



Acute hepatopancreatic necrosis disease in black tiger shrimp: evidence from the 2018 Bangladesh outbreak

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Abstract

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome, is a major threat to shrimp aquaculture. We investigated a mortality event affecting black tiger shrimp (*Penaeus monodon*) in southern Bangladesh in 2018. Hepatopancreatic tissues from 248 moribund shrimp, collected from 39 grow-out ponds on 31 farms, were examined by AP4 nested PCR, microbiological culture, phenotypic characterization, 16S rRNA gene sequencing of representative isolate MKD03, and challenge bioassays. The AHPND-associated AP4 target was detected in 168 of 248 shrimp (67.7%); at least one AP4-positive shrimp was found in 74.4% ponds and 74.2% farms. Six presumptive *Vibrio* isolates were AP4-positive. Isolate MKD03 showed 99% 16S rRNA sequence similarity to *Vibrio parahaemolyticus*, supporting its provisional taxonomic placement. Challenge with MKD03 produced 60% and 90% mortality in *Artemia* nauplii at 24 and 48 hr, respectively, and 94% mortality in *P. monodon* post-larvae at 24 hr; mortality remained substantially lower in unchallenged controls. Recovery of AP4-positive bacteria from challenged post-larvae further supported the association with the outbreak. Because histopathology and species-specific or multilocus confirmation were not performed, these findings support, but do not definitively establish, the involvement of a putative *V. parahaemolyticus* AHPND isolate in the 2018 event. The study provides outbreak-specific baseline evidence for targeted surveillance, biosecurity, and preparedness in Bangladesh shrimp aquaculture.

Keywords: acute hepatopancreatic necrosis; AP4 nested PCR; *Penaeus monodon*; pirAB; putative VpAHPND

1 | INTRODUCTION

Black tiger shrimp (*Penaeus monodon*) is an economically important aquaculture species in Bangladesh, where shrimp production supports export income and coastal

livelihoods. Shrimp farming nevertheless remains vulnerable to infectious diseases, including luminous vibriosis, white spot disease, yellow head disease, Taura syndrome, and acute hepatopancreatic necrosis disease (AHPND),

which was historically described as early mortality syndrome (Ruangpan and Kitao 1991; Hossain *et al.* 2004; Flegel 2006; Hossain *et al.* 2017).

AHPND is associated with strains of *Vibrio parahaemolyticus* and related *Vibrio* species that harbor plasmid-borne *pirA* and *pirB* toxin genes (Kumar *et al.* 2020; Kumar *et al.* 2021). Because this virulence locus can occur in more than one *Vibrio* species, detection of the AHPND-associated genotype and identification of the bacterial species carrying it are related but distinct analytical questions. Moreover, clinical signs compatible with AHPND may overlap with conventional vibriosis, viral disease, and mixed infections, making laboratory confirmation important.

AHPND primarily affects post-larvae and juveniles during the first 30 – 35 days after stocking and may cause mortality approaching 100% (World Organization for Animal Health 2023). The disease was first reported in China in 2009, and its bacterial etiology was demonstrated in 2013 (Lightner *et al.* 2012; Tran *et al.* 2013). It subsequently spread to Vietnam, Malaysia, Thailand, Mexico, and other shrimp-producing regions (Leaño and Mohan 2012; Gomez-gil *et al.* 2014; Soto-Rodriguez *et al.* 2015).

Bangladesh experienced a major mortality event among farmed *P. monodon* in 2018, with gross signs compatible with AHPND. Because comparable signs may have several causes, an outbreak investigation integrating field sampling, virulence-targeted PCR, bacterial isolation, taxonomic characterization, and experimental challenge was undertaken to evaluate the organisms associated with the event.

The contribution of this study is outbreak-specific and regional rather than methodological. It describes AP4 detection at three epidemiological levels (individual shrimp, ponds, and farms) across major shrimp-producing areas of southern Bangladesh; recovery of AP4-positive *Vibrio* isolates; 16S rRNA-based characterization of isolate MKD03; challenge experiments in *Artemia* and *P. monodon* post-larvae; and re-isolation followed by repeat AP4 testing. This field-to-laboratory design provides baseline evidence on the scale and distribution of AHPND-associated detection during the 2018 event.

