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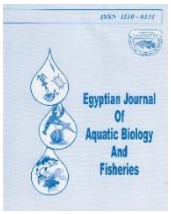

Comprehensive Assessment of Bacterial Diseases in the Shrimp: Clinical, Phenotypic, Genotypic, and Histopathological Approaches

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ABSTRACT

This study was conducted to investigate bacterial pathogens related to diseases in shrimp and their associated alterations. A total of 80 live adult Penaeid shrimps, including *Penaeus japonicus*, *Litopenaeus vannamei*, and *Penaeus semisulcatus*, with average body weight and length of 32± 3g and 14.5± 2cm, respectively, were randomly collected from fishermen in Suez Province, Egypt. The collected shrimp samples underwent comprehensive clinical, postmortem, bacteriological, and histopathological examinations. Moreover, antimicrobial profiling of the recovered bacterial isolates was conducted. PCR-based methods were also applied to shrimp tissues to identify bacterial pathogens. Clinically, affected shrimp displayed symptoms including red discoloration, soft exoskeleton, and brown to black or white spots on various body parts. Bacteriological analysis revealed the isolation and identification of *Vibrio*, *Pseudomonas*, and *Aeromonas* species, with *Vibrio* being the most prevalent (82.5%), followed by *Pseudomonas* (78.75%), and *Aeromonas* species (42.5%). The hepatopancreas was identified as the most affected organ. Antibiotic susceptibility of bacterial isolates showed high susceptibility to gentamicin, trimethoprim/sulfamethoxazole and tetracycline among the recovered bacterial isolates. Moreover, analysis of *16S r*RNA, *PA-GS*, and *Ae*r genes (species- specific genes) confirmed the presence of these species within shrimp tissues, with *Vibrio* species being the most dominant. Histopathological examination revealed degenerative changes in muscle, hepatopancreas, and uropods, accompanied by edema and hemocytic influx throughout the infected tissues. In conclusion, applying molecular techniques is the most precise and rapid method to diagnose bacterial pathogens in shrimp tissues, which is an essential step for ensuring sustainability in the shrimp industry.

INTRODUCTION

Shrimp stands as the most valuable traded seafood commodity worldwide, with a substantial increase in global aquaculture production. According to **FAO (2020)**, shrimp production has experienced significant growth, rising from 0.673 million tons in 1990 to 6.004 million tons in 2018, marking an almost tenfold increase. The growth trend continued into 2022, with production reaching a record of 3.3 million tons for shrimps and lobsters **(FAO, 2024)**. In

numerous tropical developing countries, the shrimp industry holds a prominent position as the most profitable sector within fisheries. Shrimp fisheries not only contribute substantially to food resources but also play a crucial role as significant economic assets in nations like Egypt and other developing regions **(Wati, 2018)**. Commercially important shrimp species fall under the superfamily Penaeoidea. Although all members are marine species, certain juvenile Penaeidae species are known to inhabit brackish water and can occasionally be found in environments with nearly freshwater conditions **(Karuppasamy *et al.*, 2020)**. For instance, the white-leg shrimp, *Penaeus vannamei,* is widely distributed in diverse regions. The growth of the shrimp farming industry in Egypt is steadily progressing, with the implementation of numerous large-scale projects aiming at building a resilient and thriving sector **(Elgendy *et al.*, 2015)**.

Over the last forty years, shrimp farming has undergone substantial growth to meet the increasing demand for high-quality protein. However, ensuring the sustainability of shrimp production requires maintaining proper management conditions, particularly the prevalence of bacterial infections that cause significant mortalities and economic losses. Consequently, there is a growing emphasis on employing closed-life cycle rearing systems to prioritize biosecurity and prevent disease transmissions **(Martínez-Córdova *et al.*, 2015)**.

The shrimp farming industry has encountered significant challenges due to bacterial infections caused by various pathogens, including *Vibrio* and *Aeromonas* **(Thornber *et al.*, 2020; Vaiyapuri *et al.*, 2021)**. Vibriosis, a bacterial infection caused by Gram-negative bacteria, is widespread in marine aquaculture farms and ecosystems, especially exhibiting pathogenicity under stressful conditions. It is the most prevalent disease, significantly impacting mortality rates in cultured aquatic crustaceans worldwide **(de Souza Valente & Wan, 2021)**. Moreover, Pseudomonas, typically present in the normal microflora of shellfish, can transform into opportunistic pathogens, increasing and spreading in stressed shrimp populations. Additionally, it contributes to the decomposition of shrimp **(Cholewińska *et al.*, 2022)**. In certain scenarios, Pseudomonas strains, notably *Pseudomonas aeruginosa,* have the potential to cause infections in humans **(Knipe *et al.*, 2021)**. Furthermore, bacterial black disease, caused by *Aeromonas* spp. causes black lesions on the cuticle, predominantly observed on the dorsal side and pereopods. Through molecular identification, the most virulent strain was determined to be *A. hydrophila*, a motile, Gram-negative, rod-shaped bacterium **(Saejung *et al.*, 2014)**. Affected shrimp exhibit abnormal swimming behavior and typically perish within 24 to 48 hours of post-bacterial infection **(Thaimuangphol *et al.*, 2022)**.

Standard biochemical techniques for identifying bacterial pathogens are not only highly reliable but also time-consuming. Molecular approaches have become widely employed for screening various pathogenic bacteria due to their convenience, rapidity, and high sensitivity. PCR detection techniques targeting the causative agents of these diseases are commonly recommended **(Law *et al.*, 2015)**. Hence, the development of a PCR method capable of targeting specific nucleotide sequences unique to *Vibrio* could enhance its detection and differentiation from closely related *Vibrio* species **(Tang *et al.*, 2020)**.

Thus, the study aimed to identify prevalent bacterial diseases in shrimp, focusing particularly on molecular detection of these pathogens in shrimp tissue, along with investigating the associated histopathological changes.

