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Original Article

Virulence profiling and integrated pathogenic risk assessment of bacterial isolates from freshwater-farmed Asian seabass (*Lates calcarifer*)

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Abstract
Asian seabass (*Lates calcarifer*) is one of the most commercially important fish species because of its rapid growth and tolerance to a wide range of salinities. However, bacterial diseases remain a major constraint to sustainable production, and the virulence and antimicrobial resistance status of associated bacterial pathogens in freshwater farming systems remain poorly characterized. This study aimed to identify bacterial isolates from freshwater-farmed Asian seabass and evaluate their virulence potential and antibiotic resistance status for pathogenic risk assessment. Diseased fish were collected from freshwater farms across four Indian states, and the bacterial isolates were characterized using biochemical tests and 16S rRNA gene sequencing. *Aeromonas* (42%), *Vibrio* (20%) and *Plesiomonas* (18%) were the most predominant genera, with *Aeromonas veronii* comprising 22% of identified isolates. Gelatinase was the most prevalent trait (100% in *Aeromonas*, 90% in *Vibrio*), followed by DNase (90% and 70%), amylase (76% and 90%) and hemolytic activity (86% and 70%), respectively. PCR detected *act* (95%) and *aerA* (38%) genes in *Aeromonas*, *hlyA* and *rtxA* (90%) in *Vibrio*, while all *Plesiomonas* isolates carried *astA* and *aphA*. Mean virulence scores were highest for *Vibrio* (5.60) and *Aeromonas* (5.57). Multiple antibiotic resistance (MAR) index analysis revealed that 71.4% of *Aeromonas* isolates exceeded the 0.2, and several *Aeromonas* isolates exhibited high virulence scores and elevated MAR indices. The isolation of *Vibrio cholerae* from food fish highlights an additional 'one health' concern. These findings underscore the need for integrated pathogen monitoring, prudent antimicrobial use and robust biosecurity practices in freshwater Asian seabass aquaculture.

Keywords: *Aeromonas veronii*; Asian seabass; freshwater aquaculture; multiple antibiotic resistance; *Vibrio cholerae*; virulence genes

1 | INTRODUCTION

Lates calcarifer, commonly known as Asian seabass or barramundi, is one of the most economically valuable aquaculture species across tropical and sub-tropical regions of Asia-Pacific (Munilkumar *et al*. 2013). Global production of Asian seabass has expanded substantially over the past two decades, rising from 18.1 thousand tonnes in 2000 to 105.8 thousand tonnes in 2020 (FAO 2022), with

projected market growth at a compound annual growth rate of 4.5%, reaching an estimated USD 1,567 million by 2034 (Detcharoen *et al*. 2025). India, the world's second-largest producer of aquatic animals (FAO 2026), recorded a production of 19,030 metric tonnes of Asian seabass in 2022–2023 (MPEDA 2023). In recent years, the culture of the Asian seabass in the freshwater environment has been widely adopted by fish farmers due to its euryhaline nature and high market demand (Ghosh 2019), where it can thrive under artificial feeding regimes (Orbán *et al*. 2021).

Despite remarkable production gains, disease outbreaks are the major constraint for aquaculture production resulting in mass mortality of cultured fishes (Hutson 2013). Bacterial diseases pose a significant challenge to fish health management in intensive culture systems (Mishra *et al*. 2017). Common bacterial diseases recorded in the Asian seabass aquaculture include Vibriosis (Krupesha Sharma *et al*. 2012); Photobacteriosis (Kanchanopas-Barnette *et al*. 2009); Streptococcosis (Awate *et al*. 2023); Aeromoniasis (Kathirkaman *et al*. 2018) and Columnaris (Kerddee *et al*. 2020) disease. Many of these are caused by opportunistic pathogens that preferentially infect environmentally stressed or immunocompromised fish (Sandeep *et al*. 2016), and their prevalence and pathotypic diversity may differ markedly across culture systems and geographic regions (Rathinam *et al*. 2021; Irshath *et al*. 2023).

Virulence, defined as the relative capacity of a microorganism to cause host damage, is mediated through a spectrum of mechanisms including extracellular enzyme production, toxin secretion, adherence, immune evasion and biofilm formation (Casadevall and Pirofski 2003; Abdulateef *et al*. 2023). Among gram-negative fish pathogens, gelatinase, DNase, amylase and hemolysin are well-established virulence attributes that facilitate tissue invasion, nutrient acquisition and cytotoxicity (Pemberton *et al*. 1997; Too and Masila 2024). Molecular screening for virulence-associated genes provides genotypic evidence of pathogenic potential that complements phenotypic assessments. An additional dimension of pathogenic risk in aquaculture settings is antimicrobial resistance (AMR). Bacteria exhibiting both high virulence and elevated MAR indices represent a compounded public health and aquaculture biosecurity threat a concern that aligns directly with the One Health framework linking animal, human and environmental health (Nagar *et al*. 2025). Despite the increasing intensification of freshwater Asian seabass aquaculture in India and neighbouring countries, systematic studies on the virulence characteristics and AMR profiles of associated bacterial pathogens remain scarce. The present study was therefore undertaken to isolate and molecularly characterize bacterial communities associated with diseased freshwater-farmed Asian seabass in India, evaluate phenotypic and genotypic virulence profiles of representative isolates, determine multiple antibiotic resistance indices and conduct an integrated pathogenic risk assessment by examining the co-occurrence of virulence determinants and AMR profiles.

2 | METHODOLOGY

2.1 Sample collection and bacterial isolation

The study was conducted from February 2024 to September 2024 at the Department of Aquatic Animal Health, ICAR–Central Institute of Fisheries Education, Mumbai, India. During this period, a total of 60 live and moribund Asian seabass exhibiting clinical signs of bacterial disease (lethargy, exophthalmia, haemorrhages, skin ulceration, erratic swimming) were collected from different freshwater farms located in Andhra Pradesh, Gujarat, Karnataka and Maharashtra. Five fish were sampled directly from each farm during field visits. The geographical locations of the sampled farms are shown in Figure 1. The mean body weight and total length of the sampled fish were 40±10.4 g and 16±4.2 cm, respectively. Fish were transported to the laboratory in oxygenated containers for further bacteriological analysis. Fish were anesthetized by immersion in chilled water, followed by euthanasia using an overdose of buffered tricaine methanesulfonate (MS-222; 0.2 mg mL^{-1}) prior to aseptic dissection, in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA 2020). Tissue samples were aseptically collected from kidney, liver and eyes under sterile conditions. Samples were inoculated into Brain Heart Infusion (BHI) broth (HiMedia, Mumbai) and incubated at 28±2°C for 24 hr. Enriched cultures were sub-cultured onto BHI agar and incubated under the same conditions for 24 hr. Morphologically distinct colonies were purified by repeated streaking on fresh BHI agar.

2.2 Phenotypic characterization

Colony morphology, gram staining, catalase activity (3% H_2O_2) and oxidase activity (HiMedia Ltd, Mumbai, India) were assessed for all isolates. Biochemical characterization included indole production, triple sugar iron (TSI) agar reactions, hydrogen sulfide (H_2S) production and citrate utilization, conducted according to standard protocols (Lehman 2005; MacWilliams 2009b, 2009a). Based on overall biochemical profiles, 50 representative isolates were selected for molecular identification.