Recent shrimp-pathogen surveillance has shown that combining molecular testing with pathological and epidemiological information broadens pathogen detection and improves the interpretation of positive molecular results (Intriago *et al.* 2024). Accordingly, the present investigation is interpreted within its diagnostic limits and emphasizes targeted surveillance, rapid laboratory confirmation, and practical biosecurity rather than claiming a novel diagnostic method.

2 | METHODOLOGY

2.1 Sample collection

Moribund or weak shrimp ($n = 248$) observed near pond

margins were collected using a hand net. Samples were obtained from 39 grow-out ponds on 31 farms in four subdistricts (Debhata, Dumuria, Rampal, and Paikgachha) across Satkhira, Bagerhat, and Khulna districts, Bangladesh (Table S1). All sampled shrimp showed gross signs compatible with AHPND, including a pale hepatopancreas and an empty stomach and gut. Samples were transported on dry ice and delivered to the laboratory within 6 hr of collection.

2.2 Total DNA extraction from hepatopancreatic homogenates

Hepatopancreatic tissues were aseptically dissected from moribund shrimp and homogenized in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 ; pH 7.4). The homogenates were centrifuged at $500 \times g$ for 5 min at 4°C (Tomy MX-305 High-Speed Refrigerated Microcentrifuge, Japan) to remove coarse debris. The resulting supernatant was assigned the corresponding sample identifier and, when AP4 testing was positive, divided into two aliquots: one for molecular testing and one for microbiological analysis.

For total DNA extraction, 1 mL of each labeled homogenate was centrifuged at $10,000 \times g$ for 10 min at 4°C. The pellet was washed three times by resuspending in 1 mL PBS, followed by centrifugation at $10,000 \times g$ for 5 min at 4°C. After the final wash, the pellet was resuspended in 200 μL nuclease-free water. Total DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions and used as the template for PCR analysis.

2.3 AP4 nested-PCR detection of the AHPND-associated *pirAB* target

The AHPND-associated virulence target was detected using the AP4 nested-PCR assay developed by Dangtip *et al.* (2015) and recognized in the WOA Aquatic Manual (Dangtip *et al.* 2015; World Organization for Animal Health 2023). The 230-bp nested product spans part of the *pirA* region, the intervening spacer, and the adjacent *pirB* region; thus, AP4 positivity supports the presence of the characteristic contiguous AHPND-associated target but does not represent sequencing of the complete *pirA* and *pirB* genes. DNA templates (100 – 150 ng) were obtained from shrimp tissues or bacterial isolates recovered from challenged *Artemia* nauplii or *P. monodon* post-larvae.

Two primer pairs were used. First-step primers: Forward: AP4-F1, 5'-ATG AGT AAC AAT ATA AAA CAT GAA AC-3'; Reverse: AP4-R1, 5'-ACG ATT TCG ACG TTC CCC AA-3'; Second-step primers: Forward AP4-F2, 5'-TTG AGA ATA CGG GAC GTG G-3'; Reverse AP4-R2, 5'-GTT AGT CAT GTG AGC ACC TTC-3'.

The thermal cycling conditions for the first-step PCR included an initial denaturation at 94°C for 5 min, fol-

lowed by 30 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C, with a final extension at 72°C for 10 min. For the second step, a portion (10% of the reaction mix) was used as the template, with the annealing temperature adjusted to 60°C. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide and documented using a UV transilluminator (microDOC, Cleaver Scientific, USA).

2.4 Isolation and presumptive identification of AHPND-associated bacterial isolates

Homogenates selected for microbiological analysis after AP4 positivity were enriched in alkaline peptone water (APW) for 2 hr at 37°C. A 100 µL aliquot was then plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK) and incubated at 30°C for 24 hr. Pure isolates were subcultured on CHROMagar, XLD agar, and MacConkey agar. Presumptive identification followed standard procedures, including Gram staining and tests for catalase, oxidase, motility, and urease; glucose fermentation; and H₂S production on Kligler iron agar; as well as indole, citrate, methyl red, and Voges-Proskauer tests (Jones *et al.* 2012).