MATERIALS AND METHODS

Sample collection, clinical and postmortem examination

A total of 80 adult Penaeid shrimps, including *Penaeus japonicus, Litopenaeus vannamei*, and *Penaeus semisulcatus*, with an average body weight of 32± 3g and a total body length of 14.5± 2cm, were randomly collected alive seasonally from fishing men. Shrimp specimens were subjected to a full external inspection and underwent a postmortem examination for any gross lesions or abnormalities following the procedures outlined by **Lightner (1996)**.

Bacteriological isolation and phenotypic identification of the bacterial isolates

Bacterial species were recovered from freshly dead shrimp specimens following euthanasia, from hepatopancreas, muscles and uropodes of naturally infected shrimp (n= 80). Bacterial inocula were enriched into tryptic soy broth with 2% NaCl and incubated for 24 hours at 28°C. The initial isolation was carried out on TCBS (Thiosulfate Citrate Bile Salts Sucrose agar, Oxoid), PAB (Pseudomonas Agar Base, LAB M), and ASA (Aeromonas Selective Agar, HIMEDIA) following the methods described by **Bergey (1994)**. The straked plates were incubated at 28°C for 24h. The growth was observed to determine whether the colonies were dense and virtually pure (with distinct morphology) or mixed (containing multiple colony types). Suspected colonies with characteristic features were selected and purified for further identification based on their culture morphology. The identification of the isolated *Vibrio*, *pseudomonas* and *Aeromonas* strains were performed according to the criteria outlined in Bergey's Manual (9st Edition). Gram staining was conducted on smear preparations from young 24hr- aged colonies **(Coico, 2006)**. The oxidase test used Oxoid strips (Oxoid), and positive results was determined by the development of dark purple or blue color within 30 seconds, as outlined by **Shields and Cathcart (2010)**. The catalase test, according to **Reiner (2010)**, involved streaking a pure colony on a glass slide adding hydrogen peroxide (H_2O_2) and examining the production of O_2 froths. Salt tolerance studies assess growth in different NaCl concentrations **(Aly *et al.*, 2023)**. The sensitivity test against the vibriostatic agent (Novobiocin 30µg) was conducted using the disc diffusion method.

Antibiogram pattern of the retrieved bacterial isolates

The susceptibility of bacterial isolates to antibiotics was assessed by the Disk Diffusion Susceptibility method following **Bauer *et al.* (1966)** against the antibiotics disks (Oxoid) including; tetracycline (30µg), ampicillin (10µg), trimethoprim/sulfamethoxazole (1.23/23.75µg), kanamycin (30µg), erythromycin (15µg), and gentamicin (10µg). The inhibition zones were then measured using a millimeter ruler. The interpretation of inhibition zones followed the guidelines provided in the Clinical Laboratory Standards **(CLSI, 2021)**.

Molecular detection of the bacterial pathogens in shrimp tissue specimens

The shrimp specimens (n=80) underwent DNA extraction using the QIAamp DNA mini kit from QIAGEN® (USA) following the manufacturer's instructions. For PCR, three genus-specific primers were used, including *V 16S r*RNA (for *Vibrio* species), *PA-GS* (for *Pseudomonas* species), and *Aer* (for *Aeromonas* species), each with specified PCR conditions detailed in Table 1). The gene segment amplification was conducted using the DreamTaq Green PCR master mix (2x) kit from Thermo Scientific. Moreover, the simplex PCR method was employed for each pathogen in a total volume of 25µl, while a 50µl volume was used for duplex PCR targeting *Aeromonas* and *Pseudomonas* species. Visualization of the amplification products was carried out on a 1.5% agarose gel by a gel documentation system (Alpha Innotech, Biometra).

Table 1. A list of primers used in the present study with their corresponding PCR conditions

Target	Genus-specific PCR primers Sequence5′-3′	Product size (bp)	PCR thermal profile (35 cycles)			Reference
			Denaturation	Annealing	Extension	
***Vibrio* spp.**	*V.16S* F: CGGTGAAATGCGTAGAGAT *V.16S* R: TTACTAGCGATTCCGAGTTC	663	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec.	**(Tarr Cheryl *et al.*, 2007)**
***Pseudomonas* spp.**	PA-GS-F: GACGGGTGAGTAATGCCTA PA-GS-R: CACTGGTGTTCCTTCCTATA	618	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	**(Spilker *et al.*, 2004)**
***Aeromonas* spp.**	*Aer* F: CTACTTTTGCCGGCGAGCGG *Aer* R: TGATTCCCGAAGGCACTCCC	953				***(*Gordon *et al.*, 2007)**

Histopathological examination

Specimens of subcuticular tissues, muscles, hepatopancreas, and uropods obtained from freshly dead shrimp samples were preserved in Davidson's fixative **(Lightner, 1996)**. Subsequently, these samples underwent histological processing and were stained with hematoxylin and eosin. The tissue samples were dehydrated using sequential ethyl alcohol dilutions (30, 50, 70, 90%, and absolute), followed by clearing with xylene and embedding in paraffin wax. Sections of the paraffin-embedded tissues, with a thickness of 5µm, were stained using hematoxylin and eosin, as per the method described by **Suvarna *et al.* (2018)**. The stained

tissue sections were mounted using Canada balsam, and subsequently examined under light microscopy. The stained tissue slides were examined and photographed using a digital camera fitted with a Leica microscope (DM 1750, version 3.6.0, United States).

Ethics statement

The present study was conducted following the ARRIVE guidelines. Shrimp handling and experimental procedures were approved by the Scientific Research Committee (Animal Ethics Review Committee) of the Faculty of Veterinary Medicine, Suez Canal University, Egypt, with approved Code: 201806.

