**Research Article****Isolation and molecular identification of diversified *Vibrios* from cultured shrimp (*Penaeus monodon*) from the south-western region of Bangladesh**

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Aquaculture is a fast-growing food producing industry that plays a significant role in employment generation and protein supply. In Bangladesh, the southwestern region, especially the districts of Khulna, Satkhira and Bagerhat, is a major hub of shrimp aquaculture. This study was conducted to isolate and identify the *Vibrio* diversity in cultured *Penaeus monodon* via classical microbiological and molecular approaches. Samples were collected from shrimp farms in the three southwestern districts of Khulna, Satkhira, and Bagerhat. Bacterial isolation was performed using the conventional agar plate method, followed by molecular identification through 16S rRNA gene sequencing. A total of 80 bacterial isolates were initially screened, and 21 representative isolates were selected for molecular identification. Based on nucleotide BLAST analysis using the NCBI database, six isolates were identified as *Vibrio mediterranei*, while four isolates each were identified as *V. cholerae*, and *V. parahaemolyticus*. One isolate each was identified as *Photobacterium damsela* subsp. *damsela*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi*, *V. plantisponsor*, and *Vibrio* sp. In addition, one strain of *Bacillus subtilis* was isolated and identified, reflecting the widespread use of probiotics in contemporary shrimp farming practices. A phylogenetic tree was constructed using the maximum likelihood method in MEGA 12 software to confirm the evolutionary relationships between the bacteria isolates and reference strains. Our results provide valuable insight into the diversity of *Vibrio* species present in shrimp farms, including potentially pathogenic species, and offer scientific evidence to support disease management and control in shrimp aquaculture.

**Introduction**

Aquaculture is one of the industries with the fastest employment growth and animal protein supply worldwide (FAO, 2018). Among the aquaculture sub-sectors, Bangladesh's shrimp industry is vital to the country's economy because of its potential for

exports and GDP contributions (Washim et al., 2020). For almost 8.5 million people, it is a major source of income, especially for those who live near the coast (Jamal et al., 2023). The majority of shrimp production in Bangladesh is concentrated in

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southwestern districts, particularly Bagerhat, Khulna, and Satkhira, which account for almost 75% of total shrimp production annually (DoF, 2022). However, multiple viruses and pathogenic bacteria hinder the rapid development and shrimp sector growth (El-Saadony et al., 2022). Each year, these pathogens cause major yield reductions and severe financial losses (Paul and Vogl, 2012). The success and sustainability of the shrimp aquaculture sector depend on addressing disease prevalence.

A common bacterial group in coastal areas is *Vibrio* species (Baker-Austin et al., 2018). *Vibrio* species known to harm shrimp include *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* (Yildirim-Aksoy et al., 2022). Other *Vibrio* and non-*Vibrio* species known to harm shrimp include *V. azureus*, *V. fluvialis*, *V. vulnificus*, *V. cholerae*, *Photobacterium damsela* subsp. *damsela* and *Aeromonas hydrophila* (Rahman et al., 2020). Additionally, one of the most common contagious diseases in shrimp aquaculture is acute hepatopancreatic necrosis disease (AHPND), which causes mass mortality. The causative agent is the binary toxin *pirA/B* gene-bearing *V. parahaemolyticus* (Huynh et al., 2025). In addition, luminescent *V. harveyi* has been identified as a major cause of mass mortality in penaeid shrimp hatcheries worldwide (Bagthasingh et al., 2019).

Furthermore, *V. vulnificus* causes vibriosis in farmed fish and humans and is transmitted via contact or ingestion (Ceccarelli et al., 2019). Hemorrhagic septicemia is the most severe form of vibriosis, with a high risk of death in humans with elevated blood iron or in fish exposed to 1-1.5% salinity and temperatures exceeding 25° C (Amaro et al., 2015; Ceccarelli et al., 2019). Infections caused by other genera from the Vibrionaceae family, such as *P. damsela* subsp. *damsela* and *P. damsela* subsp. *piscida*, also substantially affect the mariculture industry (Sharma et al., 2017; Mohamad et al., 2019). Other human-

pathogenic *Vibrio* species also pose food safety risks. *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are three major human-pathogenic *Vibrio* species (Neetoo et al., 2022). These dangerous pathogens pose a major threat to the shrimp farming industry and public health. Resolving these issues should improve the industry's overall sustainability, profitability, and productivity (Seibert et al., 2012).