2.5 DNA extraction from bacterial cultures

Bacterial isolates were grown on LB agar for 16 hr at 37°C. A single colony was suspended in 100 µL of sterile deionized water and vortexed. DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and stored at -20°C until use.

2.6 16S rRNA gene sequencing

Genomic DNA (200 – 250 ng) from isolate MKD03 was used as the template to amplify the 16S rRNA gene with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTACGACTT-3') and Taq 2X Master Mix (New England Biolabs, Hertfordshire, UK) (Frank *et al.* 2008). Amplification was performed in an Aeris PCR Thermal Cycler (ESCO, UK) with initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 90 sec, 55°C for 60 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min. Products were resolved on 1% agarose gels with a 1-kb Plus DNA ladder (New England Biolabs, USA), stained with ethidium bromide, and visualized under UV illumination (microDOC, Cleaver Scientific, USA).

PCR products were purified using the PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions and sequenced bidirectionally with primers 27F and 1492R using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA).

2.7 Phylogenetic analysis

Partial sequences were assembled into full-length sequences (1300–1400 bp) using SeqMan Genome Assem-

bler (DNASTar, Madison, WI, USA). These sequences were compared with the GenBank database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/GenBank) using the Basic Local Alignment Search Tool (BLAST) to identify closely related taxa. The final dataset included study sequence, representative *V. parahaemolyticus* strains from different sources/locations, closely related *Vibrio* species, and *Photobacterium phosphoreum* ATCC 11040 (NR_115205.1) as the outgroup. Multiple sequence alignment was performed using ClustalW. A phylogenetic tree was constructed using the neighbor-joining algorithm (Saitou and Nei 1987; Tamura and Nei 1993; Thompson *et al.* 1994), with 1000 bootstrap replicates using MEGA 12 (Version 12.1).

2.8 Artemia lethality bioassay

A brine shrimp lethality bioassay was performed to evaluate the cytotoxicity of the pathogenic isolates against *Artemia* nauplii, a model eukaryotic organism for in vivo pathogenicity studies. *Artemia* nauplii (Red Jungle Pro 80, East Coast Marine Products & Supplies, USA) were cultured for 48 hours before the experiment. Groups of 10 nauplii were transferred into 25 mL flasks containing 9 mL of filtered artificial seawater (salinity: 3.5%). Each flask was inoculated with 1 mL of bacterial culture (1×10^4 CFU mL⁻¹) of the identified pathogen. One flask containing 10 nauplii was left as a control without bacterial inoculation. *Artemia* mortality was recorded 24 hr and 48 hr post-inoculation. This experiment was independently replicated five times to ensure the reliability and reproducibility of the results.

2.9 Penaeus monodon post-larval bioassay

Post-larvae of *P. monodon* were obtained from Balaka Hatchery, Cox's Bazar, Bangladesh, rinsed with sterile seawater, and acclimatized for 3 – 5 hr at 28°C in 5-L beakers containing sterile seawater at 15 ppt. They were fed commercial aquafeed (Japonica, Nahar Agro, Bangladesh) (Hossain *et al.* 2017). Each challenge replicate contained 10 post-larvae in 270 mL rearing water to which 30 mL bacterial suspension (1×10^4 CFU mL⁻¹) was added, producing a 300-mL working volume, a 10% (v/v) inoculum, and a nominal final concentration of 1×10^3 CFU mL⁻¹. Control replicates contained 10 post-larvae under the same conditions without bacterial inoculum. Challenge and control conditions were tested in triplicate, and mortality was recorded after 24 hr.

2.10 Data analysis

Mortality percentages from challenged and control replicates were compared using two-tailed unpaired Student's *t*-tests in VassarStats. Normality and homogeneity of variance were assessed before testing. Statistical significance was defined as $p < 0.05$.

3 | RESULTS

3.1 AP4 nested-PCR detection of the AHPND-associated target

Total DNA was extracted from the hepatopancreatic homogenates of 248 moribund *P. monodon*. AP4 nested PCR produced the expected 230-bp amplicon in 168 of 248 shrimp (67.7%), indicating detection of the AHPND-associated contiguous *pirA-pirB* target (Figure 1A – 1C; Table S1). Because the sampling was restricted to mori-

bund or weak shrimp from outbreak-affected farms, this value is reported as the AP4-positive proportion among the sampled shrimp, not as population prevalence.