RESULTS

Clinical and postmortem lesions

The clinical examination detected various disease lesions, such as red discoloration, sloughing of uropods and pleopods, muscle and cuticle softening, along with an empty gut. Black to brown spots were observed on various parts of the exoskeleton, including the pleopods, uropods, and telsons (Fig. 1A-B), as well as within the underlying muscle tissue (Fig. 1C). Additionally, some shrimps exhibited white spots on the carapace. Some shrimp specimens displayed black and melanized gills (Fig. 1D), while others had a sticky texture with red discoloration. Moreover, the hepatopancreas exhibited congestion and, in some cases, melanization (Fig. 1E).

Bacteriological examination

Presumptive phenotypical identification of bacterial isolates recovered from the hepatopancreas, muscle, and uropod of freshly dead shrimp, cultured on various selective media, was recorded. Colonies with a yellow or dark-centered green appearance, measuring 2-3mm with swarming activity, retrieved from TCBS, were identified as *Vibrio* species. Furthermore, some colonies exhibited fluorescein pigments on PAB, presumptively indicating *Pseudomonas* species. Additionally, translucent, round colonies on ASA were presumptively identified as *Aeromonas* species. Biochemically, all bacterial isolates were Gram-negative, motile, and positive for oxidase and catalase (Table 2). They were also capable of fermenting glucose and were facultative anaerobic. *Vibrio* isolates were sensitive to the vibriostatic agent novobiocin, while *Pseudomonas* were aerobic and produced diffused fluorescent pigment.

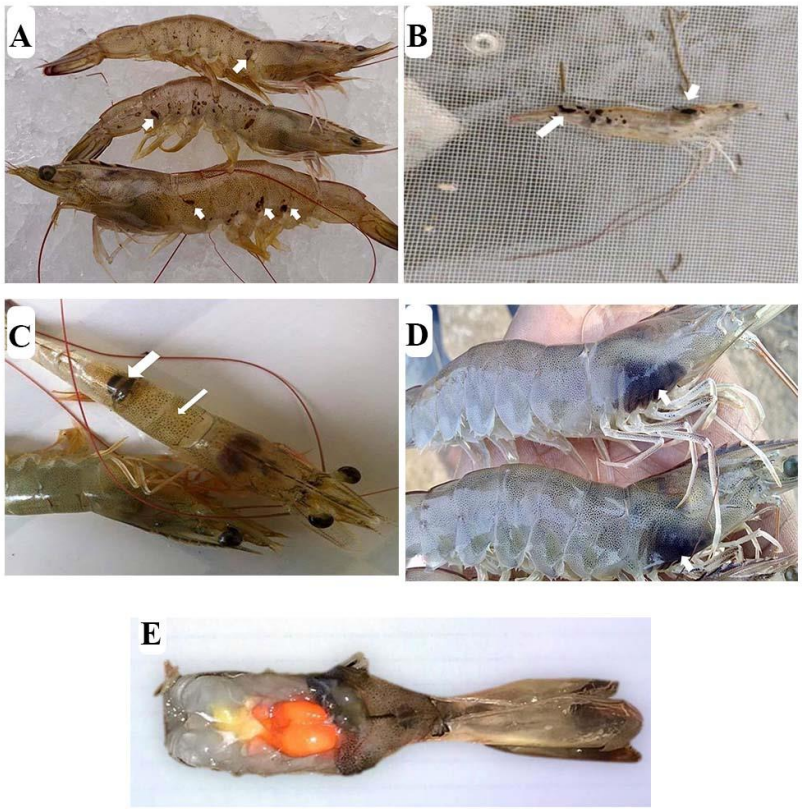

Fig. 1. Naturally infected shrimp specimens. **A &B:** Gross lesions of *Litopenaeus vannamei* showing dark brown spots with dark brown patches (arrow), **C:** Gross lesions of *Penaeus semisulcatus* showing black streaks (thick arrow) and empty gut (thin arrow), **D:** Gross lesions of infected *Litopenaeus semisulcatus* showing black gills (arrow), and **E:** Gross lesions of *Penaeus semisulcatus* showing dark orange coloration of hepatopancreas

Table 2. Biochemistry profile of recovered bacterial isolates

Phenotypic profile	***Vibrio* species**	***Pseudomonas* species**	***Aeromonas* species**
Culture on selective media	Yellow and green colonies on TCBS	straw-coloured colonies on PAB	Translucent, round colonies on ASA
Gram stain	- Ve	- Ve	- Ve
Shape	Rods or comma	Single rod	Single or paired rod
Cytochrome oxidase	+ Ve	+ Ve	+ Ve
Catalase	+Ve	+Ve	+Ve
Novobiocin (30µg)	Sensitive	Sensitive	Resist
Pigment production	-Ve	-/+ Ve	-Ve
Salt tolerance			
0%	-/+ Ve	+ Ve	+ Ve
2%	+Ve	+ Ve	+ Ve
4%	+ Ve	-	-
6%	+Ve	-	-

* PAB (*Pseudomonas* agar base), ASA (*Aeromonas* selective agar).

The prevalence of bacterial isolates in shrimp tissues through bacteriological analysis

The total occurrence of bacterial pathogens in infected shrimp tissues is illustrated in Fig. 2). *Vibrio* species constitute the majority, accounting for 45.94%, followed by *Pseudomonas* and *Aeromonas* species at 40.54 and 13.51%, respectively. The hepatopancreas and muscles served as the predictable sites for *Vibrio* pathogens, while *Pseudomonas* was predominantly found in muscles and *Aeromonas* in the hepatopancreas. Among the various tissues, the hepatopancreas showed the highest occurrence of bacterial pathogens at 37.83%, followed by the muscle (35.14%) and the uropod (27.03%).

