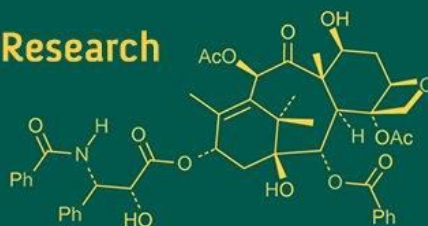


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(1): 1278-1284
www.biochemjournal.com
Received: 24-10-2025
Accepted: 26-11-2025

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Phenotypic antimicrobial resistance traits of *Aeromonas* isolated from freshwater-farmed Asian seabass, *Lates calcarifer*

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i1p.7248>

Abstract

The objective of this study was to investigate antibiotic resistance patterns and phenotyping of *Aeromonas* species isolated from freshwater farmed Asian seabass. A total of 21 *Aeromonas* were identified through biochemical and molecular characterization. Antimicrobial susceptibility tests for various antibiotics revealed high levels of multi-antibiotic resistance among isolates. Antibiotic resistance profile of all *Aeromonas* isolates showed high resistance rates for Oxacillin (100%) and Methicillin (100%), Ampicillin (90%), Cefazolin (86%), Ticarcillin (57%) and Nalidixic acid (57%). The Multiple Antibiotic Resistance index for isolates ranged from 0.11 to 0.50. Antibiotics phenotyping of the *Aeromonas* isolates revealed 21 different phenotypes of the isolates. Bacterial pathogens exhibited distinct resistance patterns across first-, second-, and third-generation antibiotics. This study establishes essential baseline data that can aid in predicting antimicrobial resistance (AMR) trends in freshwater Asian seabass farming.

Keywords: *Aeromonas*, antibiotic resistance, Asian seabass, freshwater, multi-antibiotic resistance

Introduction

Aeromonadaceae is family of Gram-negative, rod-shaped bacteria that have been isolated from various sources such as aquatic environment, soil, fish, higher vertebrates, agricultural products and several other food products. They are ubiquitous in nature and are known to cause diseases particularly when the host is under stress. Aeromonads are recognized as emerging human pathogens, as they are associated with a wide range of food- and water-borne infections. These include gastroenteritis, diarrhoea, urinary tract infections, necrotizing fasciitis, septicaemia and respiratory tract infections (Fernández-Bravo and Figueras, 2020) [18].

For several decades, antibiotics have been used to treat bacterial infections. While antibiotics play a crucial role in disease management, the growing threat of antimicrobial resistance (AMR) has emerged as a major global concern (Dodds, 2017) [16]. Alarming, various species of *Aeromonas*, including both clinical and environmental isolates have been found to exhibit multidrug resistance (Odeyemi and Ahmad, 2017) [34]. The emergence and spread of these resistant bacteria can make the treatment difficult and less effective. It is estimated that drug-resistant diseases could cause 10 million deaths each year by 2050 (Alara and Alara, 2024) [2].

Aeromonas species are among the most important disease-causing bacteria in freshwater aquaculture systems. Several *Aeromonas* species have been associated with fish diseases, including *A. hydrophila* (Junior *et al.*, 2019) [21], *A. veronii* (Li *et al.*, 2020) [27], *A. caviae* (Sharma *et al.*, 2022) [38], *A. dhakensis* (Carriero *et al.*, 2016) [7], *A. schubertii* (Liu and Li, 2012), *A. salmonicida* (Cipriano and Bullock, 2001) [9], *A. jandaei* (Assane *et al.*, 2021) [4] and *A. sobria* (Wahli *et al.*, 2005). Frequent disease outbreaks in aquaculture compel farmers to rely heavily on antibiotics for disease management (Preena *et al.*, 2020) [36].

