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ORIGINAL RESEARCH ARTICLE

Molecular and Histopathological Detection of *Vibrio harveyi* Associated with Mortality in Asian Sea Bass Cultured in Sri Lanka

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ABSTRACT

Asian sea bass (*Lates calcarifer*), commonly known as barramundi, is an economically important food fish inhabiting coastal and brackish waters. In Sri Lanka, demand for commercial and recreational fishing has increased due to its mild flavor and culinary versatility. However, disease outbreaks pose significant challenges to aquaculture production, particularly in cage culture systems. This study investigated the causative agent of mass mortality events in Asian sea bass farms in Trincomalee, Sri Lanka. Fish exhibiting clinical signs, including skin ulcers, fin rot, exophthalmia, and lethargy, were collected for examination. Samples from the kidney, liver, and spleen were cultured on Tryptic Soy Agar (TSA) and Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar at 37 °C for 24-48 hr. Isolates produced small to medium, round, convex colonies on TSA and green colonies on TCBS. Biochemical characterization revealed Gram-negative, oxidase-positive, catalase-positive, motile rods that fermented glucose without gas production. Molecular identification using 16S rRNA polymerase chain reaction confirmed the pathogen as *Vibrio harveyi* with 99.44% similarity to strain CP014038.2. The sequence was deposited in GenBank (accession number OR351233). Histopathological examination revealed tubular injury, necrotic epithelial cells, and macrophage-lymphocyte infiltration in liver and kidney tissues. This study confirms that *Vibrio harveyi* is strongly associated with Vibriosis, causing mass mortality in Asian sea bass cage culture in Trincomalee. These findings provide essential baseline data for developing effective disease prevention and control strategies in Sri Lankan aquaculture systems.

Keywords: *Aquaculture disease, Asian sea bass (Lates calcarifer), Molecular identification, Vibrio harveyi, Vibriosis*

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INTRODUCTION

Sea-cage aquaculture has emerged as a vital economic sector along the eastern coast of Sri Lanka, with Asian sea bass (*Lates calcarifer*), locally known as Modha, representing a cornerstone species for both domestic consumption and export markets. The species' rapid growth rate, adaptability to a wide range of salinities, excellent flesh quality, and high market value have positioned it as a preferred candidate for intensive mariculture development in the region (Araujo et al., 2022; Parappurathu et al., 2023). Following the establishment of Sri Lanka's first commercial offshore finfish farm by Oceanpick (Pvt) Ltd in Cod Bay, Trincomalee, in 2012, the industry has experienced substantial expansion, with current production capacity approaching 1,200 tonnes annually and projected targets of 3,000 tonnes per year (Udugama et al., 2025).

Among the diverse pathogens affecting Asian sea bass aquaculture, bacterial infections are the predominant cause of morbidity and mortality, with Vibriosis among the most economically significant diseases worldwide (Yue and Guo, 2025). *Vibrio harveyi*, a Gram-negative, bioluminescent, halophilic bacterium belonging to the family Vibrionaceae, has been identified as a primary etiological agent of Vibriosis in cultured Asian sea bass across the Asia-Pacific regions (Campbell et al., 2026; Idris et al., 2022). The pathogen exhibits broad host specificity, infecting various life stages from larvae to market-size adults, and demonstrates high virulence under conducive environmental conditions. Clinical manifestations of *V. harveyi* infection are diverse and often severe, encompassing external signs such as skin ulceration, hemorrhagic lesions, fin and tail rot, exophthalmia (pop-eye), and scale loss, alongside systemic indicators including lethargy, anorexia, abdominal distension,

erratic swimming behavior, and acute mortality events that can exceed 80% in naïve populations (Li et al., 2025; Liu et al., 2024; Yue, 2025).

The epidemiology of *V. harveyi* infections in aquaculture is significantly influenced by environmental and management factors. Elevated water temperatures (25–30 °C) characteristic of tropical and subtropical coastal waters create optimal conditions for bacterial proliferation and virulence expression (Guo et al., 2022). Additional risk factors include high organic loading, poor water quality parameters (elevated ammonia, nitrite, and organic matter), excessive stocking densities, inadequate biosecurity measures, handling stress, nutritional deficiencies, and concurrent infections with other pathogens that compromise host immunity (Chen et al., 2025; Radwan et al., 2023). The economic impact of Vibriosis extends beyond direct mortality losses to encompass treatment costs, reduced growth performance, compromised product quality, trade restrictions, and market price fluctuations, collectively threatening the financial sustainability of commercial operations (Sanches-Fernandes et al., 2022).

Accurate and timely diagnosis of *V. harveyi* infections is paramount for implementing effective disease management strategies and mitigating production losses. Conventional diagnostic approaches rely primarily on bacterial isolation through culture-based methods using selective media such as Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, followed by phenotypic characterization through Gram staining and biochemical profiling including oxidase, catalase, motility, and carbohydrate fermentation tests (Hafezi and Khamar, 2024). While these traditional techniques provide foundational diagnostic information, they possess inherent limitations including time requirements of 24–72 hr for culture and identification, potential for contamination by faster-growing organisms, inability to detect viable but non-culturable (VBNC) bacterial states, and most critically, phenotypic similarities among *Vibrio* species that may lead to misidentification or inadequate taxonomic resolution (Morgado et al., 2026).

The advent of molecular diagnostic technologies has revolutionized bacterial pathogen identification by enabling rapid, specific, and sensitive detection independent of cultivation requirements. Polymerase chain reaction (PCR)-based methods targeting conserved genetic markers such as the 16S ribosomal RNA (rRNA) gene provide robust tools for *V. harveyi* identification and phylogenetic characterization (Leunda-Esnaola et al., 2024; Worden et al., 2022). These molecular approaches offer multiple advantages, including superior specificity through sequence-based confirmation, enhanced sensitivity capable of detecting low pathogen loads, reduced turnaround time (hours versus days), and the capacity to identify organisms directly from clinical specimens or environmental samples (El-Zamkan et al., 2023; Ellahi Babar et al., 2022).

Despite the diagnostic power of molecular methods, pathogen detection alone provides limited insight into disease pathogenesis, tissue tropism, and host response dynamics. Histopathological examination is an indispensable complementary approach that reveals microscopic tissue alterations, cellular injury patterns, inflammatory responses, and sites of bacterial colonization, thereby establishing causality between pathogen presence and disease manifestation (Blackmon et al., 2025). In vibriosis cases, characteristic histological findings include necrosis of tubular epithelial cells in the kidney, hepatocellular degeneration and necrosis in the liver, infiltration of inflammatory cells (macrophages, lymphocytes, and granulocytes) in affected organs, vascular congestion, hemorrhage, and occasionally bacterial colonies within tissues (Uma, 2025; Varalakshmi et al., 2022).

