalakshmi *et al.* (2006)** and **Fadel and El-Lamie (2019)** who found that, 9.41% and 20.2% of tested shrimp samples collected from Suez Governorate in Egypt and India were infected with *Aeromonas hydrophila* in summer and autumn, respectively. Additionally, **Rahimi *et al.* (2014)** showed that the summer season had the highest seasonal prevalence of *A. hydrophila* in shrimp obtained off the south coast of Iran (21.3%), whereas spring, fall, and winter had lower seasonal prevalence (10.9%, 9.6%, and 4.2%, respectively). Overall, 9.9% of all shrimp samples tested positive for *A. hydrophila*. These differences in prevalence may be attributed to different locations and/or different species. On the other hand, our results revealed that the prevalence peak was in the autumn season which disagrees with **Colakoglu *et al.* (2006)**, **Karipatas *et al.* (2009)** and **Rahimi *et al.* (2014)** results. This could be due to the increased coastal water pollution resulting from land runoff, municipal sewage outflows and stormwater surges during the monsoon season (**Vivekanandhan *et al.*, 2005**). Other studies showed that there were no apparent patterns observed in shrimp samples in the seasonality of *A. hydrophila* prevalence (**Ottaviani *et al.*, 2006**). However, our results are higher than the incidence of *A. hydrophila* which was reported by **Thayumanavan *et al.* (2003)** 37% of the seafood gathered from the four sample locations in India during the course of the year-long investigation including *A. hydrophila* contamination. The prevalence of *A. hydrophila* was about 35.6% in prawns. They noted that household sewage is the main cause of the large number of diseases that are present in the aquatic environment.

For the histopathological studies, our results revealed that the histopathological examination for hepatopancreas of infected *L. vannamei* shrimp revealed cellular degeneration with connective tissue detachment, necrosis, distended abnormal Lumen, vacules and flattened hyperchromatic cells with sloughing of hepatopancrease cells; while, others showed melanisation of cells and loss of tubules. These results agree with **Zhou *et al.* (2019)** who found that, the disrupted organization of the hepatopancreatic tubules and inflammatory cell

infiltration in the naturally infected hepatopancreas were revealed by histological abnormalities in the atrophic hepatopancreas of affected *L. vannamei* with *A. hydrophila*. However, the gills revealed sloughed off, atrophy of the haemolymphatic lacuna of branchial lamellae and cell proliferation. The musculature of infected shrimp showed broken muscle fibers with haemocytic infiltration. Finally, the subcuticular gut epithelium recorded haemocytic plug, eosinophilic inclusion bodies, necrotic inflammation and haemocytic infiltration, and this matches with the finding of **Abdolnabi et al. (2015)** who denoted that, *A. hydrophila* infection caused histological abnormalities in the gills, hepatopancreas, and heart of giant freshwater prawns raised in east Malaysia. Focused necrosis, haemoglobin infiltration, and hyperplasia were all visible in the tissue. Notably 1- 6h after injection, there was muscle injury and a minor to significant haemocyte reactivity.

CONCLUSION

Aeromonas hydrophila infection in cultured shrimp increased in the autumn season during temperature fluctuation. It could be as a result of stresses which indeed predispose to increasing infection. Moreover, PCR is the best tool for the detection of bacteria, especially highly virulent strains, which exposed the shrimp culture systems to high losses. Thus, it was deduced that pathogomonic dark colored lesions along with histopathological findings are good markers that might aid in the detection of *A. hydrophila* infection in cultured shrimp.

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