Fish farms and aquaculture systems have increasingly been identified as hotspots for antibiotic resistant pathogens (Watts *et al.*, 2017) [41]. There are numerous studies reporting the presence of multi-antibiotic resistant *Aeromonas* strains isolated from widely cultured

fish species such as catfish, tilapia and carps with presence of various AMR genes among them (Nguyen *et al.*, 2014; Suresh and Pillai, 2023) [32, 39]. This is alarming as resistant genes can spread among bacteria and it has direct implications for human health, as resistant bacteria can enter the food chain and are extremely difficult to manage once established (Čížek *et al.*, 2010) [10]. Despite the growing concern, there is a lack of data on the antibiotic resistance patterns of bacterial pathogens from freshwater fish culture systems. Further, the adoption of Asian seabass culture in freshwater is new and therefore, if we record the resistance profile of the bacterial pathogens at this stage of the culture, this data would help us to understand as well as to formulate culture policies that can be followed to obtain a sustainable production of Asian seabass from freshwater culture system without increasing the current resistance pattern of the bacterial pathogens associated with freshwater culture system of Asian seabass. Therefore, the objective of this study was to investigate antibiotics resistance patterns and phenotyping of *Aeromonas* species isolated from freshwater farmed Asian seabass. The findings are expected to provide baseline data on antimicrobial resistance bacteria in freshwater aquaculture systems and contribute to ongoing efforts in controlling antimicrobial resistance.

Materials and Methods

Ethical statement: The study complies with the current animal welfare laws in India. The care of sampled animals used in this study were following the guidelines of 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] on care and use of animals in scientific research. The study was approved by the board of Studies and authorities of the ICAR-Central Institute of Fisheries Education, Mumbai, India. As the sampled animal *L. calcarifer* is not an endangered fish, the provisions of the govt. of India's Wildlife Protection Act of 1972 are not applicable for research on this fish.

Sample collection and isolation of bacteria

Freshwater farmed Asian seabass samples (n = 60) were collected from 12 different freshwater farms located in

Andhra Pradesh, Gujarat, Karnataka and Maharashtra, February, 2024 to July, 2024. Moribund fish exhibiting clinical symptoms were transported in live condition to the laboratory for microbiological analysis. Swab samples were collected from kidney, liver and eyes in aseptic condition and inoculated into 5ml Brain Heart Infusion broth (HiMedia Ltd, Mumbai). After 24hrs incubation at 28±2 °C, they were streaked onto BHI agar (HiMedia Ltd, Mumbai). Following an 18-24 hr incubation at 28±2 °C, distinct colonies from the culture plates were selected and sub-cultured to obtain pure isolates for further examination.

Biochemical and molecular characterization of bacterial isolates

Isolates were subjected to presumptive identification based on colony morphology, gram staining, catalase and oxidase (HiMedia, India) tests. Biochemical tests such as citrate utilization (MacWilliams, 2009a), indole production (MacWilliams, 2009b) and carbohydrate fermentation (Lehman, 2005) were performed to aid in bacterial species identification.

Genomic DNA extraction from all bacterial isolates were done using the CTAB (Cetyltrimethylammonium bromide) method (Chachaty and Saulnier, 2000) [8]. PCR was performed to amplify 16S rRNA gene for the bacterial isolates using specific primers (Table 1). Annealing temperature for PCR was determined by performing gradient PCR. Cycling conditions under which PCR was performed are summarised in Table 1. 1.5% (w/v) agarose gel electrophoresis with ethidium bromide dye was used to check the quality of amplified product. The PCR product was extracted from gel using the Purelink Quick Gel Extraction Kit (Invitrogen, Thermo Fisher Scientific, USA) in accordance with the manufacturer's protocol. The PCR amplified product was then sent for Sanger's dideoxy sequencing (Eurofins Genomics India Private Limited, Karnataka, India). The obtained sequences were trimmed by using BioEdit software and were compared to the NCBI nucleotide database using BLASTn. The trimmed sequences were submitted to the GenBank database of the National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1: PCR cycling conditions for 16S rRNA gene amplification