Given the critical importance of accurate pathogen identification and the documented advantages of integrating multiple diagnostic modalities, this study was designed to comprehensively investigate *V. harveyi* infections in Asian sea bass cultured in sea-cage systems on the east coast of Sri Lanka. The specific objectives were: (1) to isolate and characterize bacterial pathogens from clinically affected fish through conventional culture-based and biochemical methods; (2) to confirm species identification and genetic characterization of isolates using PCR amplification and sequencing of 16S rRN; (3) to document histopathological alterations in major organ systems (liver, kidney, spleen, gills, and muscle) associated with infection; and (4) to correlate molecular, microbiological, and histopathological findings to establish definitive etiological diagnosis and inform evidence-based disease control recommendations.

MATERIALS AND METHODS

Description of the study area

The study area was located in Trincomalee, a coastal region on the northeastern seaboard of Sri Lanka known for its extensive marine aquaculture activities (Figure 1). Sampling was carried out at offshore sea-cage culture facilities during two mass mortality events affecting Asian sea bass stocks. The area is characterized by a tropical maritime climate with relatively stable high temperatures and saline conditions that favor intensive fish farming. These environmental and operational conditions can predispose cultured fish to stress and increased susceptibility to bacterial infections, making the site suitable for investigating disease outbreaks under commercial aquaculture settings.

Isolation of bacteria

Bacteriological investigations were conducted at the Centre for Aquatic Animal Disease Diagnosis and Research (CAADDR) in accordance with standard microbiological protocols under strict aseptic conditions. Twenty Asian sea bass (*Lates calcarifer*) exhibiting clinical signs indicative of bacterial infection, including skin ulceration, abdominal distension (dropsy), and fin rot, were collected from two mass mortality episodes at sea-cage culture facilities in Trincomalee, Sri Lanka. Affected fish were immediately transported to the laboratory in insulated containers with ice packs to maintain cold chain integrity and processed within 2–4 hr of collection. Since this study also incorporates genetic analysis, the morphological and molecular data serve as mutually reinforcing lines of evidence. The combined dataset strengthens conclusions even with a moderate morphological sample size, as genetic markers can resolve population structure that morphology alone might not detect at small sample sizes.

Before necropsy, fish were euthanized humanely in accordance with ethical guidelines, and their external surfaces were disinfected with 70% ethanol to prevent contamination. Necropsy was performed aseptically using sterile instruments, which were flame-sterilized between each sample. Internal organs, including the kidney, liver, and spleen, as well as material from necrotic skin ulcers, were sampled using sterile cotton swabs or inoculation loops. Samples were immediately streaked onto three types of culture media: (1) Tryptic Soy Agar (TSA; Oxoid, England) supplemented with 3% sodium chloride (NaCl) to support halophilic bacterial growth; (2) Thiosulfate Citrate Bile Salts Sucrose agar (TCBS; Himedia, India) as a selective medium for *Vibrio* species; and (3) 5% sheep blood agar (Oxoid, England) to assess hemolytic activity and support fastidious organisms. All culture plates were prepared in duplicate.

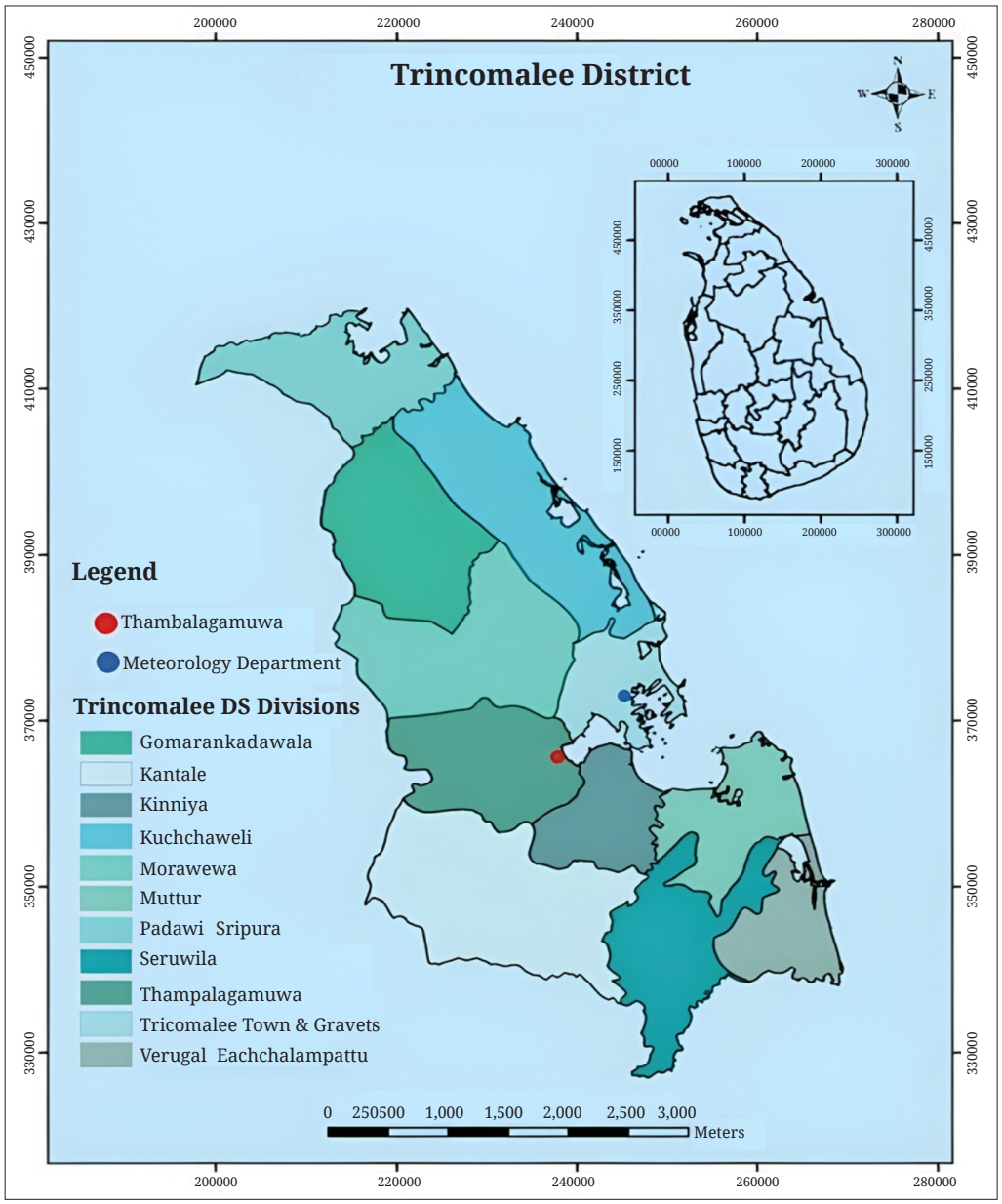

Figure 1. Location of the study area.