At the pond level, 29 of 39 sampled grow-out ponds (74.4%) contained at least one AP4-positive shrimp. At the farm level, 23 of 31 farms (74.2%) contained at least one positive pond. These sample-, pond-, and farm-level detection frequencies use different denominators and are therefore not interchangeable (Figure 1D; Table S1).

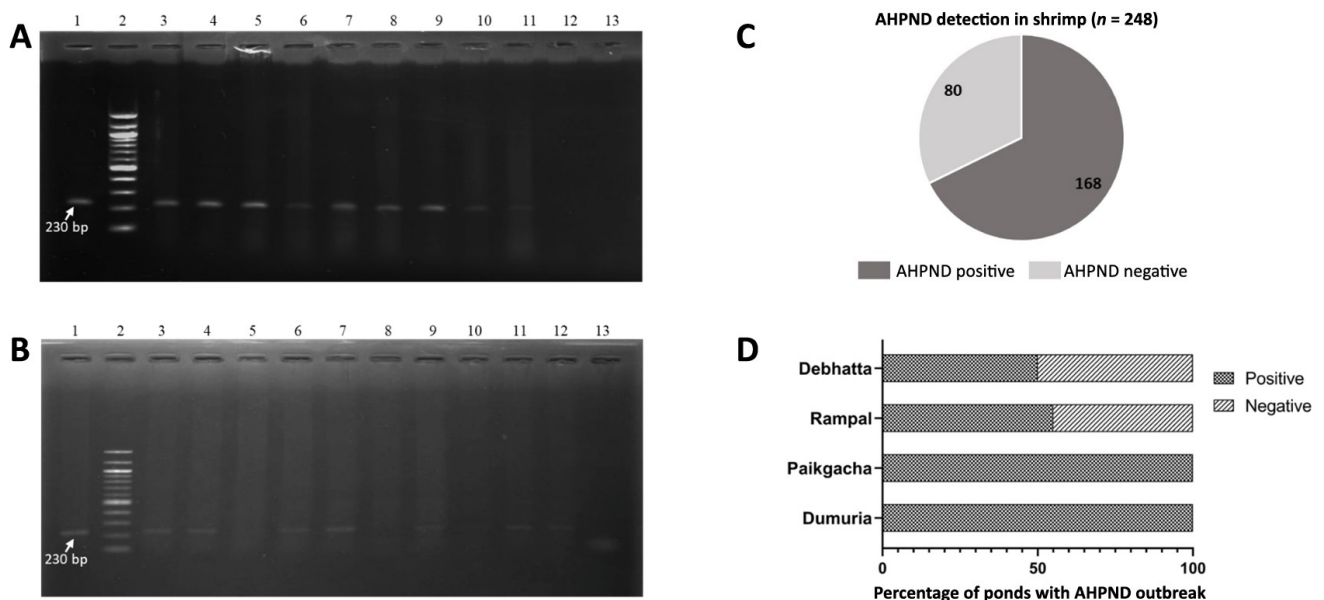


FIGURE 1 AP4 nested-PCR detection of the AHPND-associated target in moribund *Penaeus monodon*. Panels A and B show representative 230-bp AP4 products from sampled shrimps collected in Dumuria and Debhata, respectively. In each gel, lane 1 contains the positive control (a synthetic plasmid carrying the target amplicon), lane 2 contains the DNA ladder, lanes 3–12 contain sample DNA, and lane 13 contains the negative control. Panel C is defined as sample-level AP4 detection, whereas Panel D is defined as the proportion of sampled ponds with at least one AP4-positive shrimp. Lane assignments and denominators are stated explicitly.

3.2 Isolation and characterization of AP4-positive *Vibrio* isolates

Bacteriological culture was performed on 90 AP4-positive homogenates selected from the 29 AP4-positive ponds. Six pure presumptive *Vibrio* isolates were recovered, corresponding to an isolate recovery rate of 6.7% (6/90). All six isolates exhibited similar colony morphology and biochemical characteristics and produced the expected 230-bp AP4 amplicon. One randomly selected representative isolate, MKD03, was subsequently identified as *V. parahaemolyticus* by 16S rRNA gene sequencing, showing 99% sequence identity. BLAST analysis of the 16S rDNA sequence revealed 99% similarity between strain MKD03 and *V. parahaemolyticus*. The phylogenetic analysis placed MKD03 within the genus *Vibrio*, clustering with 16S rDNA sequences from other *V. parahaemolyticus* strains (Figure 2). The isolate was subsequently assigned an accession number, OQ780863, in the GenBank data-

base.