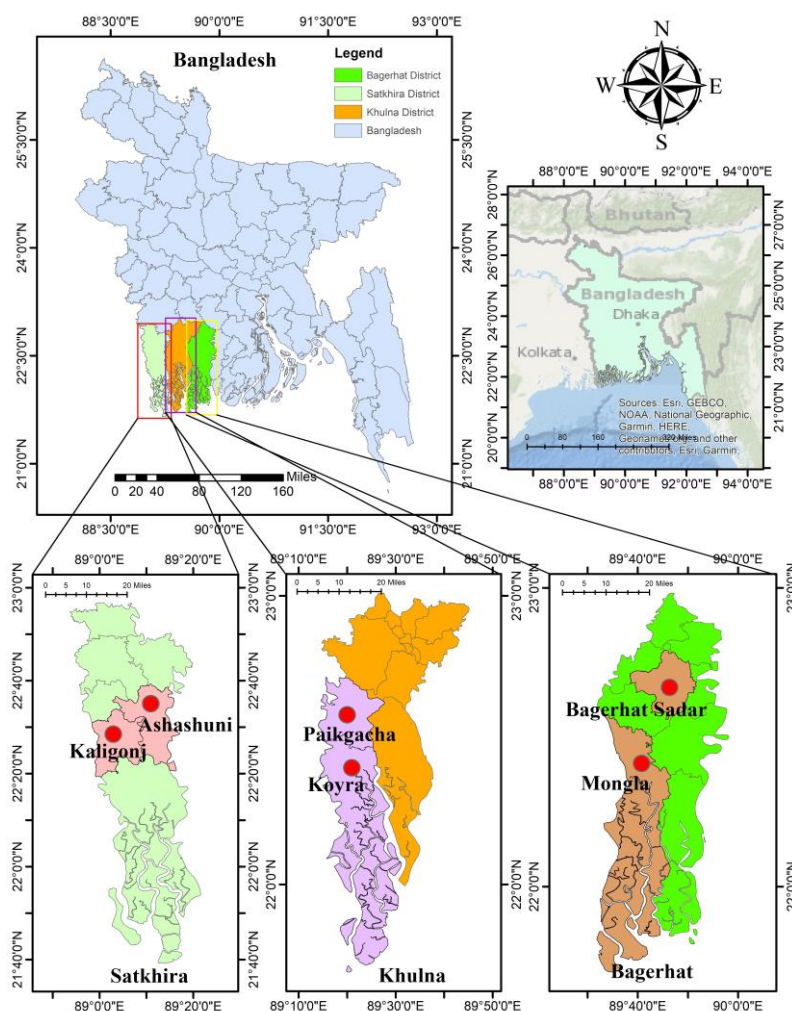
However, excessive antibiotic use to control these shrimp diseases creates selective pressure that drives antimicrobial resistance (Ievy et al., 2020; Tawwab et al., 2020). Antibiotic-resistant bacteria proliferate in the environment because of indiscriminate antibiotic use and insufficient public awareness of proper antimicrobial stewardship (Hossain et al., 2020). To reduce indiscriminate uses and ensure proper antibiotic application, clinicians must diagnose pathogens quickly and accurately. Besides classical microbiological investigation, researchers have identified numerous molecular markers over recent decades that enable specific microbial identification and phylogenetic classification (Bartual et al., 2005). The 16S rRNA gene, about 1500 base pairs long and encoding a catalytic RNA component of the 30S ribosomal subunit, has several characteristics that have established it as the 'gold standard' molecular marker in bacterial taxonomy. Because it is universally present in prokaryotes, the gene contains both highly conserved and variable regions that evolve at distinct rates, enabling simultaneous universal PCR amplification and phylogenetic analysis across closely and distantly related taxa (Clarridge, 2004). These features facilitate taxonomic assignment at the genus level and frequently at the species level, supported by comprehensive curated databases containing near-complete sequences from numerous strains with established taxonomic classifications (Conlan et al., 2012). Therefore, this study aimed to isolate and identify bacterial diversity in cultured *P. monodon* using classical (agar plate) and molecular (16S rRNA gene sequencing) approaches.

## Materials and Methods

### Sample collection

A total of 20 samples were collected from both extensive and semi-intensive black tiger shrimp (*Penaeus monodon*) farms across six upazilas in three southwestern districts of Bangladesh (Satkhira, Khulna, and Bagerhat) as shown in Fig.1. The samples included 16 shrimp specimens (both healthy and diseased individuals), three soil samples, and one water sample. From Khulna, we collected five shrimp samples (two from semi-intensive and three from extensive farms) and one soil sample from a semi-intensive farm. Additionally, five shrimp samples (four from semi-intensive and one from

extensive farms), one soil sample, and one water sample from semi-intensive farms were collected from Satkhira. We also collected about six shrimp samples (three from semi-intensive and three from extensive farms), along with one soil sample from a semi-intensive farm, were obtained from Bagerhat. We packed the shrimp and soil samples in zipper bags, and collected the water sample in a 500 mL sterile bottle. We collected all samples early in the morning from March to August 2023 and transferred them to a sample box on ice (~4°C). Finally, we transported the samples to the laboratory for further analysis. The samples were subsequently excised for bacteriology and molecular analysis.



**Fig. 1. Location maps. Maps indicating the locations of studied shrimp farms of six upazilla from three districts (Khulna, Satkhira and Bagerhat) created via ArcGIS version 10.8.**

### Microbiological investigation

We prepared shrimp and water samples to isolate different bacteria. The samples were homogenized with 0.9% NaCl solution or physiological saline separately in sterile zipper bags. We dissected the hepatopancreas from the collected shrimp samples. For the water samples, a 1 mL was mixed with 9 mL of physiological saline; for the shrimp samples, 1 g was homogenized with 9 mL of physiological saline in a sterile zipper bag. Then, we took 100 µL of each sample mixture, diluted it with 900 µL of sterile physiological saline in an eppendorf tube, vortexed, and further diluted to  $10^{-2}$  dilutions. Then, 100 µL of the homogenized sample was used for bacterial culture to isolate pathogenic bacteria. We used APW, TSB + 2% salt, TCBS, and TSA + 2% salt media for bacterial culture. To isolate different pathogenic bacteria, we inoculated 100 µL of homogenized sample onto sterile APW and TSB + 2% salt, then incubated the media at 37°C for 24 hrs. After 6 hrs of incubation, cultured bacteria from APW were streaked on TCBS agar and TSA + 2% salt agar plates, and were transferred to an incubator at 37°C for 24 hrs. The screw-cap bottles of APW were also transferred into an incubator. After 24 hrs, the bacteria cultured from APW and TSB + 2% salt were streaked on TCBS agar and TSA + 2% salt agar plates. We incubated the plates at 37°C for 24 hrs. After incubation, the plates had discrete colonies. From the TCBS agar and TSA + 2% salt agar plates, we subcultured selected colonies on TCBS. We observed colony morphology, including formation, elevation, shape and color. We selected discrete colonies based on colony morphology. Overall, 24 colonies were selected primarily and sub-cultured on the respective selective agar plates. We removed a selected colony from the respective agar plate with a sterile loop, streaked on a fresh TCBS plate, and incubated it at 37°C for 24 hrs to isolate single colonies for pure culture and further work. Only one colony of each strain was considered pure. We

picked single pure colonies and stored them in cryovials containing LB with 30% glycerol at -80°C to maintain a stock culture.