Inoculated plates were incubated under aerobic conditions at two temperature regimes: room temperature (28–30 °C) and 37 °C, for 24–48 hr. Plates were examined at 24-hr intervals for bacterial growth. Distinct colony morphologies were recorded, including colony size, shape, color, texture, elevation, and margin characteristics. Presumptive Vibrio isolates displaying characteristic green or yellow colonies on TCBS agar were subcultured onto fresh TSA supplemented with 3% NaCl to obtain pure cultures. Pure cultures were maintained on TSA slants at 4 °C for short-term storage and in 20% glycerol stocks at -80 °C for long-term preservation before further characterization.

Phenotypic and biochemical identification of bacteria

Bacterial isolates were characterized based on their morphological and biochemical properties following standard microbiological protocols (Parasuraman et al., 2024). All colonies exhibiting distinct morphological characteristics on primary culture plates were subcultured onto fresh TSA supplemented with 3% NaCl to obtain pure cultures for further identification. Colony morphology was systematically documented, including observations of size, shape, color, surface texture, elevation, margin, and consistency.

Pure cultures were subjected to Gram staining using the standard Hucker's modification method (Paray et al., 2023). Heat-fixed bacterial smears were sequentially stained with crystal violet, treated with Gram's iodine, decolorized with 95% ethanol, and counterstained with safranin. Stained preparations were examined under oil-immersion microscopy at 1000× magnification to determine the Gram reaction, cellular morphology (rods, cocci, or curved forms), and arrangement patterns. A comprehensive panel of biochemical tests was performed to characterize the metabolic and enzymatic profiles of the isolates:

Catalase test: A loopful of fresh bacterial culture was emulsified in 3% hydrogen peroxide (H_2O_2) solution on a clean glass slide. Immediate effervescence indicating oxygen liberation was recorded as a positive result.

Oxidase test: Cytochrome oxidase activity was determined using commercially prepared oxidase reagent discs (Oxoid, England). A bacterial colony was transferred onto the reagent disc using a platinum loop. Development of a dark purple color within 10–30 seconds indicated a positive reaction.

Sulfide Indole Motility (SIM) test: The Sulfide Indole Motility (SIM) test was performed to assess three distinct biochemical parameters simultaneously. First, hydrogen sulfide (H_2S) production was evaluated by observing blackening of the medium due to ferrous sulfide formation; this was negative in our isolates. Second, indole production was assessed by adding Kovac's reagent, which produced a red ring at the surface of the medium, confirming a positive indole result consistent with *Vibrio harveyi*. Third, motility was determined by observing diffuse turbid growth radiating outward from the stab inoculation line, confirming that our isolates were motile, which is a characteristic feature of *Vibrio* spp. Isolates were stab-inoculated into SIM medium (Himedia, India) and incubated at 30 °C for 24–48 hr. Motility was assessed by observing diffuse turbidity extending from the stab line. Hydrogen sulfide production was indicated by black precipitate formation along the stab line. Indole production was tested as described above.

Citrate Utilization Test: Isolates were streaked onto Simmons citrate agar slants (Himedia, India) and incubated at 30 °C for 24–96 hr. A color change from green to Prussian blue, indicating citrate utilization and alkalinization, was considered positive. Standard SIM medium does not contain NaCl, which may potentially inhibit or reduce the growth of halophilic Vibrio isolates, affecting the reliability of the results. Therefore, in future studies, SIM medium supplemented with 1–3% NaCl will be used to ensure consistent and optimal growth conditions.

Triple Sugar Iron (TSI) Agar Test: Bacterial cultures were inoculated into TSI agar slants (Himedia, India) by stabbing the butt and streaking the slant surface. Tubes were incubated at 30 °C for 24–48 hr and examined for carbohydrate fermentation patterns (slant and butt color changes), gas production (cracks or bubbles), and hydrogen sulfide (H_2S) production (black precipitate).

Oxidation-Fermentation (O-F) Test: The carbohydrate metabolism pathway was determined using Hugh and Leifson's O-F glucose medium (Himedia, India). Two tubes were inoculated by stabbing; one tube was overlaid with sterile mineral oil to create anaerobic conditions, while the other remained open. Tubes were incubated at 30 °C for up to 7 days. Yellow coloration in both tubes indicated fermentative metabolism, whereas yellow in the open tube indicated oxidative metabolism.

All biochemical tests were performed in duplicate with appropriate positive and negative control strains to ensure the reliability of the results. Results were interpreted according to standard microbiological criteria and recorded after designated incubation periods. Bacterial identification was performed by comparing phenotypic and biochemical profiles with established identification schemes and taxonomic keys for marine bacterial pathogens (Duman et al., 2022).

Histopathological investigations

Tissue samples from affected fish were collected for histopathological examination during necropsy. Representative specimens of brain, kidney, liver, spleen, gills, and skeletal muscle were excised aseptically and immediately fixed in 10% neutral phosphate-buffered formalin (pH 7.2–7.4) for 24–48 hr at a tissue-to-fixative ratio of 1:10 (v/v). Fixed tissues were processed following standard histological procedures (Roberts, 2012). Tissues were dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and absolute ethanol), cleared in xylene, and infiltrated with molten paraffin wax at 58–60 °C. Tissues were then embedded in paraffin blocks and sectioned at 4–5 µm thickness using a rotary microtome. Sections were mounted onto glass slides and dried overnight at 37–40 °C. Tissue sections were deparaffinized in xylene, rehydrated through descending ethanol concentrations, and stained with hematoxylin and eosin (H&E) following standard protocols. Stained sections were dehydrated, cleared in xylene, and mounted with DPX mounting medium under coverslips. Histological sections were examined under light microscopy at various magnifications (40×, 100×, 400×, and 1000×) to assess tissue architecture and identify pathological changes. Histopathological alterations evaluated included necrosis, inflammatory cell infiltration (macrophages and lymphocytes), cellular degeneration, hemorrhage, congestion, edema, and the presence of bacterial colonies. Lesion severity was graded as mild, moderate, or severe. Representative photomicrographs of significant findings were captured using a digital microscope camera system for documentation and analysis.