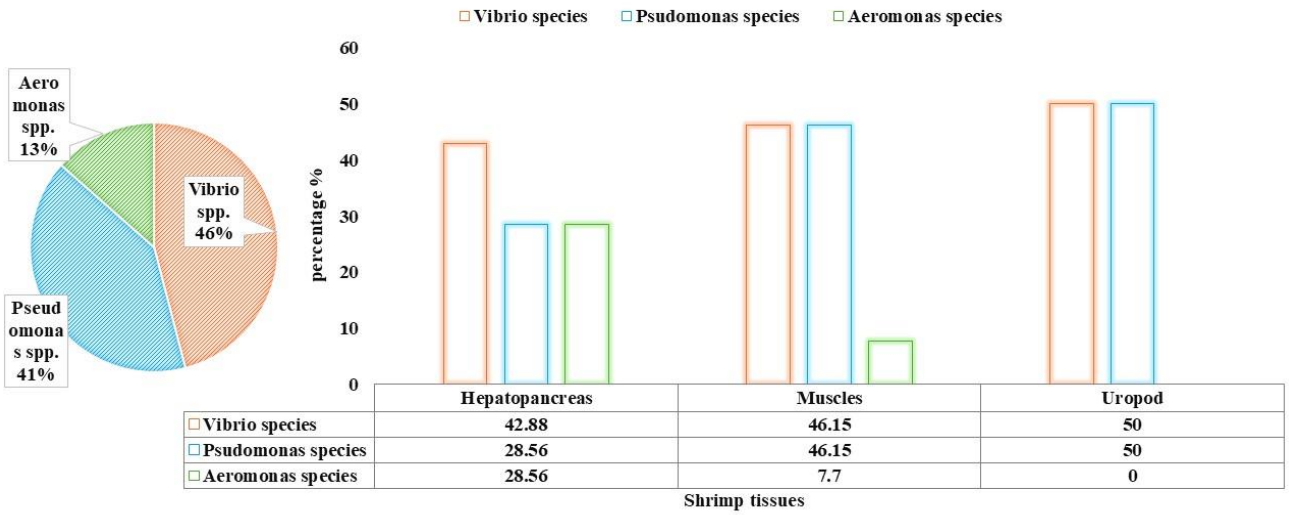

Fig. 2. Prevalence of *Vibrio*, *Pseudomonas*, and *Aeromonas* pathogens among tissues of infected shrimp

Antibiogram profile of bacterial isolates

The whole antibiotic profiling of recovered pathogens from examined shrimp is illustrated in Fig. 3**)**. The retrieved *Vibrio* isolates were highly susceptible to gentamycin (100%), followed by tetracycline (80.39%), however showing high resistance to erythromycin (80.39%) and ampicillin (60.78%), and intermediate susceptibility for kanamycin (27.45%). The *Pseudomonas* isolates exhibited the highest susceptibility to trimethoprim/ sulphamethoxazole (SXT) and gentamycin, with 100% resistance against ampicillin. Moreover, intermediate susceptibility was observed for erythromycin (66.67%). Furthermore, the *Aeromonas* isolates revealed the highest resistance to ampicillin (100%), followed by erythromycin (66.67%). While, the highest sensitivity was recorded for SXT (100%) then gentamycin (73.33%), followed by tetracycline (60%). Intermediate susceptibility was noted for kanamycin (46.67%).

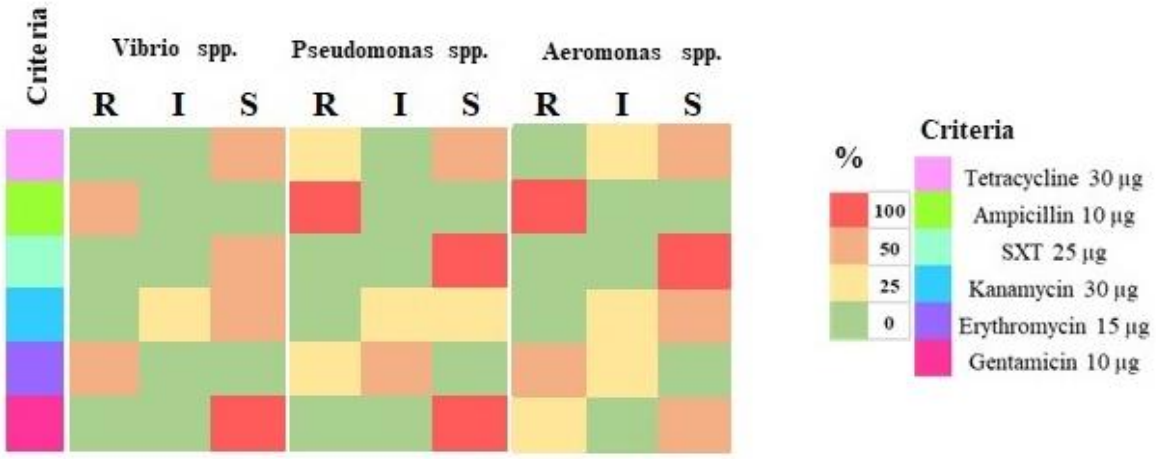

Fig. 3. The heat map illustrates the antimicrobial susceptibility patterns of retrieved *Vibrio*, *Pseudomonas*, and *Aeromonas* isolates (n= 111)

Molecular identification of the bacterial pathogens in shrimp tissue specimens

PCR-generated bands specific for *Vibrio*, *Pseudomonas*, and *Aeromonas* species recovered from the infected shrimp specimens are illustrated in Fig. 4, 5) The universal *Vibrio 16S r*RNA gene PCR generated 663-bp bands size characteristic for all detected *Vibrio* strains from all shrimp samples is illustrated in Fig. 4). Moreover, specific 618 and 953bp bands were recovered using species-specific primers for *Pseudomonas*, and *Aeromonas* bacteria by duplex PCR (Fig. 5).