### Molecular identification

The molecular identification of the isolate was achieved via 16S rRNA gene sequencing. In brief, the heat extraction method was used for DNA extraction from the representative bacterial isolates. The target DNA amplicon was amplified using the following universal primers for the 16S rRNA gene: 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-CGGTTACCTTGTTACGACTT-3' (Weisburg et al., 1991). PCR reactions were performed under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30s and extension at 72°C for 1 min 30 s, followed by a final extension at 72°C for 5 min. The amplified products were checked for the desired amplicon in a 1% agarose gel. The amplified DNA was further purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions prior to sequencing. The sequencing of 21 PCR products was performed using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions and capillary electrophoresis was performed via the ABI Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). We used Geospizas Finch TV version 1.4 to view the DNA sequences. We used the Basic Local Alignment Search Tool (BLAST) to compare primary sequence information. We matched the sequences to the GenBank database using the BLAST search algorithm (Altschul et al., 1990). We aligned the 16S rRNA gene sequences of the isolates using the ClustalW algorithm (Thompson et al., 1994). We constructed a phylogenetic tree using the maximum likelihood method in MEGA-12 to compare 16S rRNA sequences of the bacterial isolates in the present study with reference sequences downloaded from the NCBI database. We drew the tree to scale, with branch lengths in the same units as

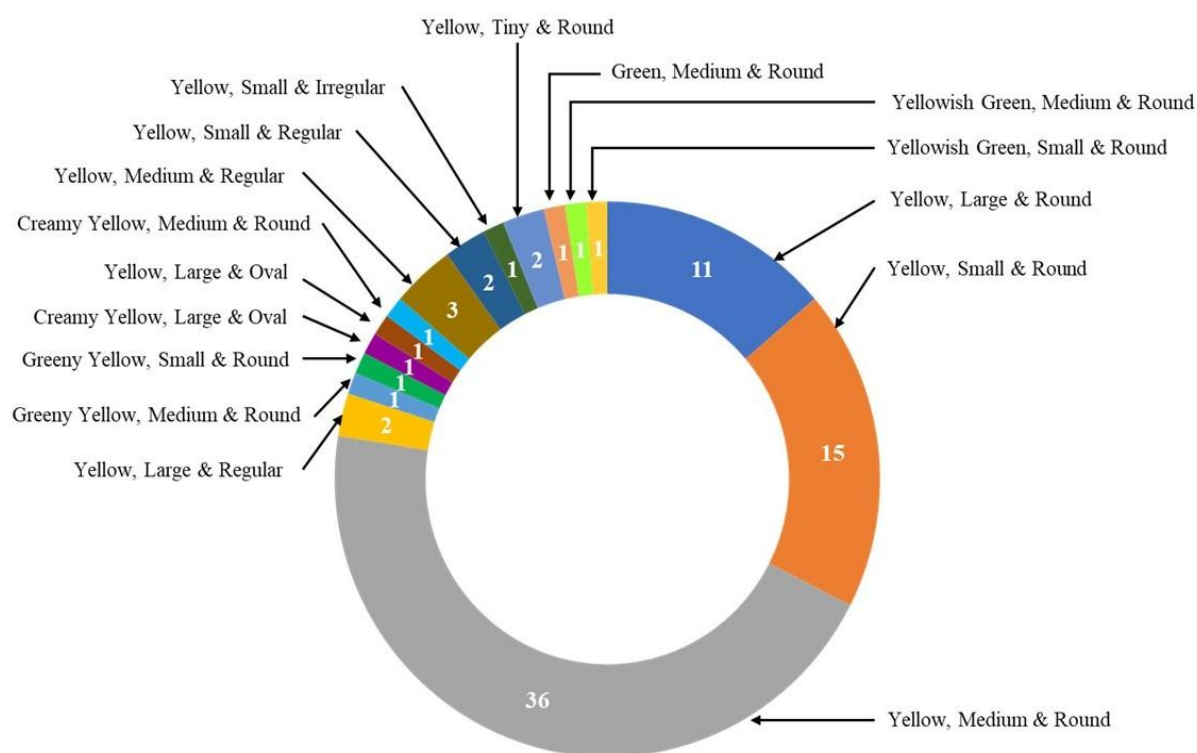
the the evolutionary distances used to infer the tree. We computed evolutionary distances among these representative isolates using the maximum-likelihood method (Kumar et al., 2024).

## Results

### Microbial investigation

In this study, we used the classical agar plate technique and observed the colony morphology of bacterial isolates cultured on TCBS agar. We classified 80 bacterial colonies by color, size, and shape. Among them, the majority were yellow in color with different morphological features (Fig. 2).

Specifically, 36 colonies were yellow, included three yellow, medium, and regular colonies; two yellow, small, and regular; and two yellow, large, and regular colonies. Creamy yellow colonies were less common, with one large, oval colony and one medium, round colony observed. We also distinguished greenish variants, including one greenish yellow, small, round colony; one greenish yellow, medium, round; one yellowish-green, small, round; and one yellowish-green, medium, round. Finally, we found a single green, medium-sized, round colony (Fig. 2). This diverse morphological distribution shows variability in colony features among the isolated strains.