2.3 Molecular identification

Genomic DNA was extracted using the CTAB method (Chachaty and Saulnier 2000). PCR amplification of 16S rRNA gene was performed using universal primers 27F and 1492R (Table 1) in a thermal cycler under the following conditions: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 45

sec; and a final extension at 72°C for 5 min. Purified amplicons (Purelink Gel Extraction Kit, Invitrogen) were subjected to Sanger sequencing (Eurofins Genomics India Pvt. Ltd., Karnataka). Sequences were edited in BioEdit, trimmed for quality and deposited in the NCBI GenBank database.

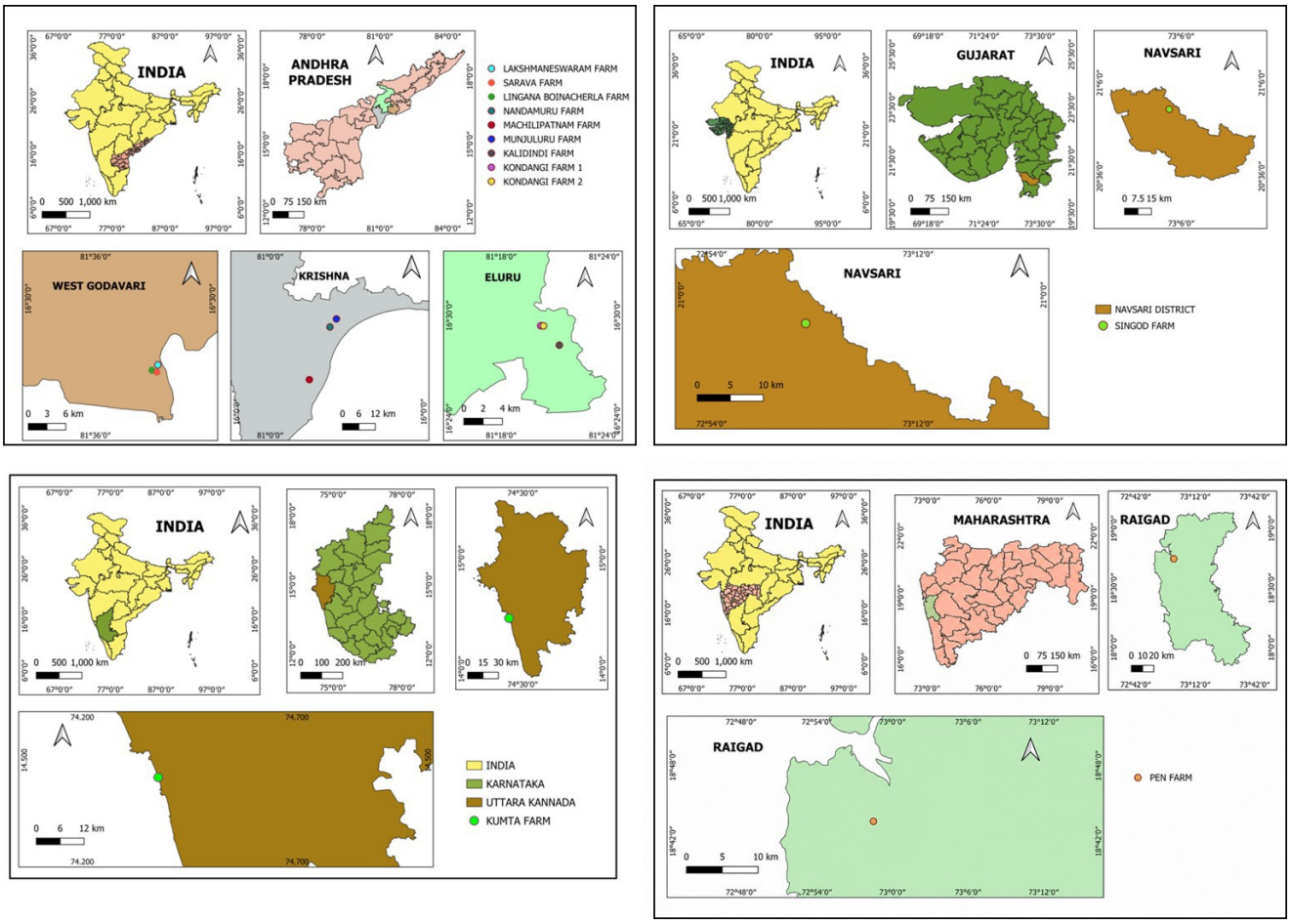

FIGURE 1 Maps showing the geographical locations of the sampling sites across four Indian states. Maps were generated using QGIS software.

2.4 Phenotypic virulence assessment

Extracellular enzyme activities and hemolytic properties were evaluated for all 50 molecularly identified isolates. DNase activity was assessed on DNase test agar containing toluidine blue (Gumasta *et al.* 2019), amylase activity was determined on starch agar with subsequent iodine flooding (Nimisha *et al*. 2019) and gelatinase activity was evaluated on nutrient gelatin agar (dela Cruz and Torres 2012). Slime production was determined using Congo red agar method (Fabres-Klein *et al*. 2015). Hemolytic activity was assessed on blood agar (HiMedia Ltd, Mumbai) and classified as α-hemolysis (partial or green zone), β-hemolysis (complete clear zone), or γ-hemolysis (no hemolysis) (Yeom *et al*. 2026).

2.5 Virulence gene detection

PCR amplification of virulence-associated genes was performed using gene-specific primers. All PCR reactions included no template negative controls and positive controls using previously confirmed positive isolates. Amplicons were resolved on 1.5% agarose gels with appropriate DNA size markers. Amplicon sizes were verified against expected product lengths (Table 1).

2.6 Virulence scoring and categorization

A cumulative virulence scoring system was applied to each isolate based on combined phenotypic and genotypic virulence data, following the approach of El-Baz *et al*. (2016). The presence of each phenotypic virulence factor and virulence-associated gene was assigned a score of 1. Hemolytic activity was scored as 2 for β-hemolysis (complete hemolysis), 1 for α-hemolysis (partial hemolysis) and 0 for γ-hemolysis (no hemolysis) (Yeom *et al*. 2026). Cumulative scores were calculated for each isolate, and mean scores were computed for each bacterial genus. This scoring approach should be interpreted as a compar-

ative index rather than an absolute measure of pathogenicity, acknowledging the limitation that individual virulence determinants may contribute differently to overall virulence. Isolates were categorized as highly virulent (score 6–8), moderately virulent (score 4–5), or low virulence (score 2–3) for comparative interpretation of cumulative virulence scores.

TABLE 1 Primers used for molecular characterization and virulence gene detection.

Primer	Primer sequence (5′–3′)	Product length	Annealing	Reference
16S rRNA 27F	AGAGTTTGATCCTGGCTCAG	1465bp	52°C/30sec	Sarkar *et al.* (2020)
16S rRNA 1492R	GGTTACCTTGTTACGACTT			
aerA-F	CAAGAACAAGTTCAAGTGGCCA	309bp	59°C/30sec	Wang *et al.* (2003)
aerA-R	ACGAAGGTGTGGTTCCAGT			
act-F	AGAAGGTGACCACCACCAAGAACA	232bp	56°C/30sec	Nawaz *et al.* (2010)
act-R	AACTGACATCGGCCTTGAACTC			
astA-F	ATGGTGAAAGCCCTCTTCCAG	243bp	55°C/30sec	Qian *et al.* (2023)
astA-R	ATTTCGGATGAACACGGCGA			
aphA-F	GCCATCAGAATCGCCGTAAT	249bp		
aphA-R	AGCATTCCGAAGAAAGTGGG			
hlyA-F	GGCAAACAGCGAAACAAATACC	736bp	60°C/30sec	Devi *et al.* (2022)
hlyA-R	CTCAGCGGGCTAATACGGTTTA			
rtxA-F	GCGATTCTCAAAGAGATGC	1366bp	54°C/30sec	
rtxA-R	CACTCATTCCGATAACCAC			
oprL-F	ATGGAAATGCTGAAATTCGGC	504bp	55°C/30sec	Xu *et al.* (2004)
oprL-R	CTTCTTCAGCTCGACGCGACG			
toxA-F	GGTAACCAGCTCAGCCACAT	352bp	55°C/30sec	Wendt *et al.* (2017)
toxA-R	TGATGTCCAGGTCATGCTTC			
recA-F	CACGCCCTAGACCCTCAATA	136bp	52°C/30sec	Bahador *et al.* (2018)
recA-R	CGATTAAATCAATTGCGCCT			
dnaK-F	GCGTTTAATTGGTCGTCGTT	105bp		
dnaK-R	ACTTCAACCCAAGCATCACC			