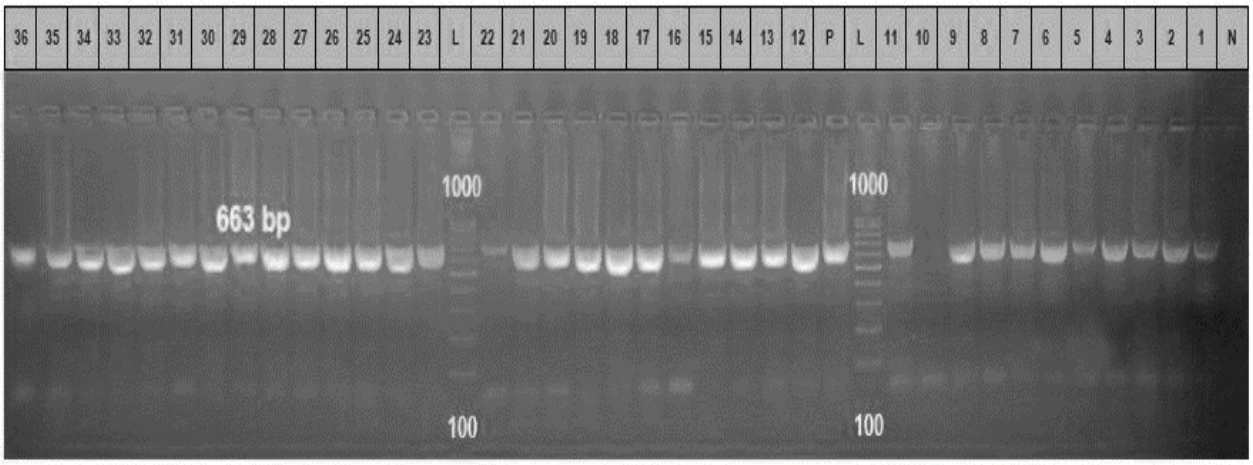

Fig. 4. Agarose gel electrophoresis of PCR products corresponding to amplification of *16S r*RNA target primers for detected Vibrio species from muscles of naturally infected shrimp. L: Molecular weight marker (Gene Direx), N:control negative, P:control positive, Lane (1-9) sample collected in autaum season infected with vibrio, Lane (12-21) sample collected in spring season infected with *Vibrio*, Lane (23-36) sample collected in summer season infected with vibrio, The 663bp fragments correspond to the known type of *16S r*RNA PCR products

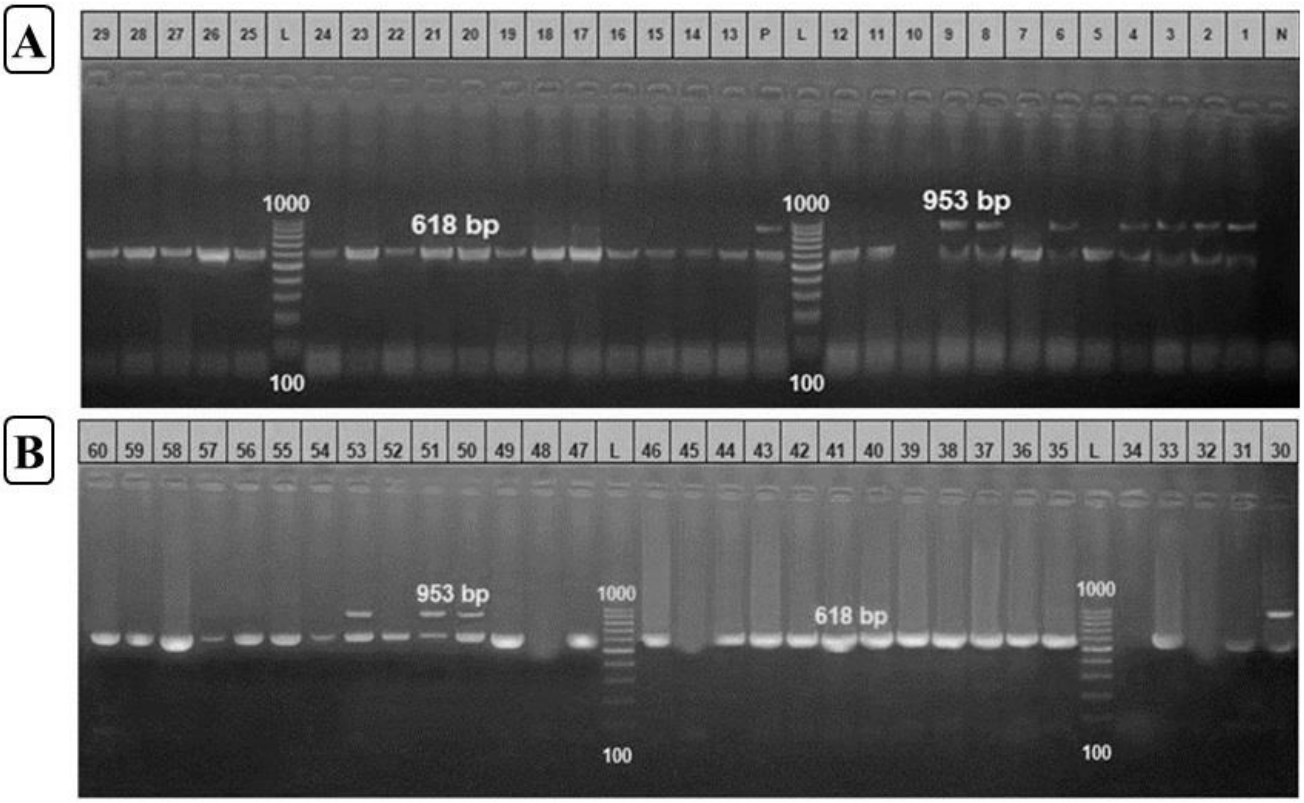

Fig. 5. Agarose gel electrophoresis of Duplex PCR products corresponding to amplification of *PA-GS*, and *Aer* primers for detected Pseudomonas and Aeromonas species from muscles of naturally infected shrimp. L: Molecular weight marker (Gene Direx), N: control negative, P: control positive, **(A)**: lane (1, 2 ,3, 4, 6, 8, and 9) showing mixed infection with *Aeromonas* and *Pseudomonas*, lane (11-29) sample infected with pseudomonas. **(B):** showing duplex PCR L: Molecular weight marker (Gene Direx), N: control negative, P: control positive, lane (30, 50, 51 and 53) mixed infection with *Aeromonas* and *Pseudomonas*, lane (31, 33, 46, and 47) (35 and 44) (49 and 60) sample infected with pseudomonas. The 618 and 953bp fragments correspond to the known type of *PA-GS*, and *Aer* primers PCR products

The prevalence of bacterial isolates in shrimp tissues through PCR-based assay

The total frequency of bacterial infection in naturally infected shrimp is shown in Table 3). *Vibrio* species constitute the majority, accounting for 82.5%, followed by *Pseudomonas* and *Aeromonas* species at 78.75 and 42.5%, respectively.