Molecular characterization of bacteria

Molecular identification using polymerase chain reaction (PCR) and DNA sequencing was employed for bacterial isolates that could not be conclusively identified to species level based on phenotypic and biochemical characteristics alone.

DNA extraction

Genomic DNA was extracted from pure bacterial isolates using the Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol. A loopful of bacterial culture from fresh growth on TSA supplemented with 3% NaCl was suspended in 200 µl of sterile distilled water and homogenized by vortexing. The bacterial suspension was then processed according to the kit instructions for DNA extraction. Extracted DNA samples were quantified using a NanoDrop spectrophotometer (or visually assessed for purity) and stored at -20 °C until further use for polymerase chain reaction (PCR) amplification.

Polymerase chain reaction

Molecular identification of *Vibrio harveyi* was performed by polymerase chain reaction (PCR) amplification targeting the 16S ribosomal RNA (rRNA) gene using universal bacterial primers as described by Segers and Grisez (2005). The primer set consisted of forward primer (5'-AGA GTT TGA TCA TGG CTC AG-3') and reverse primer (5'-GGT TAC CTT GTT ACG ACT T-3'), yielding an expected amplicon size of approximately 1,450 base pairs (bp). PCR reactions were prepared in a final volume of 50 µl containing the following components: 5 µl of 10× Taq buffer, 1.5 µl of deoxyribonucleoside triphosphate mix (dNTPs) (10 mM), 1.5 µl of each primer (10 µM), 2 µl of $MgCl_2$ (25 mM), 0.4 µl of Taq DNA polymerase (5 U/µl), 2 µl of template DNA, and 36.1 µl of nuclease-free distilled water. Negative controls containing nuclease-free water instead of template DNA were included in each PCR run to monitor for contamination.

Amplification was performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA) with the following cycling conditions: initial denaturation at 94 °C for 5 minutes; followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1.5 minutes; with a final extension at 72 °C for 10 minutes. Following amplification, reactions were held at 4 °C until analysis.

PCR products (5 µl) were analyzed by horizontal gel electrophoresis on 1.5% (w/v) agarose gel prepared in 1× Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide (EtBr) (0.5 µg/ml). Electrophoresis was conducted at 120 V for 60 minutes, and DNA bands were visualized under ultraviolet (UV) transillumination using a gel documentation system (Bio-

Rad, USA). A Quick-Load™ 100 bp DNA ladder (New England Biolabs, USA) was used as a molecular weight marker to confirm the sizes of the amplified products.

Phylogenetic analysis and sequence characterization

PCR products showing the expected amplicon size of approximately 1,450 bp were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) or GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Purified amplicons were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) to assess DNA concentration and purity based on A260/A280 ratios.

Bidirectional DNA sequencing of purified PCR products was performed using the same forward and reverse primers employed for amplification. Sequencing reactions were conducted using the Sanger dideoxy chain termination method on an automated DNA sequencer (ABI 3730xl or equivalent, Applied Biosystems, USA) at a commercial sequencing facility (BGI Genomics, Shenzhen, China).

Raw sequence chromatograms were analyzed and edited using BioEdit Sequence Alignment Editor version 7.2.5 or Chromas version 2.6.6 (Technelysium Pty Ltd, Australia) to assess sequence quality and trim ambiguous base calls from terminal regions. Forward and reverse sequences were assembled into consensus sequences, and overlapping regions were examined for discrepancies. High-quality consensus sequences ranging from 1,400 to 1,450 bp were obtained for phylogenetic analysis.

Sequence homology searches were performed using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI) GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were compared against the non-redundant nucleotide (nr/nt) database to identify closely related bacterial species based on percentage identity, query coverage, and E-values. Bacterial isolates showing ≥97% sequence similarity with reference strains were considered to belong to the same species according to established taxonomic criteria for 16S rRNA gene-based identification (Iqra et al., 2024).

Multiple sequence alignment was conducted using the ClustalW or MUSCLE algorithms in MEGA X (version 10.0; Kumar et al., 2018) or MEGA11 (Tamura et al., 2021). Representative 16S rRNA gene sequences of *Vibrio harveyi* and closely related *Vibrio* species retrieved from GenBank were included in the alignment for comparative analysis. Sequences were trimmed to equal length, and alignment was manually inspected and adjusted to remove gaps and ambiguous regions.

Phylogenetic trees were constructed using three different methods to assess tree topology robustness: (1) the neighbor-joining (NJ) method with the Kimura 2-parameter distance model, and (2) the maximum likelihood (ML) method with the best-fit nucleotide substitution model determined by ModelTest. The reliability of internal branches was assessed by bootstrap analysis with 1,000 replications (Felsenstein, 1985). Bootstrap values ≥70% were considered to indicate significant phylogenetic support. Trees were rooted using an appropriate outgroup species (Escherichia coli sequence in NCBI -NR_024570.1 or Photobacterium damselae strain sequence in NCBI -NR_1 15206.1) and visualized using MEGA X or FigTree version 1.4.4 (Noreen et al., 2023).

Genetic distances between isolates and reference strains were calculated using the Kimura 2-parameter model in MEGA X to quantify evolutionary divergence. Pairwise sequence identities were determined to assess intraspecific and interspecific variation among *Vibrio* species. The consensus 16S rRNA gene sequences of *Vibrio harveyi* isolates obtained in this study were deposited in the NCBI GenBank database. Sequence data are publicly available for future comparative studies and epidemiological investigations.

RESULT

Bacterial isolation and identification

Of the 20 Asian sea bass (*Lates calcarifer*) specimens subjected to bacteriological investigation, bacterial isolates were successfully recovered from the kidney, liver, and necrotic skin ulcers. Based on colony morphology and conventional biochemical characterization, the predominant isolates were tentatively identified as *Vibrio* species. The isolates produced small to medium-sized, round, convex, and shiny colonies on TSA supplemented with 3% NaCl, and characteristic green colonies on TCBS agar. Biochemical tests revealed that these isolates were Gram-negative rods, oxidase-positive, catalase-positive, motile, and capable of fermenting glucose without gas production, consistent with the genus Vibrio. However, one Gram-negative rod isolate could not be definitively identified to the species level using available conventional biochemical tests, necessitating molecular characterization (Table 1).

Histopathological findings

Table 1. Biochemical characteristics of bacterial isolates recovered from asian sea bass (*Lates calcarifer*).