**Fig. 2. Morphological characteristics of the eighty suspected colonies of *Vibrio* spp. isolated from samples of black tiger shrimp (*Penaeus monodon*) found in different shrimp farms of Khulna, Satkhira and Bagerhat.** Small:  $\leq 1$  mmdiameter; medium : 1.1 –  $\leq 2$  mmdiameter; large:  $> 2$  mmdiameter.

medium, and round; 15 were yellow, small, and round; and 11 were yellow, large, and round. In addition, we found two yellow, tiny, round colonies; one yellow, large, oval colony; and one yellow, small, irregular colony. The other yellow variants

### Molecular identification of representative isolates

We selected 24 representative bacterial colonies based on colony morphology on TCBS medium. The isolate numbers were PD1, PD7, PD20, PD3s, PD39, PD49, PD49b, PD65, PD67, PD68, PD77,

PD78, PD86w, PD87, PD89, PD95, PD98, PD101, PD102, PD103, PD104, PD105, PD106, and PD107. We selected all 24 bacterial isolates for PCR amplification and gel electrophoresis. After amplification, 21 PCR products presented a positive band at 1500 bp via gel electrophoresis. Gel electrophoresis showed negative (no band) results in three isolates (PD39, PD101 and PD105). As a result, we excluded PD39, PD101 and PD105, and selected 21 representative bacterial isolates for further analysis.

### 16S rRNA gene sequence based identification of the studied isolates

Table 1 shows the results of a nucleotide BLAST search of the NCBI database (National Center for Biotechnology Information) to identify the 16S rRNA gene sequences of 21 representative isolates. We matched the isolates DNA sequences with those of

the strains presented in the respective columns. Table 1 also provides the number of isolates, identified species, strain number, query length (bp), maximum score comparing the total score, E-value, query cover, percentage, percentage identity, and GenBank accession number of the matched sequences.

From the shrimp samples, we identified representative isolates PD1, PD7, PD20 and PD49b as *V. parahaemolyticus*, PD65, PD68, PD86w (sourced from pond water), as *V. cholerae*; and PD87 as *V. cholerae*. The isolates PD67, PD78, PD89, PD98, PD104, and PD106 were recognized as *V. mediterranei*. In addition, PD3s was found as *V. alginolyticus* from pond soil, PD107 as *V. vulnificus*, and PD103 as *P. damsela* subsp. *damsela*. The representative isolates PD77, PD95, and PD102 were confirmed as *V. harveyi*, *V. plantisponsor*, and *Vibrio* sp., respectively. In addition, PD49 was identified as *Bacillus subtilis* (Table 1).

**Table 1. Identification of twenty one representative bacterial isolates via nucleotide BLAST searches of NCBI database using 16S rRNA gene sequences.**

| Serial no. | Isolate no. (Strain designation) | Identified species                      | Most relevant strain name | Max score | Total score | Query length | Query cover | E value | Identify match. | GenBank accession no. of corresponding sequence | GenBank accession no. of studied strains |
|------------|----------------------------------|-----------------------------------------|---------------------------|-----------|-------------|--------------|-------------|---------|-----------------|-------------------------------------------------|------------------------------------------|
| 01         | PD1                              | <i>V. parahaemolyticus</i>              | TV24                      | 2507      | 2507        | 1357         | 100%        | 0       | 100%            | MT549171.1                                      | PP967804                                 |
| 02         | PD3s                             | <i>V. alginolyticus</i>                 | SK1.2                     | 2567      | 2567        | 1394         | 100%        | 0       | 99.93%          | MT299658.1                                      | PP967805                                 |
| 03         | PD7                              | <i>V. parahaemolyticus</i>              | TV24                      | 2551      | 2551        | 1381         | 100%        | 0       | 100%            | MT549171.1                                      | PP967807                                 |
| 04         | PD20                             | <i>V. parahaemolyticus</i>              | MC32                      | 2571      | 2571        | 1399         | 100%        | 0       | 99.86%          | MT534026.1                                      | PP967809                                 |
| 05         | PD49                             | <i>Bacillus subtilis</i>                | ZIM3                      | 2427      | 2427        | 1314         | 100%        | 0       | 100%            | MT539995.1                                      | PP967810                                 |
| 06         | PD49b                            | <i>V. parahaemolyticus</i>              | IsC24A                    | 2543      | 27739       | 1377         | 100%        | 0       | 100%            | CP099946.1                                      | PP967811                                 |
| 07         | PD65                             | <i>V. cholerae</i>                      | SL4G                      | 2564      | 25371       | 1388         | 100%        | 0       | 100%            | CP053796.1                                      | PP967812                                 |
| 08         | PD67                             | <i>V. mediterranei</i>                  | YPL4-52                   | 2562      | 2562        | 1390         | 100%        | 0       | 100.00%         | MZ333454.1                                      | PP967813                                 |
| 09         | PD68                             | <i>V. cholerae</i>                      | W10G                      | 2555      | 25461       | 1383         | 100%        | 0       | 100.00%         | CP053794.1                                      | PP967814                                 |
| 10         | PD77                             | <i>V. harveyi</i>                       | 2011V1164                 | 2475      | 27095       | 1364         | 100%        | 0       | 100.00%         | CP035693.1                                      | PP967816                                 |
| 11         | PD78                             | <i>V. mediterranei</i>                  | YPL4-52                   | 2531      | 2531        | 1383         | 100%        | 0       | 99.93%          | MT368024.1                                      | PP967817                                 |
| 12         | PD86W                            | <i>V. cholerae</i>                      | W10G                      | 2601      | 25923       | 1408         | 100%        | 0       | 100.00%         | CP053794.1                                      | PP967822                                 |
| 13         | PD87                             | <i>V. cholerae</i>                      | FBC847                    | 2586      | 2586        | 1403         | 100%        | 0       | 100.00%         | OP630653.1                                      | PP967823                                 |
| 14         | PD89                             | <i>V. mediterranei</i>                  | YPL4-52                   | 2610      | 2610        | 1424         | 99%         | 0       | 100.00%         | MT368024.1                                      | PP967824                                 |
| 15         | PD95                             | <i>V. plantisponsor</i>                 | SF-SG 1B                  | 2490      | 2490        | 1356         | 99%         | 0       | 99.85%          | OL656923.1                                      | PP967827                                 |
| 16         | PD98                             | <i>V. mediterranei</i>                  | YPL2-19                   | 2569      | 2569        | 1397         | 100%        | 0       | 99.86%          | MT368016.1                                      | PP967829                                 |
| 17         | PD102                            | <i>Vibrio</i> sp.                       | 201709CJKOP-32            | 2599      | 2599        | 1413         | 100%        | 0       | 99.86%          | MG867483.1                                      | PP967833                                 |
| 18         | PD103                            | <i>P. damsela</i> subsp. <i>damsela</i> | CSTC-G1                   | 2603      | 2603        | 1409         | 100%        | 0       | 100.00%         | MK482014.1                                      | PP967834                                 |
| 19         | PD104                            | <i>V. mediterranei</i>                  | YPL4-52                   | 2615      | 2615        | 1419         | 100%        | 0       | 99.93%          | MT368024.1                                      | PP967835                                 |
| 20         | PD106                            | <i>V. mediterranei</i>                  | YPL4-52                   | 1190      | 1190        | 1433         | 51%         | 0       | 96.04%          | MT368024.1                                      | Not Submitted*                           |
| 21         | PD107                            | <i>V. vulnificus</i>                    | DC1                       | 2556      | 2556        | 1387         | 100%        | 0       | 99.93%          | MF774041.1                                      | PP967836                                 |