Table 3. Molecular detection of the total number and percentage (%) of infections among examined shrimp specimens (n=80)

Bacterial infection	***Vibrio* spp.**	***Pseudomonas* spp.**	***Aeromonas* spp.**
Total number of specimens	80	80	80
Number of positive specimens	66	63	34
Percentage (%)	82.5%	78.75%	42.5%

Histopathological examination

The histopathological changes caused by bacterial pathogens in naturally infected shrimp are depicted in Fig. 6). The cuticle and subcuticular tissues of the examined shrimp exhibited a variety of conditions ranging from normal cuticle with intact epicuticle, exocuticle, and endocuticle, to damaged epicuticle with subcuticular edema and hemocytes infiltration in the epidermis. Moreover, the underlying subcuticular muscle showed muscular degeneration, Zenker's necrosis, accompanied by hemocytes influx, and inter-muscular oedema (Fig. 6A). Along with degenerative changes with inflammatory cell infiltration were observed in the abdominal muscles. Intramuscular oedema and necrosis were evident in the muscle fibers (Fig. 6 B).

The hepatopancreas tissue showed sloughing and various degrees of degeneration, ranging from mild to severe vacuolation in the majority of hepatopancreatic cells, with necrosis observed in some cells. Degeneration of the hepatopancreatic tubules was evident, accompanied by the collapse of the tubular lumen (Fig. 6C, D). Also, degeneration was observed in the pleopod, affecting the epidermis, and the underlying tissue layers including the epicuticle, exocuticle, endocuticle, and muscles (Fig. 6E).