Test parameter	Isolate 1 (*Vibrio* spp.)	Isolate 2 (*Vibrio* spp.)	Unidentified isolate
Gram reaction	Negative	Negative	Negative
Cell morphology	Rod	Rod	Rod
Motility	Positive	Positive	Positive
Oxidase test	Positive	Positive	Negative
Catalase test	Positive	Positive	Positive
Glucose fermentation	Positive (no gas)	Positive (no gas)	Positive
Sucrose fermentation (TCBS)	Positive (green colonies)	Positive (green colonies)	Negative
NaCl tolerance (3%)	Growth	Growth	Variable
Indole production	Positive	Negative	Negative
Methyl Red (MR)	Negative	Negative	Positive
Voges–Proskauer (VP)	Negative	Negative	Negative
Citrate utilization	Positive	Positive	Negative
H_2S production	Negative	Negative	Negative
Urease test	Negative	Negative	Negative
TCBS agar colony color	Green	Green	No growth / atypical

Histopathological examination of tissue sections from affected fish revealed severe pathological alterations in multiple organs, particularly the kidney and liver, consistent with systemic bacterial infection.

Kidney: Renal tissue sections displayed moderate to severe tubular injury characterized by necrosis and degeneration of tubular epithelial cells. Extensive inflammatory cell infiltration, predominantly comprising macrophages and lymphocytes, was observed in the interstitial tissue and surrounding necrotic tubules. Areas of tubular necrosis showed cellular debris and loss of normal tubular architecture (Figure 2A).

Liver: Hepatic tissue sections exhibited pronounced steatohepatitis with severe inflammatory changes and hepatocellular damage. Marked hepatic lipid accumulation (fatty degeneration) was evident, with hepatocyte cytoplasm containing numerous lipid vacuoles that displaced nuclei to the periphery, creating a prominent perinuclear halo. Large vacuoles were observed throughout the parenchyma, particularly surrounding blood vessels. Multifocal hepatocyte necrosis and severe vacuolar degeneration were prominent features. Inflammatory cell infiltration was present in periportal and perivascular regions (Figure 2B).

These histopathological changes were consistent with acute bacterial septicemia and provided supportive evidence for systemic Vibriosis in the affected fish population.

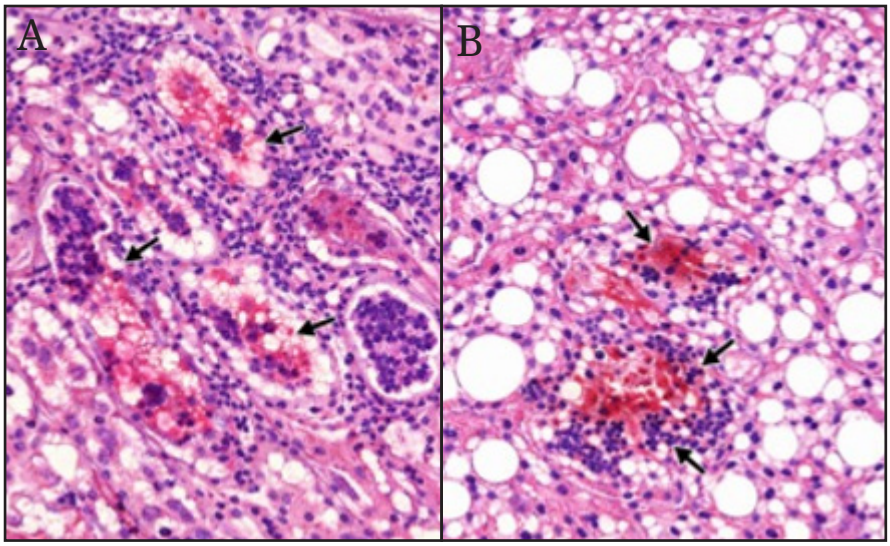

Figure 2. Histopathological images: (A) Kidney; (B) Liver.

Molecular identification and phylogenetic analysis

Twenty representative bacterial isolates—one tentatively identified as *Vibrio* sp. and one unidentified Gram-negative rod—were selected for molecular characterization through PCR amplification and sequencing of the 16S rRNA gene. Both isolates yielded amplicons of approximately 1,450 bp, consistent with the expected product size (Figure 3).

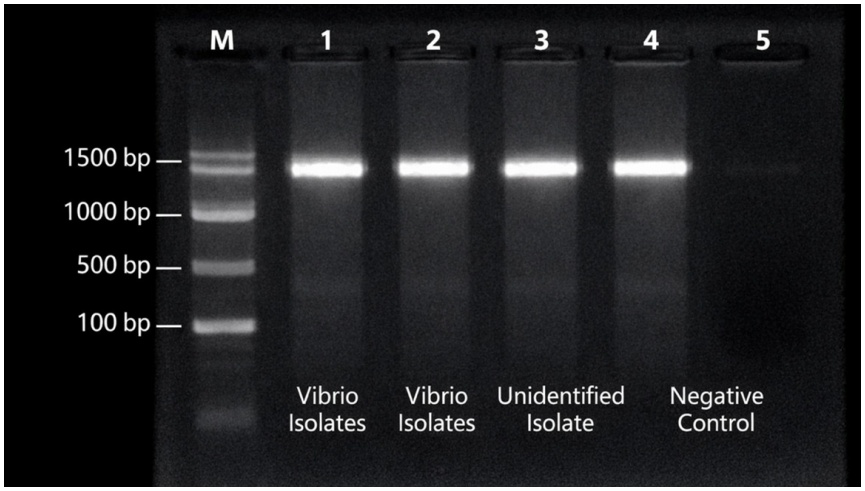

Figure 3. PCR amplification of 16S rDNA.

Following purification and bidirectional sequencing, high-quality consensus sequences were obtained and subjected to BLAST analysis against the NCBI GenBank database. The molecular identification results were as follows:

Isolate 1: The 16S rRNA gene sequence exhibited 99.44% nucleotide identity with *Vibrio harveyi* strain CP014038.2, confirming the species-level identification of this isolate as *Vibrio harveyi*. This high percentage identity (>97% threshold) provided definitive confirmation of the bacterial species responsible for the observed disease outbreak. The sequence was deposited in GenBank under accession number OR351233 (Figure 4).

Isolate 2: The 16S rRNA gene sequence showed 99.29% nucleotide identity with Shewanella algae strain XCY62,

identifying the previously uncharacterized Gram-negative rod as Shewanella algae. This species is recognized as an opportunistic pathogen in aquatic environments and may have contributed to the disease complex or served as a secondary invader.