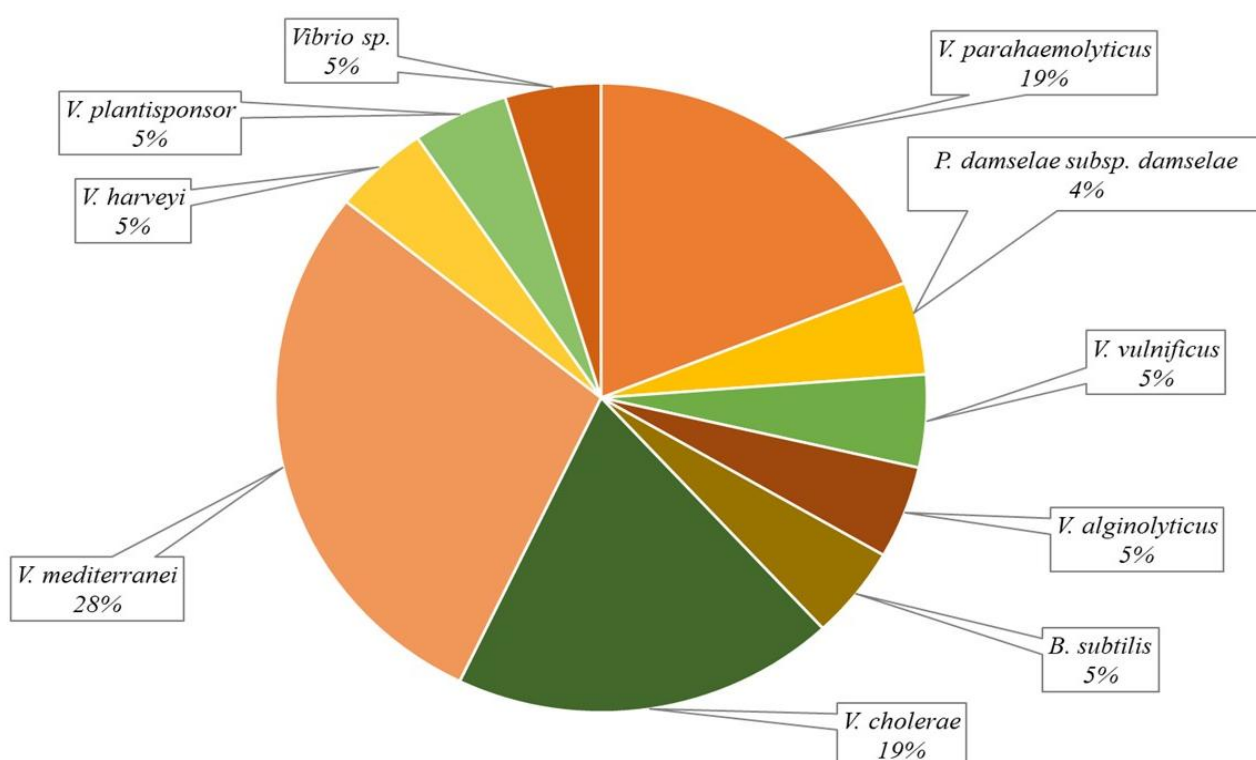
\* 16S rRNA gene sequence of PD106 strain was not submitted to the GenBank database because of its poor chromatograms, and was only used for the identification of the species.

Among all the representative isolates, six (28%) were identified as *V. mediterranei*, four (19%) as *V. cholerae*, four (19%) as *V. parahaemolyticus*, one (5%) as *V. vulnificus*, one (5%) as *P. damsela* subsp. *damsela*, one (5%) as *V. alginolyticus*, one (5%) as *V. harveyi*, one (5%) as *V. plantisponsor*, one (5%) as *Vibrio* sp., and one (5%) as *Bacillus subtilis* (Fig. 3). Among the isolates, PD106 was not used for further analysis and was not submitted to the GenBank database because of its poor chromatograms, and was only used for the identification of the species.

Notably, another manuscript on *P. damsela* subsp. *damsela* and *V. vulnificus* with detailed characterization and antibiotic susceptibility testing, is under preparation.