2.7 Antimicrobial susceptibility testing and MAR index

Antimicrobial susceptibility of *Vibrio* isolates was determined by the Kirby-Bauer disk diffusion method (Hudzicki 2009) on Mueller-Hinton agar (HiMedia), following Clinical and Laboratory Standards Institute (CLSI 2016) guidelines. Isolates were tested against oxacilllin, methicillin, piperacillin, cefoxitin, cefepime, cefotaxime, ceftazidime, meropenem, doxycycline, tetracycline, chloramphenicol, sulphamethizole and trimethoprim. The MAR index was calculated as the ratio of the number of antibiotics to which an isolate was resistant to the total number of antibiotics tested (Sandhu *et al*. 2016). MAR indices of *Aeromonas* isolates were obtained from our previous study (Shrivastava *et al*. 2026) and used for comparative pathogenic risk assessment. MAR index values > 0.2 were regarded as indicative of origin from high-antibiotic-use environments (Sandhu *et al*. 2016).

2.8 Data analysis

Statistical analyses were performed in R version 4.5.1 (R Core Team 2025). Fisher's exact tests were used to compare the prevalence of phenotypic virulence traits across bacterial genera. The Kruskal–Wallis test followed by Dunn's post hoc test with Bonferroni correction was used to compare cumulative virulence scores among bacterial genera. A significance level of $\alpha = 0.05$ was used for all statistical analyses, and p-values < 0.05 were considered statistically significant. Figures were generated using ggplot2 package in R.

3 | RESULTS

3.1 Clinical signs and bacterial Isolation

Sampled fish exhibited multiple clinical signs consistent with bacterial infection, including lethargy, erratic swimming, exophthalmia, skin discolouration, head ulceration and hemorrhages on the body surface and eyes (Figure S1). A total of 96 bacterial isolates were obtained, comprising 84 gram-negative and 12 gram-positive organisms.

3.2 Molecular identification and prevalence

Fifty representative isolates, encompassing all observed biochemical profiles, colony morphotypes and sampling locations among the 96 recovered isolates (Table S1–S2)

were selected for molecular characterization, thereby including representatives from all distinct phenotypic groups identified during primary characterization. Among them, *Aeromonas* was the predominant genus (42%), followed by *Vibrio* (20%) and *Plesiomonas* (18%) (Figure 2). *Bacillus* and *Pseudomonas* each accounted for 8% of identified isolates, while *Acinetobacter* and *Exiguobacterium* each contributed 2%. Within *Aeromonas*, *A. veronii* was the most prevalent species, representing 22% of all identified isolates. *Vibrio cholerae* was the sole *Vibrio* species identified, while *P. shigelloides* comprised all *Plesiomonas* isolates. GenBank accession numbers for all deposited sequences are listed in Table 2.

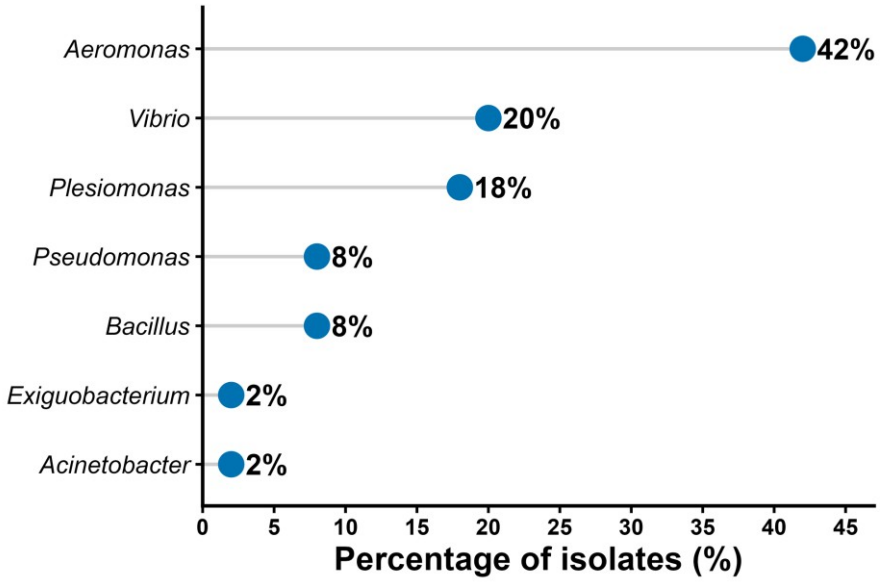

FIGURE 2 Prevalence (%) of bacterial genera identified among the 50 representative bacterial isolates recovered from diseased freshwater-farmed Asian seabass.

Table 2. NCBI Accession numbers of bacterial 16S rRNA gene partial sequences

Isolate code	Identified organism	NCBI accession ID	Isolate code	Identified organism	NCBI accession ID
A11	*A. schubertii*	PQ305827	A61	*V. cholerae*	PQ002437
A12	*A. caviae*	PP990188	A62	*P. shigelloides*	PP990192
A14	*V. cholerae*	PQ002430	A65	*P. shigelloides*	PQ295856
A21	*P. shigelloides*	PQ002435	A71	*V. cholerae*	PP990196
A22	*P. shigelloides*	PP990189	A72	*A. veronii*	PQ002428
A23	*Bacillus cereus*	PQ305828	A78	*V. cholerae*	PQ242666
A27	*P. shigelloides*	PP825364	A81	*A. veronii*	PP990193
A28	*B. kochii*	PQ242667	A84	*A. veronii*	PP825363
A211	*B. cereus*	PQ242665	A91	*P. shigelloides*	PQ295859
A31	*V. cholerae*	PQ002438	A95	*P. shigelloides*	PP990194
A32	*V. cholerae*	PQ242668	A96	*V. cholerae*	PP990197
A33	*V. cholerae*	PQ002431	A911	*P. shigelloides*	PQ295858
A313	*V. cholerae*	PP990190	A912	*B. cereus*	PQ295857
A315	*Aeromonas* sp.	PQ002429	G1	*A. veronii*	PQ243116
A317	*A. hydrophila*	PQ002432	G2	*A. caviae*	PQ242625
A41	*A. veronii*	PP990191	G3	*A. veronii*	PQ242624
A43	*A. hydrophila*	PQ295860	G5	*A. veronii*	PQ242623
A44	*A. veronii*	PQ002433	G9	*Aeromonas* sp.	PQ243118
A46	*Pseudomonas* sp.	PP990195	G15	*Aeromonas* sp.	PQ242622
A47	*A. dhakensis*	PQ002427	G16	*P. shigelloides*	PQ302310
A48	*A. hydrophila*	PQ002434	G22	*V. cholerae*	PQ243117
A49	*Pseudomonas* sp.	PQ002436	K1	*A. veronii*	PQ242621
A411	*P. otitidis*	PQ243119	K2	*A. veronii*	PQ226006
A51	*A.baumannii*	PQ242669	MN4	*A. veronii*	PQ363294
A52	*Exiguobacterium* sp.	PQ243120	MN5	*P. putida*	PQ363295

3.3 Tissue-wise distribution

Bacterial isolates were most frequently recovered from kidney tissues (*n* = 34), followed by liver (*n* = 14) and eyes (*n* = 2). *Aeromonas* was isolated from all three tissue types, with the highest numbers from kidney (*n* = 13). *Vibrio* and *Plesiomonas* isolates were exclusively obtained from kidney and liver. Only *A. veronii* was recovered from eye tissue (Table 3).