Phylogenetic analysis using the neighbor-joining method with 1,000 bootstrap replicates confirmed the taxonomic placement of both isolates within their respective genera, with the *V. harveyi* isolate clustering closely with other pathogenic *V. harveyi* strains from marine fish (Figures 5 and 6). These molecular findings, combined with characteristic clinical signs, biochemical profiles, and histopathological lesions, confirmed *Vibrio harveyi* as the primary etiological agent responsible for mass mortality events in Asian sea bass cage culture on the east coast of Sri Lanka.

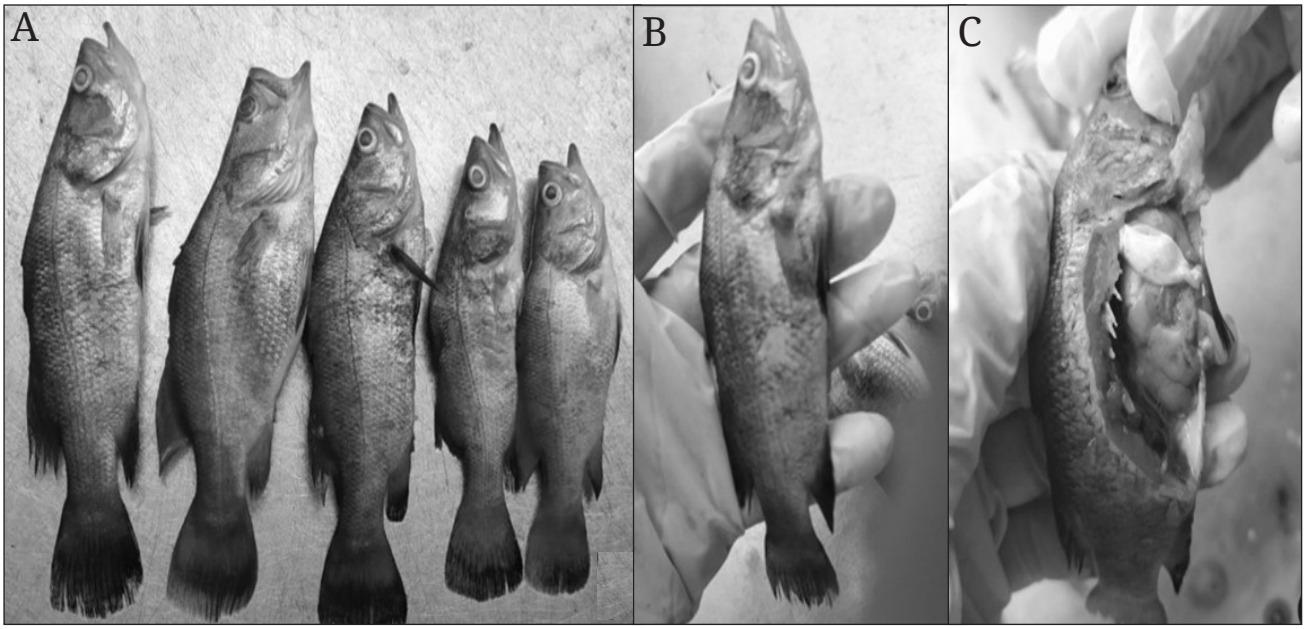

Figure 4. Morphological characteristics of diseased sea bass specimens showing clinical signs of Vibriosis: (A) Colony infection, (B) Individual, (C) After dissection.

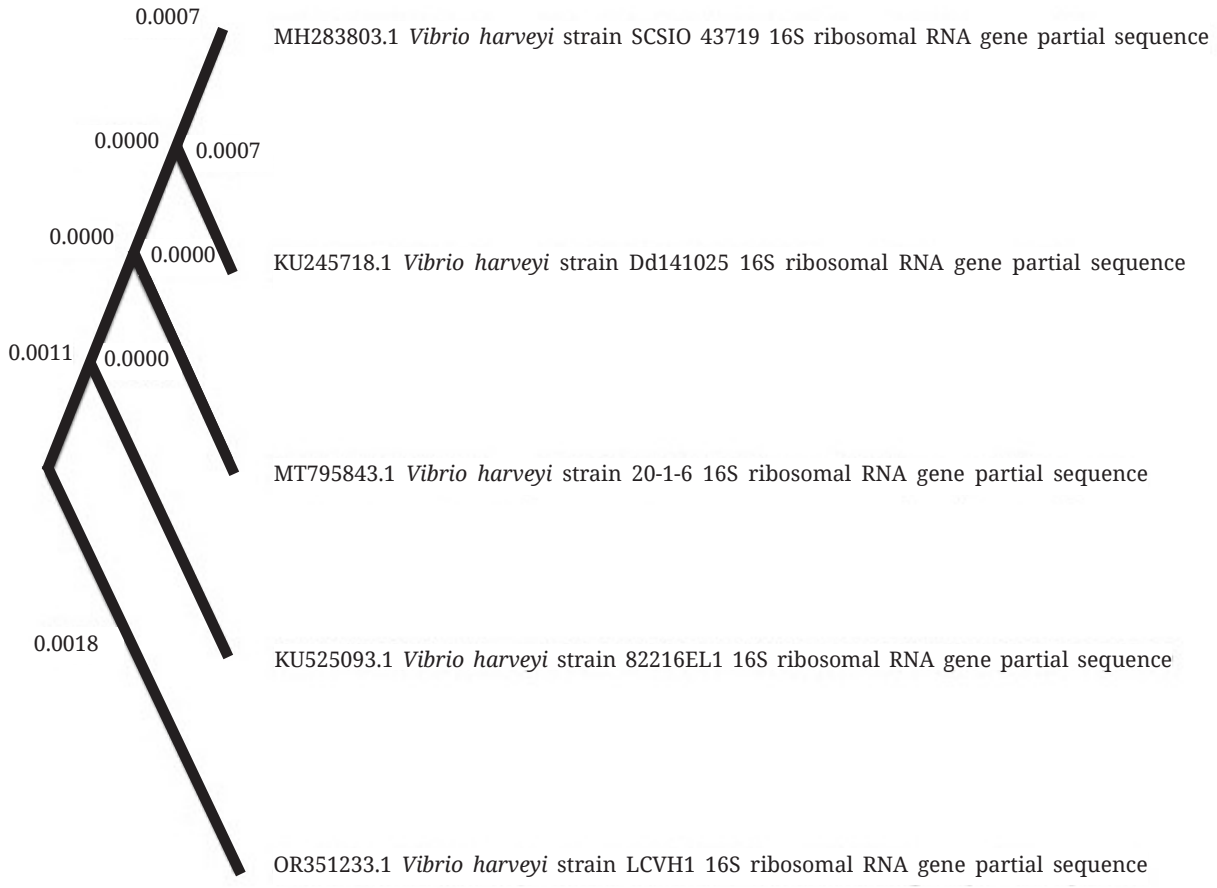

Figure 5. Phylogenetic analysis of *Vibrio harveyi* 16S ribosomal RNA gene sequences.