### Bacterial classifications of the studied isolates

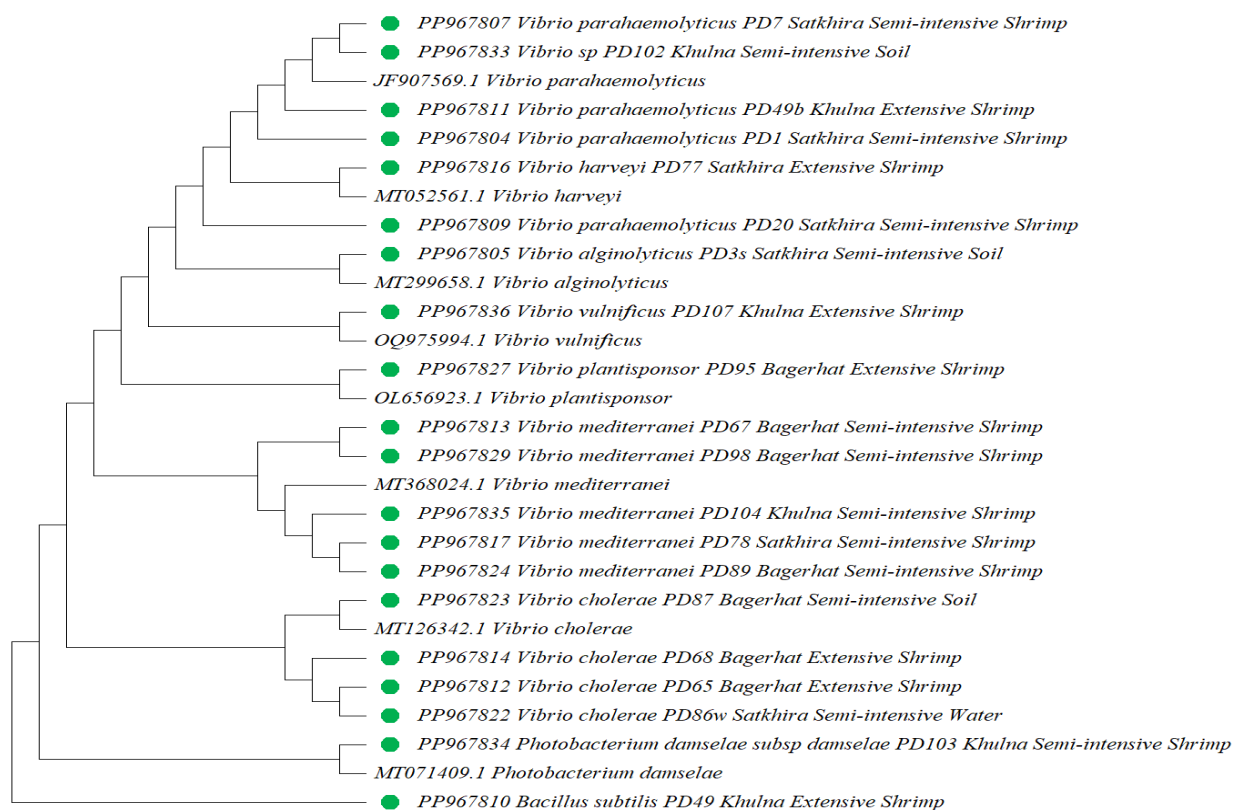
Table 2 classifies the identified bacteria by phylum, genus, and species. The bacteria are classified into two phyla: Proteobacteria and Firmicutes. Proteobacteria are classified into two genera, *Vibrio* and *Photobacterium*. Eight species were identified from the genus *Vibrio*, viz., *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *V. alginolyticus*, *V. mediterranei*, *V. harveyi*, *V. plantisponsor*, and *Vibrio* sp. The subspecies *Photobacterium damsela* subsp. *damsela* was identified as *Photobacterium damsela* and assigned to the genus *Photobacterium*. In addition, we identified *Bacillus* from the phylum Firmicutes. We identified a single species, *Bacillus subtilis*, within the genus *Bacillus*.



**Fig. 3. Diversity of the bacterial strains in cultured shrimp (*P. monodon*) of Bangladesh identified in the present study through 16S rRNA gene sequencing.**

**Table 2. Classifications of the representative diverse bacteria isolated from shrimp samples.**

| Phylum         | Genus                 | Species                        | Sub species                                         |
|----------------|-----------------------|--------------------------------|-----------------------------------------------------|
| Proteobacteria | <i>Vibrio</i>         | <i>Vibrio parahaemolyticus</i> |                                                     |
|                |                       | <i>Vibrio vulnificus</i>       |                                                     |
|                |                       | <i>Vibrio cholerae</i>         |                                                     |
|                |                       | <i>Vibrio alginolyticus</i>    |                                                     |
|                |                       | <i>Vibrio mediterranei</i>     |                                                     |
|                |                       | <i>Vibrio harveyi</i>          |                                                     |
|                |                       | <i>Vibrio plantisponsor</i>    |                                                     |
|                |                       | <i>Vibrio</i> sp.              |                                                     |
| Firmicutes     | <i>Photobacterium</i> | <i>Photobacterium damsela</i>  | <i>Photobacterium damsela</i> subsp. <i>damsela</i> |
|                | <i>Bacillus</i>       | <i>Bacillus subtilis</i>       |                                                     |



**Fig. 4.** A phylogenetic tree was constructed via MEGA 12 software, using the maximum likelihood method based on the 16S rRNA gene sequences of 19 representative isolates of the Vibrionaceae family and one isolate used as an outgroup from the genus *Bacillus*. Taxa labeled with green circles indicate the bacterial strains isolated in this study; other taxa were obtained from the NCBI database. The designation of taxa [PP967804 *Vibrio parahaemolyticus* PD1 Satkhira Semi-intensive Shrimp] denotes accession number of the strain, species name, strain code, sample collection place, farm type and sample

### Phylogenetic analysis of the studied isolates

We build a phylogenetic tree using 16S rRNA gene sequences from 19 representative isolates from the family Vibrionaceae and one isolate from the genus *Bacillus*, using MEGA 12 and the maximum likelihood method. One of our *B. subtilis* strains was used as an outgroup to confirm the taxonomic position of the isolates (Fig. 4).