3.3 Mortality following challenge with isolate MKD03

Artemia nauplii challenged with MKD03 showed a mean mortality of 60% (SD = 3.43) at 24 h and 90% (SD = 3.22) at 48 hr. Challenged *P. monodon* post-larvae showed 94% mortality (SD = 4.33) at 24 hr (Figure 3). Mortality was substantially lower in the corresponding unchallenged control groups; therefore, the control results are interpreted as evidence of a challenge-associated difference rather than proof that the animals were generally disease-free. Challenged-versus-control differences were statistically significant at $p < 0.05$ under the reported analysis.

To evaluate recovery of the challenge organism, bacterial isolates obtained from dead *P. monodon* post-larvae were tested by AP4 nested PCR. All tested re-isolates produced the expected 230-bp product spanning the

AHPND-associated *pirA-pirB* target (Figure 4). No bacterial growth was recovered on TCBS agar from the unchallenged control post-larvae. Together with the mortality difference, these findings support an association between challenge with MKD03 and the observed post-larval mor-

talities; in the absence of histopathology and species-specific re-identification of the re-isolates, they are not interpreted as definitive lesion-level confirmation of AHPND.

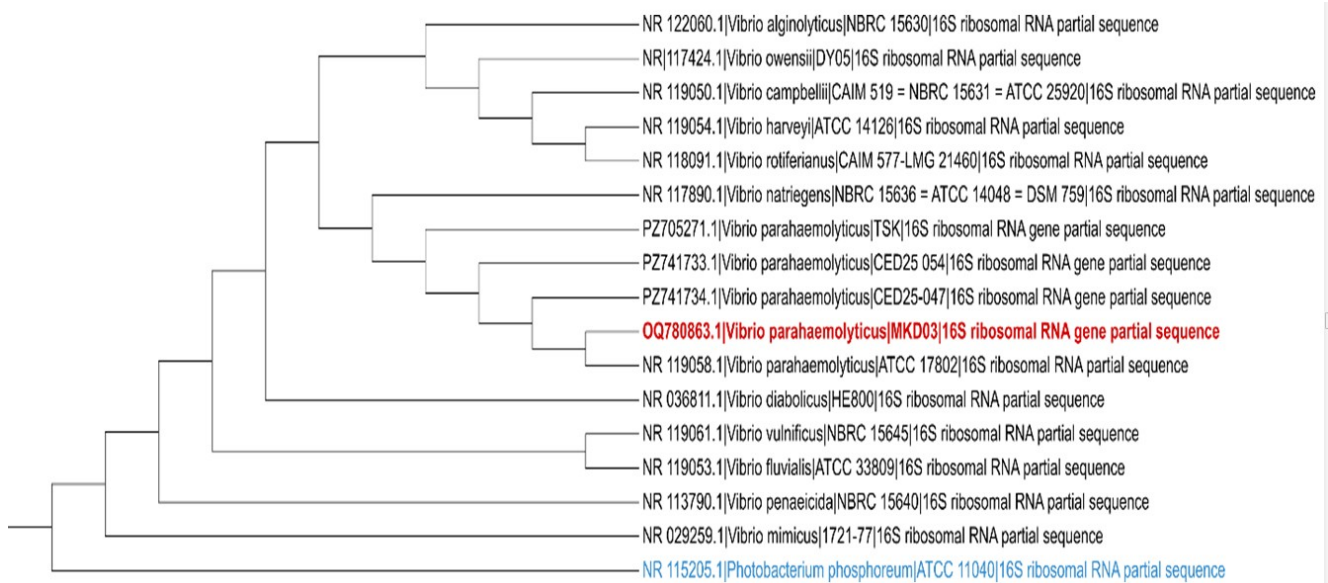


FIGURE 2 Phylogenetic placement of *Vibrio parahaemolyticus* isolate MKD03 based on partial 16S rRNA gene sequences. The tree was reconstructed using MKD03 (OQ780863.1) (Red), non-redundant accessioned *Vibrio parahaemolyticus* reference strains, closely related *Vibrio* species, and *Photobacterium phosphoreum* ATCC 11040 (NR_115205.1) (blue) as the outgroup. The evolutionary distances were estimated using neighbour joining model, and branch support was assessed with 1,000 bootstrap replicates. Bootstrap values $\geq 70\%$ are shown at branch nodes. The scale bar represents substitutions per site.