TABLE 3 Tissue-wise distribution of bacterial genera isolated from kidney, liver and eyes.

Bacterial genera	Tissue (*n*)		
	Kidney	Liver	Eyes
Aeromonas	13	6	2
Vibrio	6	4	-
Plesiomonas	6	3	-
Bacillus	3	1	-
Pseudomonas	4	-	-
Acinetobacter	1	-	-
Exiguobacterium	1	-	-
Total	34	14	2

Values expressed as '*n*' represent the number of bacterial isolates from a given tissue. A dash (-) indicates that no isolates of the species were recovered from that tissue.

3.4 Phenotypic virulence assessment

Marked variation in virulence trait prevalence was observed across bacterial genera (Figure 3). Among *Aeromonas* isolates, gelatinase activity was detected in 100%, DNase activity in 90%, hemolytic activity (α or β) in 86%, amylase activity in 76% and slime formation in 29% of isolates. Among *Vibrio* isolates, amylase and gelatinase activities were each present in 90%, while DNase and hemolytic activities were detected in 70% of isolates. *Plesiomonas* isolates showed a more restricted virulence phenotype, with DNase activity being the most prevalent trait (67%). Slime formation was detected in 44% of *Plesiomonas* isolates. *Pseudomonas* and *Bacillus* isolates showed moderate virulence, with gelatinase activity present in 75% of isolates from each genus. Fisher's exact test demonstrated significant differences in the prevalence of amylase activity ($p < 0.001$), gelatinase activity ($p < 0.001$) and hemolytic activity ($p = 0.014$) among *Aeromonas*, *Vibrio* and *Plesiomonas* genera. In contrast, DNase activity ($p = 0.225$) and slime formation ($p = 0.537$) did not differ significantly among these genera.

3.5 Virulence gene distribution

PCR screening performed with appropriate positive and negative controls, confirmed the presence of multiple virulence-associated genes across all bacterial genera (Figure 4). Among *Aeromonas* isolates, the *act* gene was detected in 95% and *aerA* in 38% of isolates. In *Vibrio* isolates, both *rtxA* and *hlyA* genes were detected in 90% of isolates. All *Plesiomonas* isolates (100%) were positive for both *astA* and *aphA* genes. All *Pseudomonas* isolates harboured the *oprL* gene, while none were positive for *toxA*. Both *dnaK* and *recA* genes were confirmed in the sole *A. baumannii* isolate (Figure 5).

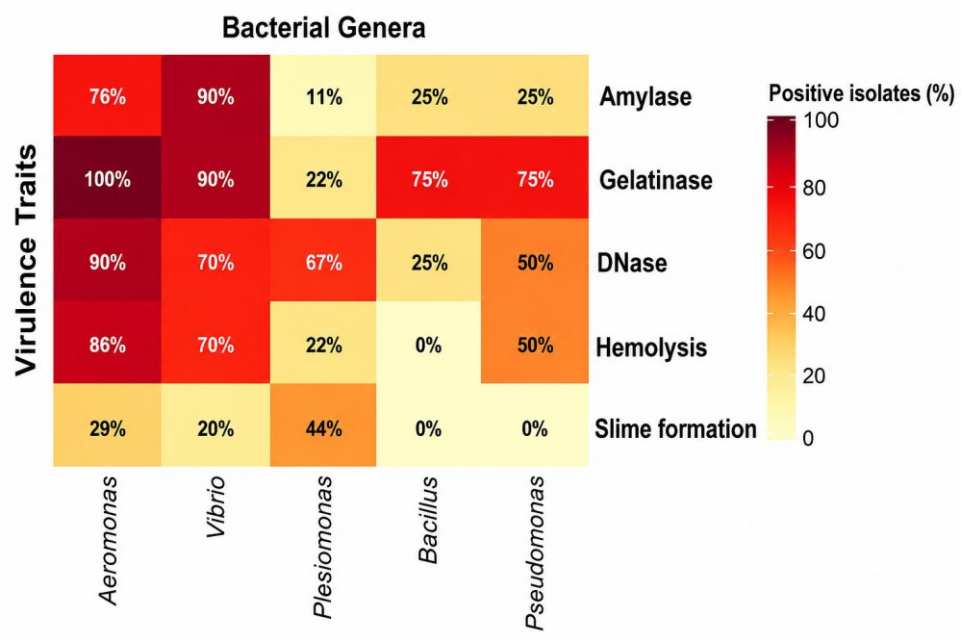

FIGURE 3 Heatmap showing the percentage of isolates positive for amylase, gelatinase, DNase, hemolysis and slime formation across the bacterial genera, including *Aeromonas* (*n* = 21), *Vibrio* (*n* = 10), *Plesiomonas* (*n* = 9), *Bacillus* (*n* = 4), and *Pseudomonas* (*n* = 4).