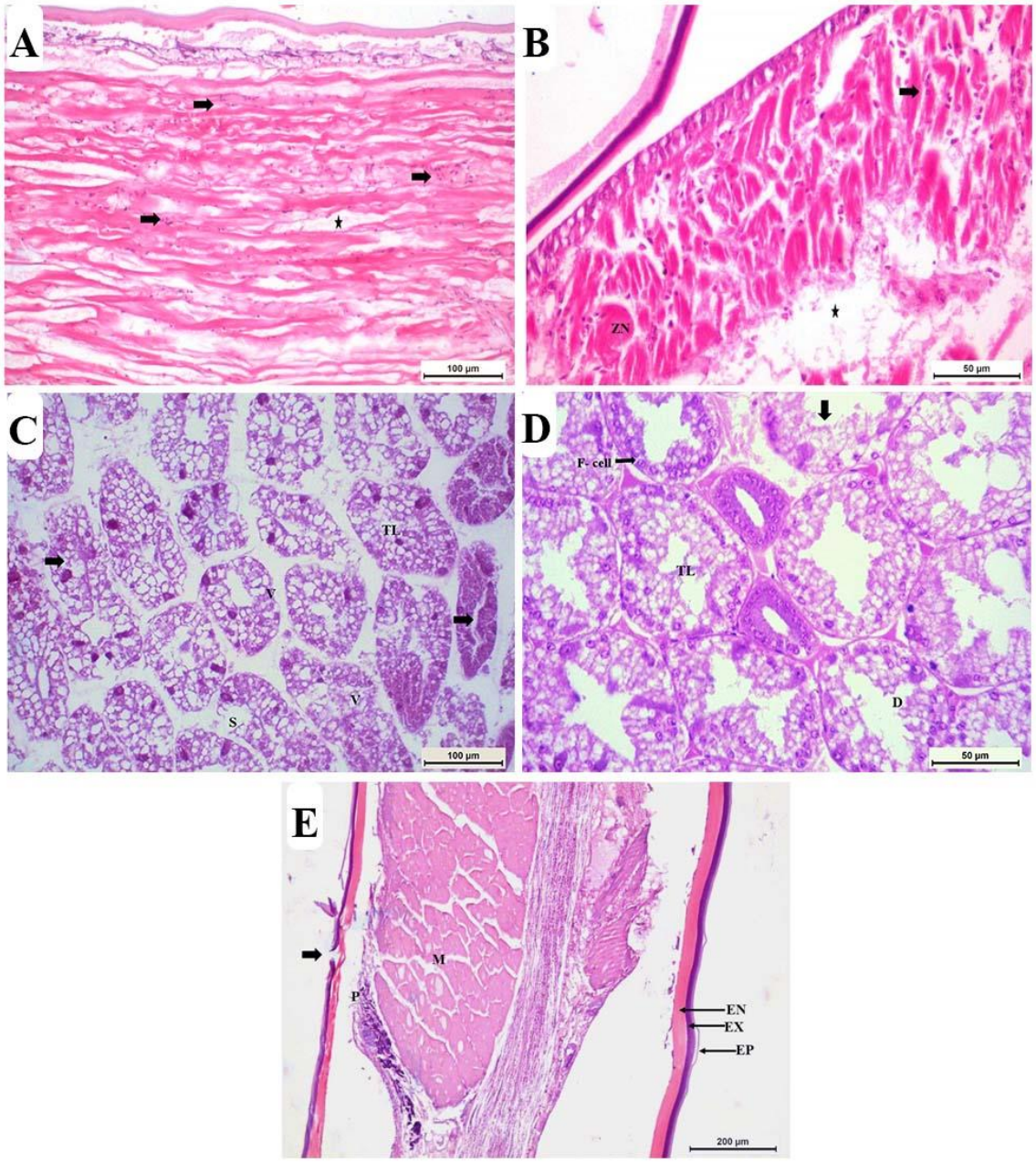

Fig. 6. Photomicrograph of histopathological alterations in the naturally infected shrimp, H& E staining. **A:** the subcuticular tissues and abdominal muscles shows inter-muscular edema (star) with haemocytic infiltration (arrows), **B:** the epicuticle and subcuticular tissues shows epicuticular damage, and Zenker's necrosis (ZN) in the underlining muscles accompanied by heamocytic influx (arrow), **C:** the hepatopancreas reveals intratubular edema (N) and collapse of tubular lumen, TL (arrows) and sloughing (S) with vacuolation (V) of hepatopancreas cells, **D:** the hepatopancreas tissue in shrimp demonstrates degeneration of hepatopancreas cells (D), necrosis (arrow) and collapse of tubular lumen (TL), and **E:** the pleopod displays degenerative changes in epidermis (P) and underlying tissue epicuticle, EP, exocuticle, EX (white arrow), endocuticle (EN) and muscles (M)

DISCUSSION

Shrimp bacterial pathogens posed a significant challenge to the sustainability of the shrimp industry. During the study period, diseased shrimp exhibited symptoms such as red discoloration, muscle and cuticle softening, and congestion in the hepatopancreas, that sometimes appeared melanized. Likewise, **El Zlitne *et al.* (2022)** recorded necrotic lesions and stunted growth in the Pacific white leg shrimp infected with vibriosis, along with reddening and congestion of body appendages and telson. Correspondingly, comparable observations were noted in the black tiger prawns infected with vibriosis **(Vaiyapuri *et al.*, 2021)**.

Moreover, predominant lesions observed in the examined shrimp included black to brown spots on the exoskeleton, pleopods, uropods, and telsons, some of which extended deeply into the underlying muscle. Additionally, white spots were occasionally observed on the carapace. In the same context, previous studies isolated chitinolytic *Vibrio* spp. from brown-spot lesions of diseased shrimp **(Sajana *et al.*, 2019, Yu *et al.*, 2022)**. Furthermore, **Yu *et al.* (2022)** clinically characterized vibriosis in shrimp by atrophied hepatopancreas and the presence of pale and sometimes black spots on the body, along with a soft shell and an empty midgut. **Abdolnabi *et al.* (2015)** identified focal melanin lesions on the outer body surface of *M. rosenbergii* infected with *A. hydrophila*, typically localized at the infection site, including the carapace, appendages, uropods, telson, or body cuticle. Additionally, they detected other pathogens, such as *Vibrio* and *Pseudomonas* species. In this context, *Vibrio*, *Pseudomonas*, and *Aeromonas* species were considered as the most common bacterial pathogens associated with shrimp diseases **(Farto *et al.*, 2019; Tang *et al.*, 2020)**.

In this study, bacterial pathogens were prevalent in various shrimp tissues, with the hepatopancreas harboring the highest proportion of pathogens, followed by the muscle and uropod. *Vibrio* pathogens were equally distributed between the hepatopancreas and muscles, while *Pseudomonas* isolates were mainly found in the muscle, and *Aeromonas* pathogens were primarily detected in the hepatopancreas. These findings are consistent with those reported by **Muthukrishnan *et al.* (2019)**, who observed a high recovery rate of *V. parahaemolyticus* from the hepatopancreas (83.4%) of infected *L. vannamei*. Similarly, **Eissa *et al.* (2011)** found various *Vibrio* species predominantly in the hepatopancreas, followed by the gills and musculature. Contrarily, **de Souza Valente and Wan (2021)** isolated *V. harveyi, V. alginolyticus, and V. parahaemolyticus* from *L. vannamei* exhibiting vibriosis symptoms, with the stomach being the primary site of isolation, followed by the hepatopancreas, haemolymph, muscle, and gut. These results align with previous findings by **Muthukrishnan *et al.* (2019)**, who noted a high prevalence of *V. parahaemolyticus* in the hepatopancreas (83.4%) of infected *L. vannamei*. Similarly, **Eissa *et al.* (2011)** observed various *Vibrio* species predominantly in the hepatopancreas, followed by the gills and musculature.

The antibiotic susceptibility profiles of the pathogenic *Vibrio*, *Pseudomonas*, and *Aeromonas* isolates indicated their highest susceptibility to gentamycin and tetracycline, followed by trimethoprim/sulfamethoxazole. Conversely, they exhibited the highest resistance to ampicillin and erythromycin. These findings align with those of **Elmahdi *et al.* (2016)**, who

highlighted the common usage of tetracycline antibiotics in aquaculture, particularly for managing severe *Vibrio* infections. Similarly, **Yano *et al.* (2014)** and **Srinivasan and Ramasamy (2017)** reported widespread resistance among *Vibrio* species to ampicillin, a β-lactam antibiotic, possibly due to the presence of antibiotic-resistant genes such as *pen*A and *bla*TEM-1 for β-lactam and penicillin resistance, and *tat*A, *tat*B, *tat*C, *tat*D, *tat*E, *tat*G, *tat*H, *tat*J, *tat*Y, and *tat*Z for tetracycline resistance. These genes can be transferred between bacteria through various mechanisms, including conjugation, transduction, or transformation **(Manjusha & Sarita, 2011)**.

In current study, *Vibrio*, *Pseudomonas*, and *Aeromonas* species were identified through PCR based assay targeting the species-specific genes as *16S r*RNA, aligning with these findings. **Janda and Abbott Sharon (2007)** informed that the widespread use of the *16S r*RNA gene for bacterial species detection due to its species-specific regions. Similarly, **Samiappan *et al.* (2022)** recommended applying this technique in confirmatory diagnostic approach for fish bacterial diseases, including *A. hydrophila, A. salmonicida,* and *V. anguillarum*. Vibriosis and *Pseudomonas* infections were prevalent across all shrimp species in this study, with a lower incidence of *Aeromonas* infection. Employing a particular primer that produced about 953 base pairs, facilitating the detection of *Aeromonas*. Duplex PCR proved to be a straightforward and rapid method for *Pseudomonas* and *Aeromonas* detection, which is consistent with the findings of **Fadel and El-Lamie (2019)**. The universal *Vibrio 16S r*RNA gene PCR amplification yielded 663-bp bands characteristic of all tested *vibrio* strains in shrimp samples across the study, validating the findings of **Khamesipour *et al.* (2014)**, who detected *Vibrio* spp. in Iranian aquaculture shrimp using PCR, offering a swift and accurate determination of vibriosis during early infection phases.