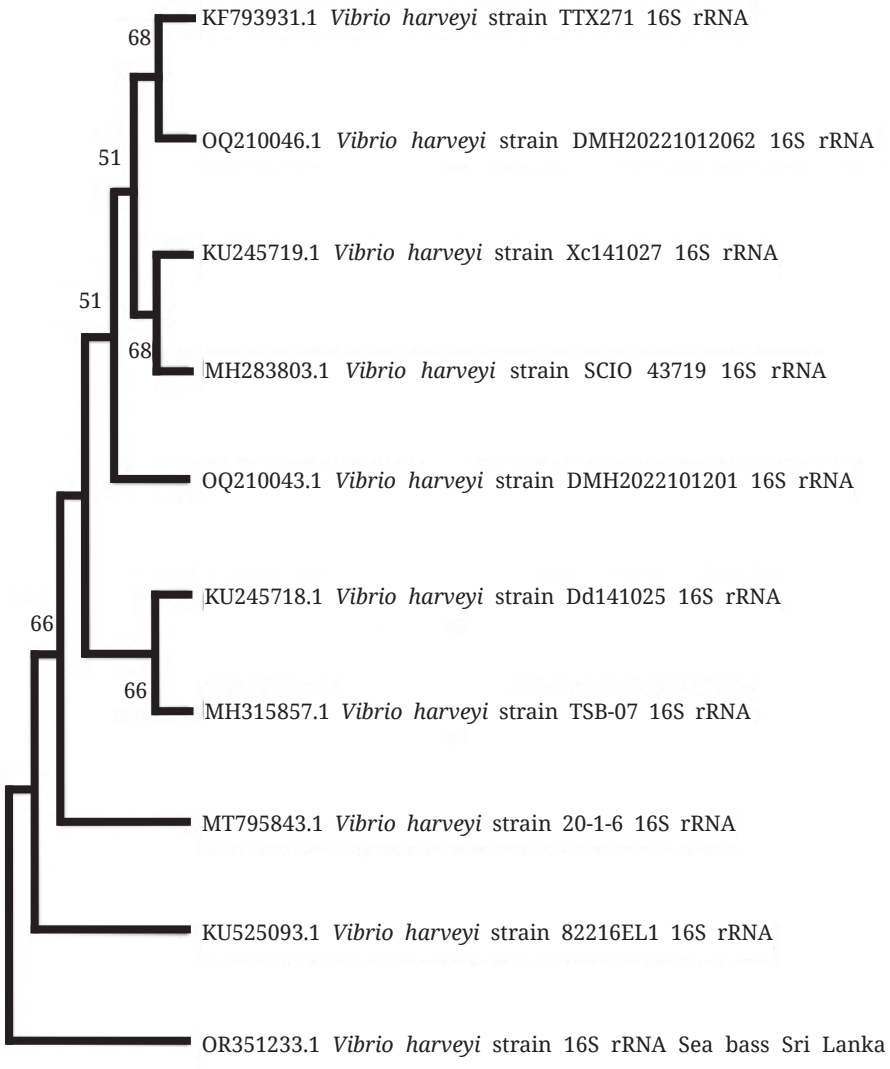

Figure 6. Bootstrap consensus phylogenetic tree of *Vibrio harveyi* 16S rRNA gene sequences.

DISCUSSION

Integrated microbiological, molecular, and histopathological evidence of bacterial pathogenicity

The convergent findings from microbiological culture, 16S rRNA gene sequencing, and histopathological examination collectively establish a robust aetiological framework for the mass mortality episodes observed in cage-cultured Asian sea bass (*Lates calcarifer*) in Sri Lankan waters. *Vibrio harveyi* was consistently isolated as the primary bacterial pathogen across multiple mortality events, with cumulative mortality rates ranging from 10% to 100% across affected cage units, underscoring not only the organism's virulence but also the variable farm-level risk factors that modulate disease severity. Shewanella algae was also implicated in a single distinct mortality episode, confirming a polymicrobial disease landscape in the study area.

Critically, the microbiological identification of *V. harveyi* from internal organs (liver, kidney, spleen, and blood) was directly corroborated by histopathological findings: hepatocyte degeneration and necrosis, renal tubular damage, and multifocal haemorrhage were consistently observed in tissues from clinically affected fish. These histopathological lesions are not merely coincidental; they are mechanistically linked to the arsenal of extracellular virulence factors produced by *V. harveyi*, including metalloproteases, haemolysins, siderophores, and cytotoxins (Austin and Zhang, 2006). Importantly, 16S rRNA gene sequencing resolved the species-level identity of isolates that could not be reliably differentiated by conventional biochemical profiling alone, given the phenotypic plasticity within the *V. harveyi* clade (Urbanczyk et al., 2013). The present approach therefore provides a methodologically integrated, three-tiered evidence base that satisfies Koch's postulates and strengthens the confidence of the aetiological diagnosis.

In *S. algae*, a corresponding histopathological picture, including branchial congestion and early hepatic vacuolation, was observed in the affected batch, consistent with its documented capacity to produce cytotoxic enzymes and iron-chelating siderophores that facilitate tissue invasion under immunocompromised conditions (Mishra et al., 2024; Yue, 2025). The detection of this pathogen in only one episode, combined with histopathological evidence of mild to moderate, rather than fulminant, tissue damage compared with *V. harveyi* episodes, suggests a lower intrinsic virulence under the prevailing environmental conditions. However, this interpretation should be treated cautiously, given the limited sample size.

Vibrio harveyi as a primary pathogen: Alignment and divergence with the literature

The dominance of *V. harveyi* in the present study aligns with global epidemiological trends, identifying this species as one of the most economically significant bacterial pathogens in tropical marine finfish aquaculture (Vandeputte et al., 2024). In Southeast Asian sea bass aquaculture, comparable prevalence and mortality profiles have been documented: Henares et al., (2020) reported vibriosis-associated cumulative mortalities exceeding 60% in cage-cultured *L. calcarifer* in Vietnam, while Lee et al. (2023) described similar haemorrhagic septicaemia outbreaks in Indonesia with mortality rates approaching 80% in fingerling cohorts figures broadly consistent with the upper range (100%) of the mortality events recorded in the present study. Notably, the most severe mortality events in our study occurred within the first 14 days following transfer from land-based nursery tanks to sea cages. This finding converges with Bondad-Reantaso et al. (2005), who identified the post-transfer period as the highest-risk interval for infectious disease in marine cage culture, attributing this to the compounding effects of handling stress, microbiome disruption, and abrupt exposure to open-water pathogen reservoirs.