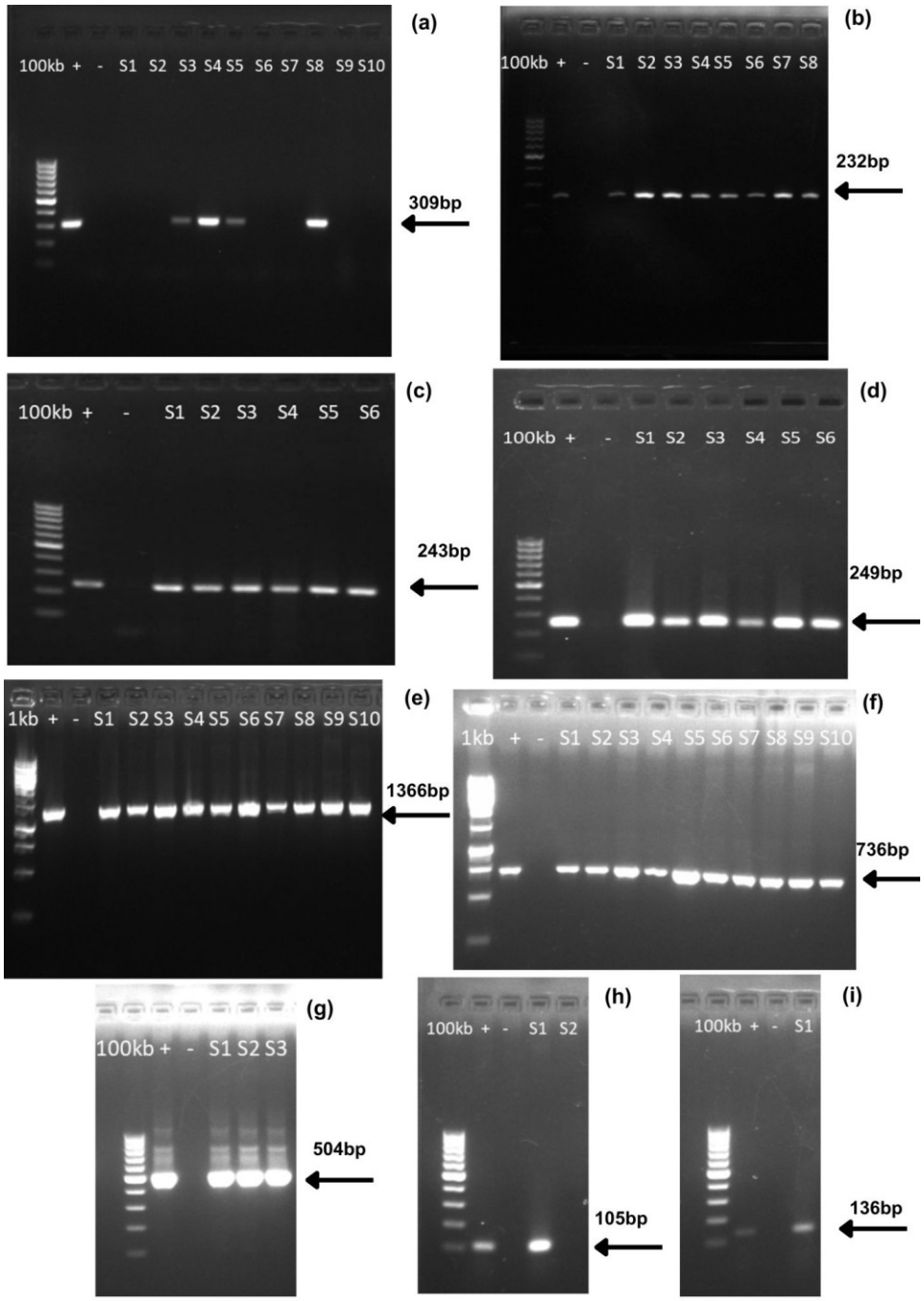

FIGURE 4 PCR amplification gel picture of virulence gene markers (a) *aerA* gene and (b) *act* gene in *Aeromonas* isolates; (c) *astA* gene and (d) *aphA* gene in *Plesiomonas* isolates; (e) *rtxA* gene and (f) *hlyA* gene in *Vibrio* isolates; (g) *oprL* gene in *Pseudomonas* isolates; (h) *dnaK* gene and (i) *recA* gene in *Acinetobacter* isolate.

3.6 Virulence scoring, categorization and risk assessment

Virulence profiling and scoring revealed considerable inter-genus variation in cumulative virulence potential (Table 4). Mean virulence scores were 5.57 for *Aeromonas* and 5.60 for *Vibrio*, compared to 3.66 for *Plesiomonas*. Among *Aeromonas*, 52% of isolates were categorized as highly virulent (score 6–8), 48% as moderately virulent and none as low virulence. Among *Vibrio* isolates, 50% were highly virulent, 40% moderately virulent and 10% low virulence. The majority of *Plesiomonas* isolates (67%) were classified in the moderate virulence category. Kruskal–Wallis analysis revealed significant differences in cumulative virulence scores among bacterial genera (H = 11.568, df = 2, p = 0.0031, $\eta^2(H)$ = 0.259, large effect size). Dunn's post-hoc test indicated significantly higher virulence scores in *Aeromonas* and *Vibrio* compared to *Plesiomonas* ($p < 0.05$, Bonferroni-adjusted).

3.7 MAR index and integrated pathogenic risk

MAR index values among *Aeromonas* isolates ranged from 0.11 to 0.50 and among *Vibrio* isolates, only one isolate (A96: MAR = 0.53) exceeded 0.2 (Figure S2, Table S3). The integrated risk assessment revealed that 71.4% *Aeromonas* isolates simultaneously exhibited moderate-

to-high virulence scores and MAR indices > 0.2 (Figure 6). In contrast, *Vibrio* isolates displaying high virulence scores generally had lower MAR indices.

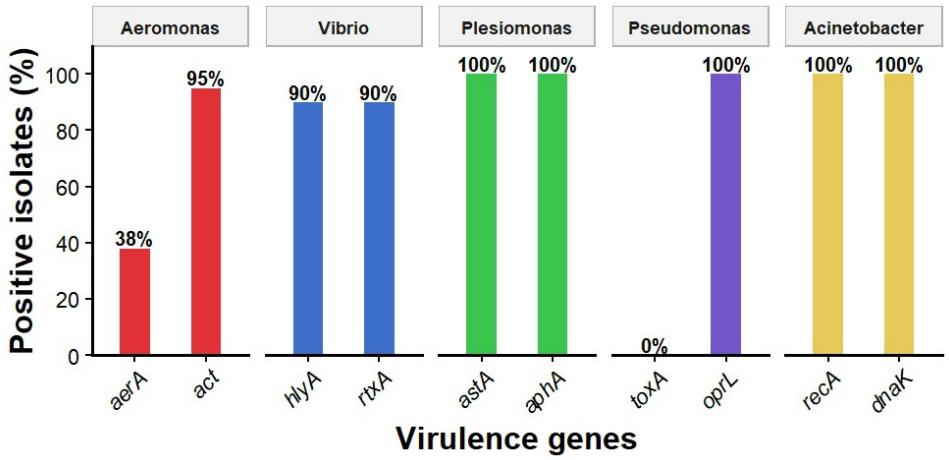

FIGURE 5 Genus-wise prevalence of virulence-associated genes in bacterial isolates.

TABLE 4 Virulence potential of *Aeromonas*, *Vibrio* and *Plesiomonas* isolates.

Isolate code	Organism	Phenotypic virulence assay					Virulence gene	Virulence score
		Amylase	Gelatinase	DNase	Slime formation	Hemolysis		
A11	*A. schubertii*	0	1	1	1	2	-	5
A12	*A. caviae*	0	1	1	1	2	*act*	6
A315	*Aeromonas* sp.	0	1	1	0	2	*aerA, act*	6
A317	*A. hydrophila*	0	1	1	0	2	*aerA, act*	6
A41	*A. veronii*	1	1	1	0	1	*aerA, act*	6
A43	*A. hydrophila*	1	1	1	0	1	*act*	5
A44	*A. veronii*	1	1	1	0	1	*act*	5
A47	*A. dhakensis*	1	1	1	0	2	*aerA, act*	7
A48	*A. hydrophila*	1	1	1	0	1	*act*	5
A72	*A. veronii*	0	1	1	0	1	*act*	4
A81	*A. veronii*	1	1	1	0	1	*act*	5
A84	*A. veronii*	1	1	1	0	2	*aerA, act*	7
G1	*A. veronii*	1	1	1	0	2	*act*	6
G2	*A. caviae*	1	1	1	0	0	*aerA, act*	5
G3	*A. veronii*	1	1	1	0	0	*act*	4
G5	*A. veronii*	1	1	0	0	0	*aerA, act*	4
G9	*Aeromonas* sp.	1	1	1	1	1	*act*	6
G15	*Aeromonas* sp.	1	1	1	1	1	*aerA, act*	7
K1	*A. veronii*	1	1	1	1	2	*act*	7
K2	*A. veronii*	1	1	1	1	2	*act*	7
MN4	*A. veronii*	1	1	0	0	1	*act*	4
Mean virulence score =								5.57
A14	*V. cholerae*	1	1	1	0	1	*rtxA, hlyA*	6
A31	*V. cholerae*	1	1	0	0	0	*rtxA, hlyA*	4
A32	*V. cholerae*	1	1	1	0	2	*rtxA, hlyA*	7
A33	*V. cholerae*	1	1	0	0	1	*rtxA, hlyA*	5
A313	*V. cholerae*	1	1	1	0	2	*rtxA, hlyA*	7
A61	*V. cholerae*	1	1	1	1	2	*rtxA, hlyA*	8
A71	*V. cholerae*	1	1	1	0	2	*rtxA, hlyA*	7
A78	*V. cholerae*	1	1	1	0	0	*rtxA, hlyA*	5

TABLE 4 Continued.