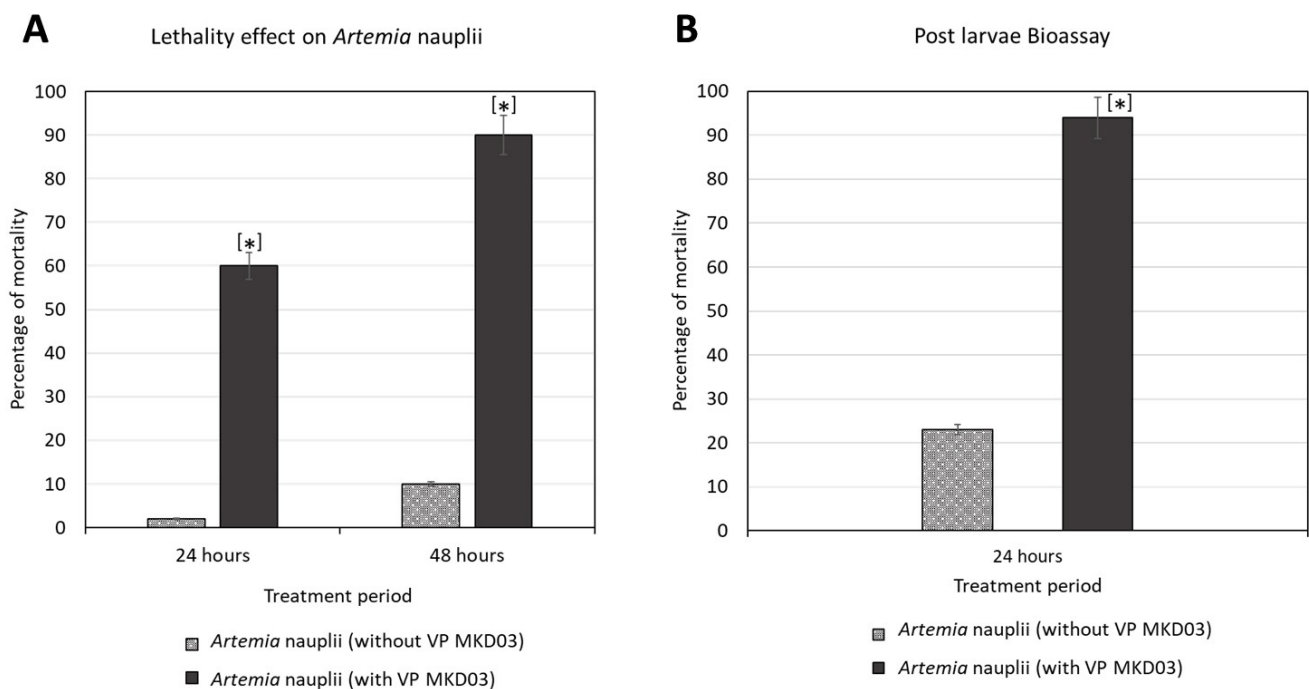


FIGURE 3 Mortality of *Artemia* nauplii (A) and black tiger shrimp post-larvae (B) following challenge with putative VpAHPND isolate MKD03. Bars show mean mortality with variability across replicates. Challenged-versus-control comparisons were statistically significant (*) at $p < 0.05$ under the analysis described in the Methodology.