The phylogenetic tree revealed that PD1, PD7, PD49b and PD102 were closely related to the Chinese *V. parahaemolyticus* strain VP1 (accession number JF907569.1), isolated from Chinese shrimp (*Fenneropenaeus chinensis*). PD3s closely matched the Indonesian *V. alginolyticus* strain SK1.2 (accession number MT299658.1), which was isolated from the north coast of Java. PD77 was found to be closely related to the Filipino *V. harveyi* strain ALM24 (accession number MT052561.1).

The phylogenetic tree further confirmed the taxonomic position of PD107, supporting its similarity to the allocated strain *V. vulnificus* VVA4 (accession number OQ975994.1), and PD95 was similar to the South Korean *V. plantisponsor* strain SF-SG 1B (accession number OL656923.1), which was isolated from the gut of sea eel in Samcheonpo. PD67, PD78, PD89, PD98, and PD104 were closely related to the Bangladeshi *V. mediterranei* strain YPL4-52 (accession number MT368024.1), isolated from Shrimp PL. PD103 is similar to the Chinese strain *P. damsela* TZH6 (accession number MT071409.1), which was isolated from water. PD65, PD68, PD86w, and PD87 are similar to the Chinese *V. cholerae* strain NQ5 (accession number MT126342.1).

### Discussion

This study successfully isolated and characterized diverse *Vibrio* species from black tiger shrimp (*Penaeus monodon*) farms in the southwestern coastal region of Bangladesh, revealing the complex microbial community associated with both diseased and apparently healthy shrimp in extensive and semi-intensive culture systems. In a study on *Vibrio*

colony morphology on TCBS agar, Kusumaningsih (2021) reported that all the bacterial colonies were yellow, with different growth rates. In another study, Tuhumury et al. (2024) reported yellow, round colonies on TCBS medium. The color turns yellow because the bacteria can ferment sucrose in the media. TCBS media, a selective medium containing thiosulfate, enhances *Vibrio* growth and reduces the development of other bacteria. Hikmawati et al. (2019) reported that 23 bacterial isolates expected to be *Vibrio* colonies were grouped together according to the similarity of their morphological features. Morphological investigations revealed that all colonies were round, yellow or green, and ranged from 2 to 5 mm in diameter. These traits are common in *Vibrio* colonies. Mailoa and Setha (2011) confirmed that green *Vibrio* colonies are caused by bacteria that cannot ferment sucrose and can decrease the pH of TCBS media. Likewise, the diverse morphological distribution shows variability in colony features among the eighty isolated suspected *Vibrio* spp. colonies in the present study, with most strains appearing yellow, followed by creamy yellow, greenish yellow, yellowish green, and green, respectively. These morphological variations align with previously documented phenotypic diversity within the genus *Vibrio*, where pigmentation differences often reflect species-level distinctions and metabolic capabilities, though morphology alone remains insufficient for definitive species identification. In a study on *Vibrio* species, Angala et al. (2025) also used PCR for 16S rRNA sequencing and reported positive bands at 1500 bp by gel electrophoresis for all six isolates. In another study, Sarjito et al. (2022) used PCR for 16S rRNA sequencing and reported positive results via gel electrophoresis at 1500 bp for eight isolates. These findings are consistent with the present study, where amplification of the 16S rRNA gene yielded PCR products of approximately 1500 bp, confirming successful amplification and supporting the reliability of the PCR protocol for molecular characterization of *Vibrio* isolates across different geographical locations and sample types.

In a study, Abdel-Raheem et al. (2025) used 16S rRNA gene sequencing among 80 samples and reported that eleven (13.75%) samples were *V. parahaemolyticus* positive. In another study using 16S rRNA gene sequencing, Chuchird et al. (2024) reported that the bacterial colonies that had been isolated from the hepatopancreas of the original diseased shrimp and grown on TCBS agar consisted of *V. parahaemolyticus* (99.93 % identity), *V. vulnificus* (97.61 % identity), *V. alginolyticus* (97.68 % identity), *V. mimicus* (97.86 % identity), and *P. damsela* (100 % identity), whereas those from the hemolymph were *V. parahaemolyticus* (99.93 % identity), *V. vulnificus* (97.76 % identity), *V. mimicus* (99.35 % identity), *V. cholerae* (97.19 % identity), and *P. damsela* (100 % identity). Fan et al. (2023) isolated and characterized 11 *V. mediterranei* isolates via 16S rRNA sequencing and studied their pathogenicity in larvae and juveniles of *S. constricta* and *Crossostrea sikamea*. In another study, Rungrasamee et al. (2016) isolated *V. harveyi* from black tiger shrimp and Pacific white shrimp and identified it by 16S rRNA sequencing. Manan et al. (2022) identified the bacterial species *V. diazotrophicus*, *V. diabolicus*, *V. natriegens* and *V. harveyi* in another study using 16S rRNA gene sequencing and reported similarities of 98.03–99.19%. PCR analysis of 16S ribosomal RNA suggested that the organism was *V. plantisponsor* in a study by Huang et al. (2025). In another study, Hong et al. (2022) identified *B. subtilis* via 16S rRNA sequencing. Another study related to vibriosis in white shrimp from extensive shrimp ponds by Sarjito et al. (2018) used 16S rRNA sequencing to identify *V. harveyi* (SJV 03), *V. parahaemolyticus* (SJV 05) and *V. alginolyticus* (SJV 19). In the present study, the molecular identification revealed 21 bacterial isolates with identity matches ranging from 96.04% to 100% against GenBank reference sequences. Among the isolates, *Vibrio* species dominated with multiple representatives viz., *V. mediterranei* (six isolates, 96.04-100% identity), *V. cholerae* (four isolates, 100% identity), *V. parahaemolyticus* (four isolates, 99.86-100%