Isolate code	Organism	Phenotypic virulence assay					Virulence gene	Virulence score
		Amylase	Gelatinase	DNase	Slime formation	Hemolysis		
A96	*V. cholerae*	1	0	1	0	1	*rtxA, hlyA*	5
G22	*V. cholerae*	0	1	0	1	0	-	2
Mean virulence score =								5.60
A21	*P. shigelloides*	0	0	1	1	1	*astA, aphA*	5
A22	*P. shigelloides*	0	0	1	0	1	*astA, aphA*	4
A27	*P. shigelloides*	0	1	1	0	0	*astA, aphA*	4
A62	*P. shigelloides*	0	0	1	1	0	*astA, aphA*	4
A65	*P. shigelloides*	1	0	1	0	0	*astA, aphA*	4
A91	*P. shigelloides*	0	1	1	1	0	*astA, aphA*	5
A95	*P. shigelloides*	0	0	0	0	0	*astA, aphA*	2
A911	*P. shigelloides*	0	0	0	1	0	*astA, aphA*	3
G16	*P. shigelloides*	0	0	0	0	0	*astA, aphA*	2
Mean virulence score =								3.66

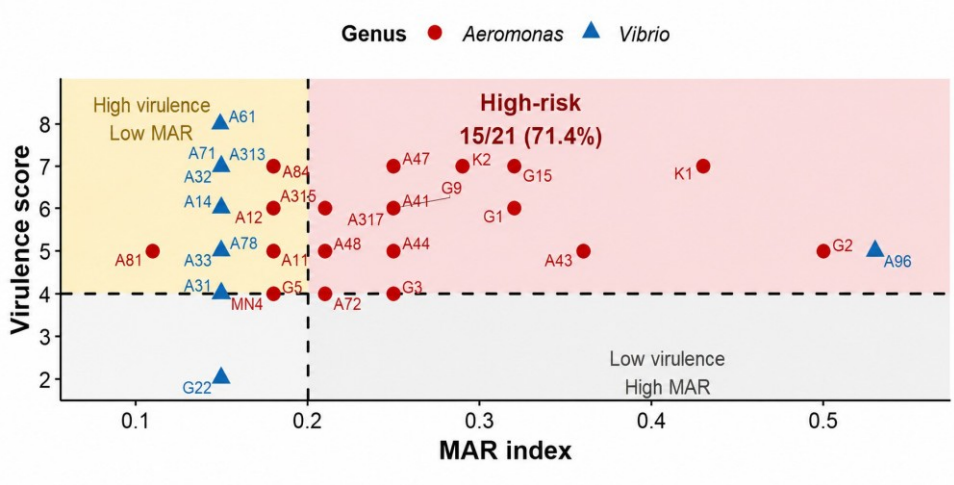

FIGURE 6 Scatter plot showing the relationship between cumulative virulence scores and multiple antibiotic resistance (MAR) indices among bacterial isolates. Dashed lines indicate the thresholds used for virulence score (4) and MAR index (0.2), dividing isolates into comparative risk categories.

4 | DISCUSSION

4.1 Molecular identification and prevalence

This study presents a systematic characterization of bacterial virulence profiles from freshwater-farmed Asian seabass in India, integrating phenotypic, genotypic and quantitative risk assessment approaches. The multi-state sampling design spanning four major aquaculture states enhances the representativeness of the findings and provides a baseline dataset for future longitudinal surveillance. The predominance of *Aeromonas* (42%), with *A. veronii* as the prevalent species (22%) is consistent with previous reports from freshwater Asian seabass in Southeast Asia (Mursalim *et al*. 2022) and from other freshwater fish species in India (Rathinam *et al*. 2021). *Aeromonas* species are ubiquitous inhabitants of freshwater environments, and their opportunistic pathogenicity is strongly influenced by fish immune status, water quality and stocking density conditions frequently suboptimal in intensive freshwater aquaculture (Sandeep *et al*. 2016). The identification of *V. cholerae* accounting for 20% of isolates, is particularly notable from a One Health perspective. *V. cholerae* is primarily recognized as a human pathogen causing cholera. However, non-O1/non-O139 serotypes are increasingly documented as opportunistic pathogens of freshwater fish (Rehulka *et al*. 2015; Devi *et al*. 2022).

4.2 Phenotypic and genotypic virulence assessment

The presence of gelatinase (100% in *Aeromonas*) and high prevalence of DNase (90%) and hemolytic activity (86%) indicate a multi-factorial virulence arsenal of *Aeromonas* species. Gelatinase facilitates extracellular matrix degradation and tissue invasiveness, while DNase provides nucleic acid substrates for bacterial growth and aids immune evasion by degrading neutrophil extracellular traps (Pemberton *et al*. 1997; Salerno *et al*. 2010). Hemolysin production, including both α- and β-hemolytic activity, is a critical determinant of systemic pathogenicity in *Aer-*

omonas, causing erythrocyte lysis and cytotoxic tissue damage (Sahu *et al*. 2012). The concordance between hemolytic phenotype and virulence gene detection (particularly *act* at 95%) further supports the utility of genotypic markers as indicators of pathogenic potential. The *act* gene, encoding cytotoxic enterotoxin, is widely regarded as one of the most reliable molecular markers for pathogenic *Aeromonas* (Sen and Rodgers 2004). Its high prevalence among the isolates examined in the present study suggests the widespread distribution of potentially pathogenic *Aeromonas* strains across the sampled farms. The detection of both *rtxA* and *hlyA* in 90% of *V. cholerae* isolates aligns with earlier reports from diseased freshwater fish in India (Devi *et al*. 2022). The MARTX toxin encoded by *rtxA* is a major virulence determinant of *V. cholerae* that disrupts host cytoskeletal integrity and facilitates intestinal colonization, while *hlyA* contributes to virulence through pore-forming hemolytic activity. The co-occurrence of these genes in most *Vibrio* isolates, combined with the detection of hemolytic phenotypes in 70% of isolates, indicates a high virulence potential that warrants close monitoring in freshwater aquaculture environments. The statistical analysis further supported these observations by demonstrating that the prevalence of amylase, gelatinase and hemolytic activities differed significantly among bacterial genera, whereas DNase activity and slime formation were comparatively conserved. These findings suggest that extracellular enzyme production and hemolytic capacity constitute the discriminatory virulence determinants among the bacterial genera isolated in the present study.

4.3 Virulence scoring, categorization and risk assessment

The virulence scoring approach employed here, while acknowledging its limitations as a relative and unvalidated index, provides a structured framework for comparative risk stratification across genera. The near-equivalent mean virulence scores of *Aeromonas* (5.57) and *Vibrio* (5.60) suggest these two genera as possess the highest virulence potential among the bacterial genera examined. The relatively lower mean virulence score of *Plesiomonas* (3.66) should not, however, obscure the significance of the universal detection of *astA* and *aphA* genes in all *P. shigelloides* isolates, as these encode enterotoxins and serine proteases associated with gastroenteritis and tissue invasion in both fish and humans (Qian *et al*. 2023). The significant differences in cumulative virulence scores among bacterial genera, confirmed by the Kruskal–Wallis test and subsequent Dunn's post hoc analysis, further support that *Aeromonas* and *Vibrio* exhibited significantly higher cumulative virulence scores than *Plesiomonas*.