In contrast, some studies from the Mediterranean and European sea bass (*Dicentrarchus labrax*) production systems report lower *V. harveyi* prevalence and milder disease outcomes (Toranzo et al., 2005), a divergence likely attributable to differences in water temperature, husbandry intensity, and the genetic composition of pathogen populations. The tropical coastal environment of Trincomalee, characterised by sea surface temperatures consistently exceeding 28 °C, provides near-optimal growth conditions for *V. harveyi*, which proliferates rapidly above 25 °C and expresses peak virulence factor production at elevated temperatures (Lee et al., 2023). This temperature-virulence relationship, absent in temperate aquaculture contexts, partially explains the higher mortalities observed in the present study relative to European reports and should inform season-specific disease surveillance protocols in Sri Lanka.

Shewanella algae: An emerging opportunistic pathogen in marine finfish

The isolation of *S. algae* from one disease episode is a noteworthy finding, as reports of this pathogen in *L. calcarifer* remain comparatively sparse in the peer-reviewed literature. Globally, *S. algae* has been documented as a cause of ulcerative skin disease in flounder (*Paralichthys olivaceus*) and haemorrhagic septicaemia in yellowtail amberjack (*Seriola quinqueradiata*) in Japan, where it was associated with cumulative mortalities of 15–30% under high-density stocking conditions (Tanimoto et al., 2024). The present episode shares this pattern of moderate but clinically significant mortality, reinforcing the view that *S. algae* is an opportunistic pathogen whose virulence is predicated upon host immune suppression rather than aggressive primary pathogenicity (Mishra et al., 2024). The molecular confirmation of *S. algae* in this study represents, to our knowledge, one of the first reports of this organism associated with disease in *L. calcarifer* in South Asian waters, emphasising the need for routine molecular characterisation of bacterial isolates rather than reliance on biochemical identification alone.

The stress-disease paradigm: Environmental and physiological drivers of disease susceptibility

The concentration of mortality events within the first two weeks post-transfer from bore-well-supplied nursery tanks to open marine cages reflects a convergence of physiological stressors that compromise immune competence and pathogen resistance. The transition from bore-well water, typically characterised by low microbial loads and stable physico-chemical parameters, to the microbiologically complex and temporally variable marine cage environment constitutes an abrupt ecological discontinuity for juvenile fish. Cortisol-mediated immunosuppression, well-established as a consequence of acute handling and confinement stress in teleosts (Tort, 2011), transiently reduces lysozyme activity, complement function, and leukocyte responsiveness, precisely the innate immune mechanisms that would otherwise limit bacterial colonisation by *Vibrio* species. The present findings are consistent with Sakai (1999), who demonstrated that cortisol concentrations in sea bass fingerlings remained significantly elevated for 7–14 days following cage transfer, overlapping almost exactly with the highest-mortality window identified in this study.

The bore-well water system, while intended to provide biosecurity through pathogen exclusion, may itself represent an indirect risk factor. Bore wells proximate to the Sri Lankan coastline are susceptible to saline intrusion, potentially introducing low levels of marine bacteria, including *V. harveyi*, into the nursery environment, thereby pre-exposing fingerlings to pathogens before their immune systems have fully matured. Furthermore, if dissolved oxygen concentrations in bore-well tanks are suboptimal due to inadequate aeration, chronic hypoxic stress will independently suppress immune function and select for stress-tolerant bacterial species. Systematic monitoring of bore-well water quality for microbial contamination, salinity intrusion, and dissolved oxygen is therefore waranted as a foundational biosecurity measure.

CONCLUSION

This study provides preliminary evidence implicating *Vibrio harveyi* as a likely causative agent associated with mass mortality in Asian sea bass (*Lates calcarifer*) cage culture systems in Trincomalee, Sri Lanka. While the findings are noteworthy, particularly this represents a first report or opportunistic documentation from an affected farm. Therefore, it is important to acknowledge that the study examined only 20 fish, with two representative isolates subjected to detailed analysis. This limited sample size and the restricted number of isolates analyzed preclude definitive causal conclusions, and the findings should be interpreted with appropriate caution given the absence of a statistically powered, widespread sampling program. Nonetheless, the pathogen was identified through a combination of cultural, biochemical, and molecular characterization, with histopathological observations revealing tissue damage in hepatic and renal organs consistent with *V. harveyi* infection. Together, these results suggest a strong association between this pathogen and the observed mortality event, contributing valuable baseline data on Vibriosis in Sri Lankan barramundi aquaculture. These preliminary findings highlight the potential threat posed by *V. harveyi* to the country's expanding Asian sea bass cage culture industry and point to the need for broader, systematic disease surveillance programs to confirm and extend these observations.

Study limitations, future research directions and challenges test:

The five-year observation period, while providing valuable longitudinal data, represents only a subset of potential disease scenarios. Seasonal variations in disease occurrence, the role of subclinical infections, and the potential presence of viral and parasitic co-infections were not comprehensively investigated. Future research should employ molecular diagnostic techniques, including PCR and whole-genome sequencing, to provide more detailed pathogen characterization and to identify virulence factors. Additionally, antimicrobial resistance patterns should be systematically monitored, given the potential for resistance development in aquaculture settings. Investigations into host-pathogen interactions, environmental risk factors, and the efficacy of various therapeutic and prophylactic interventions further contribute to the development of evidence-based disease management strategies.

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AUTHOR CONTRIBUTIONS

V. S., M. N. M. F., and F. M. M. T. M: Wrote the main manuscript text. S. S. S. S. J., R. J., and M. M. M: Prepared the table and done the analysis. M. N. M. F., and F. M. M. T: Supervised and did the final editing and all authors reviewed the manuscript.

DECLARATIONS

Informed consent statement

This study was conducted in accordance with the Declaration of Helsinki and all relevant institutional and national guidelines and regulations. Ethical approval was obtained from the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka.

Conflict of interest

The authors declare no conflict of interest.

AI Desclosure

The authors declare that no Artificial Intelligence (AI) or AI-assisted technologies were used in the preparatin of this manuscript.

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