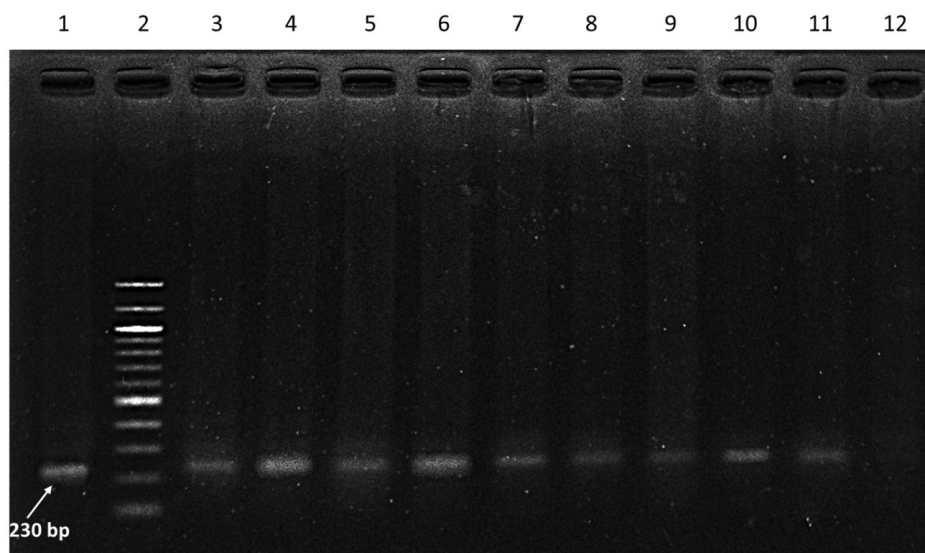


FIGURE 4 AP4 nested-PCR testing of bacterial re-isolates from challenged *Penaeus monodon* post-larvae. Lanes 3-11 contain bacterial isolates recovered from dead challenged post-larvae; the expected 230-bp AP4 product indicates the AHPND-associated target. Lane 1 contains the positive control, lane 2 the DNA ladder, and lane 12 the negative control.

4 | DISCUSSION

AHPND has substantially affected shrimp production in Asia and other regions. The 2018 mortality event among farmed *P. monodon* in southern Bangladesh, therefore, warranted an investigation to distinguish gross signs from molecular and culture-based evidence. In the present study, AP4 detection in moribund shrimp, recovery of AP4-positive *Vibrio* isolates, provisional 16S rRNA-based placement of MKD03 near *V. parahaemolyticus*, experimental challenge, bacterial re-isolation, and repeat AP4 detection collectively support the association of a putative VpAHPND isolate with the event. The evidence does not, however, warrant unequivocal species-level or histopathological confirmation.

AHPND was first reported in 2009, and its bacterial etiology was demonstrated in 2013 (Tran *et al.* 2013). Clinical signs such as lethargy, anorexia, empty gut, and a pale or atrophied hepatopancreas overlap with other bacterial and viral diseases, limiting field diagnosis (Hong *et al.* 2016; Schofield *et al.* 2021). Molecular assays targeting the plasmid-borne virulence locus therefore have an important diagnostic role. The AP4 nested-PCR assay used here targets a contiguous segment spanning the *pirA* region, the intergenic spacer, and the adjacent *pirB* region and is recognized by WOAHP as a sensitive conventional method (Dangtip *et al.* 2015; World Organization for Animal Health 2023). AP4 positivity should nevertheless be interpreted as evidence of the AHPND-associated genotype, not as independent proof of the species carrying the locus or as complete sequencing of both toxin genes.

The study extends the earlier report of AHPND-positive *V. parahaemolyticus* in Bangladesh (Eshik *et al.* 2018) by providing a geographically structured investigation of a subsequent mortality event in *P. monodon*. Among the purposively sampled moribund shrimp, 168 of 248 (67.7%) were AP4-positive. At least one positive

shrimp occurred in 29 of 39 ponds (74.4%) and 23 of 31 farms (74.2%). These proportions describe detection rates in outbreak-focused sampling and should not be generalized to population prevalence. Their value lies in documenting the distribution of AHPND-associated molecular detection across sampled production units and providing a baseline for future surveillance.

Challenge with MKD03 was followed by substantially greater mortality in both *Artemia* nauplii and *P. monodon* post-larvae than in unchallenged controls. Recovery of AP4-positive bacteria from dead challenged post-larvae provides additional support for a challenge-associated effect. However, low control mortality does not by itself establish pathogen-free status, and the absence of histopathology prevents demonstration of the characteristic hepatopancreatic lesions required for lesion-level confirmation.