4.4 MAR index and integrated pathogenic risk

The elevated MAR indices observed among *Aeromonas* isolates (71.4% exceeding 0.2) are consistent with documented patterns of antibiotic selection pressure in freshwater aquaculture systems (Mursalim *et al*. 2022). Freshwater *Aeromonas* species are known to accumulate resistance determinants rapidly through horizontal gene transfer, and their frequent exposure to anthropogenic antibiotic inputs from agricultural runoff and aquaculture practices creates ideal selection environments (Nawaz *et al*. 2010). The co-occurrence of moderate-to-high virulence scores and elevated MAR indices in 71.4% *Aeromonas* isolates represents one of the key findings of the present study and has important implications for disease management. Such isolates may represent a potential risk to fish health and reservoir for antimicrobial resistance gene dissemination in aquatic environments, with downstream implications for human and environmental health. The comparatively low MAR indices among most *Vibrio* isolates, despite their high virulence scores, suggests that virulence acquisition and AMR are not obligatorily coupled evolutionary phenomena in this genus under current aquaculture conditions. This distinction may reflect differential antibiotic exposure histories or distinct ecological niches within freshwater farm environments (Gomes 2024).

The pathogen load combined with the AMR and virulence profiles documented here, highlights the need of integrating pathogen surveillance into climate-resilient aquaculture management strategies. The One Health paradigm which recognizes the interconnection of human, animal and environmental health provides the most appropriate conceptual framework for addressing the compounded risks identified in this study. The present findings are based primarily on *in vitro* phenotypic and molecular characterization. Therefore, future studies should validate the pathogenic potential of these isolates through controlled *in vivo* challenge experiments to establish the relationship between the observed virulence profiles, disease severity and host responses.

5 | CONCLUSIONS

This study provides a systematic evaluation of the virulence and antimicrobial resistance profiles of bacterial pathogens associated with freshwater-farmed Asian seabass in India. *Aeromonas* and *V. cholerae* were the predominant bacterial genera, exhibiting multi-factorial virulence profiles encompassing extracellular enzyme activities, hemolytic traits and multiple virulence-associated genes. The co-occurrence of high virulence scores and elevated MAR indices in a subset of *Aeromonas* identified isolates with a comparatively higher risk profile, highlighting the importance of targeted disease management and antimicrobial stewardship. The isolation of *V. cholerae* from food fish further situates these findings within the One Health framework. The data generated provide a critical baseline for future longitudinal surveillance, ther-

apeutic development research and evidence-based antimicrobial stewardship in Asian seabass aquaculture. However, the integrated pathogenic risk presented in this study was inferred from laboratory-based phenotypic and molecular assays and was not validated through *in vivo* infection experiments. Therefore, controlled challenge studies are required to confirm the pathogenicity of the identified isolates.

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ETHICAL APPROVAL

The study complies with the current animal welfare laws in India. The CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt. of India) guidelines were followed while handling sampled animals. The study was approved by the authorities and board of studies of the ICAR- Central Institute of Fisheries Education, Mumbai, India (approval no. 524/GO/ReRcBiBt/S/02/CCSEA). The government of India's Wildlife Protection Act of 1972 does not apply to studies on Asian seabass, as it is not an endangered fish.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Yashwee Shrivastava: Investigation, Data curation, Formal analysis, Writing – original draft. Gayatri Tripathi: Conceptualization, Supervision, Writing – review & editing. Rajesh Bharathi Rathinam: Formal analysis, Writing – review & editing. Megha Kadam Bedekar: Formal analysis, Writing – review & editing. Arpit Acharya: Investigation, Formal analysis. Amulya Shirivanthe Girish: Investigation, Formal analysis.

AI USE STATEMENT

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to improve the language, grammar and readability of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

DATA AVAILABILITY STATEMENT

Data are contained within the article and supplementary materials.

REFERENCES

Abdulateef SA, Owaif HAA, Hussein MH (2023) Importance of virulence factors in bacterial pathogenicity: a review. International Journal of Medical Science and Clinical Research Studies 3(4): 765–769.

AVMA (2020) AVMA guidelines for the euthanasia of animals. American Veterinary Medical Association, Schaumburg, IL, USA.

Awate S, Mubarka S, Huber RG (2023) Whole genomic characterization of *Streptococcus iniae* isolates from Barramundi (*Lates calcarifer*) and preliminary evidence of cross-protective immunization. Vaccines 11(9): 1443.

Bahador A, Farshadzadeh Z, Raoofian R, Mokhtaran M, Pourakbari B, … Hashemi FB (2018) Association of virulence gene expression with colistin-resistance in *Acinetobacter baumannii*: analysis of genotype, antimicrobial susceptibility, and biofilm formation. Annals of Clinical Microbiology and Antimicrobials 17(1): 24.

Casadevall A, Pirofski L (2003) The damage-response framework of microbial pathogenesis. Nature Reviews Microbiology 1(1): 17–24.

Chachaty E, Saulnier P (2000) Isolating chromosomal DNA from Bacteria. In: The Nucleic Acid Protocols Handbook. Humana Press, Totowa, NJ, pp 29–32.

CLSI (2016) Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria (Version CLSI guideline M45, 3rd edition).

dela Cruz TEE, Torres JMO (2012) Gelatin hydrolysis test protocol. American Society for Microbiology 1: 1–10.

Detcharoen M, Khrueakaew P, Benjakul S, Romyasamit C, Suyapoh W, Saetang J (2025) Surveillance of antimicrobial resistance in the Asian seabass (*Lates calcarifer*) supply chain using nanopore sequencing. Foods 14(10): 1691.

Devi MS, Paria P, Kumar V, Parida PK, Maurye P, ... Das BK (2022) Molecular identification and pathogenicity study of virulent *Vibrio cholerae* non O1/O139 serotype associated with mortality of farmed *Labeo rohita* (Hamilton, 1822), in India. Aquaculture 547(1): 737529.

El-baz R, Rizk DE, Barwa R, Hassan R (2016) Virulence factors profile of *Staphylococcus aureus* isolated from different clinical sources. Egyptian Journal of Microbiology 43: 126–144.

Fabres-Klein MH, Caizer Santos MJ, Contelli Klein R, Nunes De Souza G, De Oliveira Barros Ribon A (2015) An association between milk and slime increases biofilm production by bovine *Staphylococcus aureus*. BMC Veterinary Research 11(1): 3.

FAO (2022) The state of world fisheries and aquaculture 2022. Food and Agriculture Organization, Rome, Italy.

FAO (2026) The state of world fisheries and aquaculture 2026. Food and Agriculture Organization, Rome, Italy.

Ghosh S (2019) Farming of Asian seabass *Lates calcarifer* in freshwater impoundments in West Bengal, India. Aquaculture Asia 23(3): 1–9.

Gomes MP (2024) The convergence of antibiotic contamination, resistance, and climate dynamics in freshwater ecosystems. Water 16(18): 2606.

Gumasta R, Nawange SR, Singh SM, Garg A, Sethi R, Yadu R (2019) Extracellular protease and DNase activities in clinical and environmental isolates of *Cryptococcus neoformans* species complex from central India. Journal of Drug Delivery and Therapeutics 9(4-A): 328–333.

Hudzicki J (2009) Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology 15(1): 1–23.