identity), *V. harveyi* (one isolate, 100% identity), *V. alginolyticus* (one isolate, 99.93% identity), *V. plantisponsor* (one isolate, 99.85% identity), and *V. vulnificus* (one isolate, 99.93% identity). Additionally, one isolate remained identified only to the genus level as *Vibrio* sp. (99.86% identity), while two non-*Vibrio* species were also identified as *Bacillus subtilis* (100% identity) and *P. damsela* subsp. *damsela* (100% identity). The high identity matches (>99% for most isolates) demonstrated the reliability of 16S rRNA gene sequencing for species-level identification, though one isolate (PD106) showed notably lower query coverage at 51% with 96.04% identity match due to poor sequence chromatograms. The findings of the present study are consistent with those reported in the aforementioned research.

In a study by Nurhafizah et al. (2021), a 16S rRNA gene-based phylogenetic tree was analyzed using neighbour-joining, maximum likelihood, and maximum parsimony methods, and all three strains in the *V. harveyi* cluster closely matched this result. In another study, Kumar et al. (2020) generated a maximum likelihood phylogenetic tree based on 16S rRNA genes from 36 *Vibrio* genomes and revealed their evolutionary relationships. Wan et al. (2025) differentiated among 79 *Vibrio* populations via a 16S rRNA gene-based phylogenetic tree. Dinçtürk et al. (2023) constructed a phylogenetic tree of the genus *Vibrio* using the maximum likelihood method, with six new sequences identified from the tissue of the sponge *Sarcotragus foetidus*. In our study, a phylogenetic tree was constructed via MEGA 12 software, using the maximum likelihood method based on 16S rRNA gene sequences of 19 representative isolates of the Vibrionaceae family and one isolate used as an outgroup from the genus *Bacillus*, revealing distinct clustering patterns among the identified *Vibrio* species and demonstrating clear genetic relationships between isolates from different geographical locations and sample types. Notably, isolates of the same species grouped together with their respective GenBank reference sequences, confirming the accuracy of molecular identification

and indicating species-level phylogenetic coherence. The *V. mediterranei* isolates (PD67, PD78, PD89, PD98, and PD104) formed a well-supported monophyletic clade, suggesting genetic similarity among strains isolated from different districts across the southwestern region. Similarly, *V. cholerae* isolates (PD65, PD68, PD86W, and PD87) clustered together, indicating genetic relatedness among strains recovered from shrimp, soil, and water samples. The *V. parahaemolyticus* isolates were distributed across multiple closely related branches, reflecting potential genetic diversity within this species despite their taxonomic classification. The clear separation of *Bacillus subtilis* (PD49) as an outgroup confirmed its phylogenetic distinctness from the *Vibrio* species and validating the tree topology. The results of the current research align with the observations documented in previous studies. This phylogenetic clustering pattern suggests that the identified *Vibrio* species may share common evolutionary origins within the region's shrimp aquaculture environment, while also highlighting the co-occurrence of multiple *Vibrio* species in black tiger shrimp farming systems.

## Conclusion

This study confirmed diverse bacterial populations in black tiger shrimp (*Penaeus monodon*) from southwestern Bangladesh. Molecular identification through 16S rRNA gene sequencing of 21 representative isolates confirmed the presence of multiple *Vibrio* species, with *V. mediterranei* (28%), *V. cholerae* (19%), and *V. parahaemolyticus* (19%) being the most prevalent, alongside other species including *V. vulnificus*, *V. alginolyticus*, *V. harveyi*, *V. plantisponsor*, and *Vibrio* sp. Additionally, non-*Vibrio* species such as *Photobacterium damsela* subsp. *damsela* and *Bacillus subtilis* were also identified, indicating a complex microbial community composition. Phylogenetic analysis revealed clear clustering patterns among species, with isolates showing close genetic relationships to reference strains. Notably, this study also detected pathogens with zoonotic potential, viz. *P. damsela* subsp. *damsela* and *V. vulnificus*, suggesting a risk

of zoonotic infection in shrimp farmers. The findings provide crucial baseline data on the bacterial diversity of *P. monodon* in Bangladesh, highlighting the co-occurrence of multiple potentially pathogenic *Vibrio* species that may threaten shrimp health and aquaculture sustainability in the region and warrant continued surveillance, good rearing practices, and overall biosecurity.

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## Authors contribution

Md Faisal Ahmed: Sampling, investigation, methodology, data interpretation and visualization, writing - original draft; Shawon Ahmmed: Data curation, formal analysis, visualization, data interpretation, validation, writing - reviewing and editing; Mst. Khadiza Begum: Investigation, methodology, visualization, writing - reviewing and editing; Mohammad Shamsur Rahman: Conceptualization, methodology, investigation, project administration, validation, fund acquisition, supervision, writing - reviewing and editing

## Conflict of interest statement

The authors declare no known conflicts of interest.

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## Ethical statement

The authors obtained animal ethics clearance from the concerned authorities. However, the protocol of this study was approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka, Bangladesh (Ref. No. 292/Biol. Scs.) and the research was conducted in accordance with the guidelines of the 'Animal Welfare Act

2019’ approved by the National Parliament of Bangladesh.

### Data availability statement

All data are included within the manuscript. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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