Environmental conditions, including water temperature and salinity, influence the abundance and persistence of pathogenic *Vibrio* populations (Takemura *et al.* 2014; Velazquez-Roman *et al.* 2014; Siddique *et al.* 2021). AHPND outbreaks have caused major production losses in regional shrimp industries, including Thailand (Joshi *et al.* 2014; Shinn *et al.* 2018). Following the 2017 report from southwestern Bangladesh (Eshik *et al.* 2018), the present detection of at least one AP4-positive pond on 23 of 31 sampled farms in 2018 indicates that AHPND-associated organisms remained an important hazard in the surveyed southern districts. The study was not designed to estimate national prevalence or determine environmental risk factors, and these questions require prospective, systematically sampled surveillance.

The findings support routine, risk-based molecular surveillance that reports results separately for individual shrimp, ponds, and farms. When feasible, virulence-targeted PCR should be paired with histopathology, spe-

cies-specific identification, and confirmatory sequencing. At the farm level, screening of incoming post-larvae and broodstock, water-quality management, movement controls, sanitation, and rapid investigation of unusual mortality can strengthen biosecurity. Recent passive-surveillance work also illustrates the value of combining multiple diagnostic modalities because molecular detection and tissue lesions may not always coincide (Intriago *et al.* 2024).

This investigation has several limitations. Only MKD03 among the six AP4-positive presumptive *Vibrio* isolates underwent 16S rRNA sequencing, so its taxonomic placement cannot be generalized to the remaining isolates. In addition, 16S rRNA alone may not distinguish closely related *Vibrio* species; species-specific chromosomal markers such as *toxR* or *tlh*, multilocus sequence analysis, or whole-genome sequencing would provide greater resolution (Kim *et al.* 1999). AP4 detected the characteristic contiguous AHPND-associated target, but the complete *pirA* and *pirB* genes were not independently sequenced. Histopathology was not performed, and outbreak-focused sampling of moribund shrimp does not support inference of population prevalence. These limitations have been incorporated into the interpretation and conclusion.

5 | CONCLUSIONS

The combined field detection, isolation, 16S rRNA-based taxonomic placement, AP4 nested-PCR positivity, experimental challenge, re-isolation, and repeat molecular detection support the association of an AHPND-causing *Vibrio* isolate, provisionally identified as *V. parahaemolyticus* MKD03, with the 2018 mortality event in black tiger shrimp in southern Bangladesh. Because species-specific or multilocus confirmation and histopathology were not performed, the findings should be interpreted as strong outbreak-associated evidence rather than definitive taxonomic and lesion-level confirmation. The study nevertheless provides baseline data for targeted molecular surveillance, standardized diagnostics, strengthened biosecurity, and preparedness in Bangladesh shrimp aquaculture.

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ETHICAL APPROVAL

As post-larvae of shrimp (*Penaeus monodon*) are not currently covered by the national regulations on animal experimentation in Bangladesh, specific ethical approval for their use was not required. Nonetheless, all bioassays were conducted in accordance with standard welfare pro-

ocols to minimize stress and suffering in experimental animals.

CONFLICT OF INTEREST

The authors declare no known competing financial interests or personal relationships that could have influenced the work. The funding organization had no role in study design, data collection/analysis, decision to publish, or manuscript preparation.

AUTHORS' CONTRIBUTION

Deb Jyoti Mukharjee: Methodology, Investigation. Samiur Rahim: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Abu Mohammed Ramim: Writing – original draft. Md. Mahmud Hasan: Formal analysis. Marufa Zerin Akhter: Supervision, Writing – review & editing. Shakila Nargis Khan: Supervision, Writing – review & editing. Muhammad Manjurul Karim: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. All authors read and approved the final manuscript and agreed to its submission.

AI-USE STATEMENT

An AI-assisted tool (Grammarly) was used solely to improve language, grammar, and readability. Generative AI was not used to generate scientific content, and the authors take full responsibility for the final manuscript.

DATA AVAILABILITY STATEMENT

All data relevant to this study are included within the paper and its Supporting Information. The partial 16S rRNA gene sequence of isolate MKD03 has been deposited in GenBank under accession number OQ780863.

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