Hutson KS (2013) Infectious diseases of Asian seabass and health management. Biology and Culture of Asian Seabass *Lates calcarifer*. CRC Press, Taylor and Francis Group, USA, 102–136.

Irshath AA, Rajan AP, Vimal S, Prabhakaran VS, Ganesan R (2023) Bacterial pathogenesis in various fish diseases: recent advances and specific challenges in vaccine development. Vaccines 11(2): 470.

Kanchanopas-Barnette P, Labella A, Alonso CM, Manchado M, Castro D, Borrego JJ (2009) The first isolation of *Photobacterium damselae* subsp. *damselae* from Asian seabass *Lates calcarifer*. Fish Pathology 44(1): 47–50.

Kathirkaman P, Ayyaru G, Serelathan MV, Singaravel V, Gunasekaran T (2018) Innate immunological responses of Asian sea bass, *Lates calcarifer* (Bloch, 1790) for experimentally challenged *Aeromonas hydrophila* infection. Comparative Clinical Pathology 27(4): 927–931.

Kerddee P, Dong HT, Chokmangmeepisarn P, Rodkhum C, Srisapoome P, … Kayansamruaj P (2020) Simultaneous detection of scale drop disease virus and *Flavobacterium columnare* from diseased freshwater-reared barramundi *Lates calcarifer*. Diseases of Aquatic Organisms 140: 119–128.

Krupesha Sharma SR, Rathore G, Verma DK, Sadhu N, Philipose KK (2012) *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. Aquaculture Research 44(1): 86–92.

Lehman D (2005) Triple sugar iron agar protocols. American Society for Microbiology, Washington, DC. pp. 1–7.

MacWilliams MP (2009a) Citrate test protocol. American Society for Microbiology, Washington, DC. pp. 1–7.

MacWilliams MP (2009b) Indole Test Protocol. American Society for Microbiology, Washington, DC. pp. 1–9.

Mishra SS, Rakesh D, Dhiman M, Choudhary P, Debbarma J, … Swain P (2017) Present status of fish disease management in freshwater aquaculture in India: state-of-the-art-review. Journal of Aquaculture and Fisheries 1(003): 14.

MPEDA (2023) Annual report 2022–23. Marine Products Export Development Authority, Kochi, India.

Munilkumar S, Sundaray JK, Bhatacharya SB, Devi GA (2013) Biology and fisheries of some brackishwater food fishes of India. Advances in Fish Research 6: 187–157.

Mursalim MF, Budiyansah H, Raharjo HM, Debnath PP, Sakulworakan R, … Rodkhum C (2022) Diversity and antimicrobial susceptibility profiles of *Aeromonas* spp. isolated from diseased freshwater fishes in Thailand. Journal of Fish Diseases 45(8): 1149–1163.

Nagar V, Ansari F, Vaiyapuri M, Joseph TC (2025) Virulent and multidrug-resistant *Aeromonas* in aquatic environments of Kerala, India: potential risks to fish and humans. Brazilian Journal of Microbiology 56(1): 303–311.

Nawaz M, Khan SA, Khan AA, Sung K, Tran Q, … Steele R (2010) Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiology 27(3): 327–331.

Nimisha P, Moksha S, Gangawane AK (2019) Amylase activity of a starch degrading bacteria isolated from soil. International Journal of Current Microbiology and Applied Sciences 8(4): 659–671.

Orbán L, Shen X, Phua N, Varga L (2021) Toward genome-based selection in Asian seabass: What can we learn from other food fishes and farm animals? Frontiers in Genetics 12: 506754.

Pemberton JM, Kidd SP, Schmidt R (1997) Secreted enzymes of *Aeromonas*. FEMS Microbiology Letters 152(1): 1–10.

Qian Q, Chen Z, Xu J, Zhu Y, Xu W, … Zhang X (2023) Pathogenicity of *Plesiomonas shigelloides* causing mass mortalities of largemouth bass (*Micropterus salmoides*) and its induced host immune response. Fish and Shellfish Immunology 132: 108487.

R Core Team (2025) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rathinam RB, Banu H, Ghode GS, Bhushan S, Bhuvaneswari R, Tripathi G (2021) High prevalence of Aeromonas and Pseudomonas infections among cage cultured Pangas catfish from the reservoirs of Maharashtra, India. Israeli Journal of Aquaculture-Bamidgeh 73: 1–18.

Rehulka J, Petras P, Marejkova M, Aldova E (2015) *Vibrio cholerae* non-O1/non-O139 infection in fish in the Czech Republic. Veterinární Medicína 60(1): 16–22.

Sahu I, Das BK, Marhual NP, Pradhan J, Sahoo DR, … Mish-

ra BK (2012) Phenotypic and genotypic methods for identifications of *Aeromonas hydrophila* strains from carp *Labeo rohita* and their virulence study. International Journal of Fisheries and Aquaculture Sciences 2: 141–156.

Salerno A, Čižnár I, Krovacek K, Conte M, Dumontet S, ... Pasquale V (2010) Phenotypic characterization and putative virulence factors of human, animal and environmental isolates of *Plesiomonas shigelloides*. Folia Microbiologica 55(6): 641–647.

Sandeep P, Chamundeswari DB, Kumar KP (2016) Present status of parasitic and bacterial diseases in fresh water fish seed farms in East Godavari District, Andhra Pradesh. International Journal of Applied and Pure Science and Agriculture 2: 117–121.

Sandhu R, Dahiya S, Sayal P (2016) Evaluation of multiple antibiotic resistance (MAR) index and Doxycycline susceptibility of *Acinetobacter* species among inpatients. Indian Journal of Microbiology Research 3(3): 299.

Sarkar SL, Hossain I, Monika SA, Sanyal SK, Roy PC, … Jahid IK (2020) Probiotic potential of *Pediococcus acidilactici* and *Enterococcus faecium* isolated from indigenous yogurt and raw goat milk. Microbiology and Biotechnology Letters 48(3): 276–286.

Sen K, Rodgers M (2004) Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. Journal of Applied Microbiology 97(5): 1077–1086.

Shrivastava Y, Tripathi G, Rathinam RB, Sahoo AK, Sheikh S (2026) Phenotypic antimicrobial resistance traits of *Aeromonas* isolated from freshwater-farmed Asian seabass, *Lates calcarifer*. International Journal of Advanced Biochemistry Research 10(1): 1278–1284.

Too E, Masila E (2024) Virulence factors, biofilm formation, and horizontal gene. *Enterococcus*: unveiling the emergence of a potent pathogen. IntechOpen, London, UK. 91 pp.

Wang G, Clark CG, Liu C, Pucknell C, Munro CK, … Rodgers FG (2003) Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. Journal of Clinical Microbiology 41(3): 1048–1054.

Wendt M, De Silva BC, Heo GJ (2017) Virulence factors and antimicrobial resistance of *Pseudomonas aeruginosa* isolated from pet turtles. Asian Journal of Animal and Veterinary Advances 12(4): 205–211.

Xu J, Moore JE, Murphy PG, Millar BC, Elborn JS (2004) Early detection of *Pseudomonas aeruginosa* – comparison of conventional versus molecular (PCR) detection directly from adult patients with cystic fibrosis (CF). Annals of Clinical Microbiology and Antimicrobials 3(1): 21.

Yeom SJ, Yang J, Yoon KN, Kim SS, Cho MJ, … Kim JK (2026) Safety and next-generation probiotic potential of *Mitsuokella multacida* AGMB01124 newly isolated from healthy swine feces. Probiotics and Antimicrobial Proteins. DOI: 10.1007/s12602-026-10925-y.

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