Likewise, the universal *Pseudomonas PA-GS* gene PCR amplification resulted in 618-bp bands characteristic of all tested Pseudomonas strains across all shrimp samples, consistent with the identification of Pseudomonas species **(Chau *et al.*, 2011)**. Through *Aeromonas* (*Aer*) gene primers, PCR amplification produced 953-bp bands characteristic of isolated *Aeromonas* from infected shrimp specimens, aligning with **Rahimi *et al.* (2014)**, who noted the higher sensitivity of PCR assays in detecting *A. hydrophila* compared to cultural methods. Overall, vibriosis and *pseudomonas* infections were prevalent across all shrimp species, with a lower incidence of *Aeromonas* spp. The highest prevalence was observed among *Vibrio* species (82.5%), followed by *Pseudomonas* species (78.75%), while *Aeromonas* species exhibited the lowest rate (42.5%) across the examined tissues. This infection might be attributed to the immunosuppression caused by stress that facilitates the invasion and outbreaks, alongside factors like high water temperature and sudden fluctuations. In this study, *Penaeus vannamei* exhibited the highest infection prevalence among shrimp species, corroborating earlier report of **Amelia *et al.* (2021)** indicating its low immunity and susceptibility to infection.

Histopathological examination of infected shrimp tissue revealed variations in the cuticle and subcuticular tissues, ranging from normal structures to epicuticular damage and subcuticular edema, and hemocytic infiltration in the epidermis. These findings align with the

descriptions of shell disease caused by shrimp vibriosis by **Abraham (2014)**, who recorded erosion and/or ulceration of the cuticle. Similarly, **Kim *et al.* (2014)** identified black spot lesions on the external skeleton of crustaceans, thus attributing the black coloration to a melanization reaction triggered by cuticular damage.

The hepatopancreas tissue exhibited sloughing, degeneration, and varying degrees of vacuolation in the majority of hepatopancreas cells, as well as necrosis in some cells. Furthermore, degeneration of hepatopancreatic tubules with a collapse of the tubular lumen was observed. These findings are consistent with those reported by **Khafagy *et al.* (2017)**, who observed intermuscular edema, inflammatory cell infiltration between muscle bundles, and degeneration and necrosis of muscles in addition to the hepatopancreas exhibiting congestion in hepatic vessels, advanced vacuolar degeneration, and nuclear pyknosis in hepatocytes among naturally infected *P. japonicas* shrimp. **Santos *et al.* (2020)** detected similar lesions in shrimp affected by Acute Hepatopancreatic Necrosis Syndrome (AHPN). In the early to mid-stage of the disease, they noted sloughing and rounding of hepatopancreatic tubule epithelial cells, while the late stage was characterized by the appearance of melanized granulomas, massive hemocyte aggregation, and infection with various bacterial colonies in the tubule lumen. **Powers *et al.* (2021)** added that in shrimp infected with AHPN, the acute phase is characterized by hepatopancreatic tubule atrophy, and sloughing of tubule epithelial cells, followed by a terminal phase with hemocytic infiltration and massive secondary bacterial infection. Similarly, **El Zlitne *et al.* (2022)** observed various degrees of degenerative changes, sloughing, and necrosis in hepatopancreatic tissues during outbreaks of vibriosis in *L. vannamei*, along with a notable influx of inflammatory hemocytes through the muscular tissues of affected shrimp.

These results are in line with those documented by **Raja *et al.* (2017)**, who observed haemocyte infiltration and nodules in the epidermis, and muscles, as well as atrophy of the excretory organ. Disrupted hepatopancreas tubules with diffuse interstitial oedema. Additionally, thickening of intertubular space, rounding, and sloughing of HP tubular epithelium, and the presence of mitotic figures with bacterial colonies and apoptotic bodies.

Moreover, **Ramalingam and Ramarani (2007)** observed vacuolation of hepatopancreatic cells with hypertrophied nuclei and notable atrophy of hepatopancreatic tubules in prawns infected with *Pseudomonas aeruginosa*. They attributed the pathogenicity of *P. aeruginosa* to its infiltration and proliferation within tissues, resulting in the release of extracellular enzymes for metabolic processes. Similar lesions were also observed in the giant freshwater prawns infected with *Aeromonas hydrophila*, as reported by **Abdolnabi *et al*. (2015).**

CONCLUSION

Rapid identification of shrimp diseases assists pathologists in devising efficient disease management strategies in aquaculture. Moreover, accurate identification of bacterial pathogens enables the targeted use of antimicrobial agents, thereby mitigating the risk of antibiotic resistance development in shrimp farms. Among naturally infected shrimp, *Vibrio* species were

the most prevalent pathogens, followed by *Pseudomonas* species, while *Aeromonas* species demonstrated the lowest incidence. The identified pathogens displayed sensitivity to gentamicin, trimethoprim/sulfamethoxazole, and tetracycline. The molecular diagnostic approach emerged as the most precise and rapid method for identifying septicemic bacterial pathogens, outperforming traditional techniques. Consequently, the quick identification of pathogens in asymptomatic or carrier shrimp aids in the development of biosecurity measures to uphold shrimp aquaculture sustainability.

Data availability

All datasets of the current study are available within the article or can be obtained from corresponding with no restriction.

Authors' contributions

All authors have significantly contributed to the study design, methodology, data analysis, drafting, and manuscript editing. All authors have read and approved the final version of the submitted manuscript.

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