Primer name	Primer sequence (5'-3')	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension	Product length	Reference
16S rRNA 27F	AGAGTTTGATCCTGGCTCAG	94 °C/ 5 min	25	94 °C/ 30 sec	52 °C/ 30 sec	72 °C/ 45 sec	72 °C/ 5 min	1465 bp	(Sarkar <i>et al.</i> , 2020) [37]
16S rRNA 1492R	GGTTACCTTGTACGACTT								

Antimicrobial susceptibility tests

Antimicrobial sensitivity testing of bacterial isolates was carried out using the Kirby-Bauer disk diffusion method (Hudzicki, 2009) on Mueller-Hinton agar (HiMedia, India) prepared following the manufacturer's instructions. Each bacterial isolate was inoculated onto the surface using the spread plate method, then antibiotic discs (Table 2) were placed onto the inoculated agar plates. After 24 hours

incubation at 28±2 °C, the diameters of the zones of inhibition around each antibiotic disk were measured in millimetres. According to CLSI standards and EUCAST breakpoints, isolates were classified as Intermediate (I), Resistant (R), or Sensitive (S) (CLSI, 2016; CLSI, 2020; CLSI, 2022; EUCAST, 2025) [11, 12, 13, 17]. A high-risk source of antibiotic exposure is indicated by a MAR index score greater than 0.2.

Table 2: Antibiotics used in the study for antibiotic sensitivity analysis of the bacterial isolates

Sl. No.	Class	Antibiotics	Short name	Concentration
1	β -lactams	Ampicillin	AMP	10 (mcg)
2		Oxacillin	OX	1 (mcg)
3		Methicillin	MET	5 (mcg)
4		Piperacillin	PIT	100/10 (mcg)
5		Ticarcillin	TCC	75/10 (mcg)
6	Cephalosporins	Cefotaxime	CTX	30 (mcg)
7		Ceftazidime	CAZ	30 (mcg)
8		Cefuroxime	CXM	30 (mcg)
9		Cefazolin	CZ	30 (mcg)
10		Cefepime	CPM	30 (mcg)
11		Cefoxitin	CX	30 (mcg)
12	Monobactams	Aztreonam	AT	30 (mcg)
13	Carbapenem	Doripenem	DOR	10 (mcg)
14		Meropenem	MRP	10 (mcg)
15	Rifamycins	Rifampicin	RIF	5 (mcg)
16	Aminoglycosides	Tobramycin	TOB	10 (mcg)
17		Gentamicin	GEN	10 (mcg)
18	Tetracyclines	Tetracycline	TE	30 (mcg)
19		Tigecycline	TGC	15 (mcg)
20	Quinolone	Norfloxacin	NX	10 (mcg)
21		Levofloxacin	LE	5 (mcg)
22		Ciprofloxacin	CIP	5 (mcg)
23		Ofloxacin	OF	5 (mcg)
24		Nalidixic acid	NA	30 (mcg)
25	Sulphonamides	Trimethoprim	TR	5 (mcg)
26	Macrolides	Erythromycin	E	15 (mcg)
27	Nitrofurans	Nitrofurantoin	NIT	300 (mcg)
28	Amphenicols	Chloramphenicol	C	30 (mcg)

Phenotypic typing of isolates

Isolates were phenotypically typed based on their antimicrobial susceptibility profiles. A numerical code was assigned to each isolate, with 3 points for resistance, 2 points for intermediate susceptibility, and 1 point for susceptibility (Odeyemi and Ahmad, 2017) ^[34].

Results

A total of 21 *Aeromonas* species were identified based on biochemical and molecular characterization (Table 3). The *Aeromonas* isolates were tested against 28 antibiotics. AST analysis is provided in Fig.1. Among isolates, the resistance to different antibiotics varied significantly. All the isolates showed resistance to oxacillin (100%) and methicillin (100%). High resistance rates were observed for ampicillin (90%), cefazolin (86%), ticarcillin (57%) and nalidixic acid (57%) while meropenem (38%), tigecycline (29%), norfloxacin (29%), trimethoprim (24%), piperacillin (19%), ceftazidime (14%), rifampicin (14%), cefoxitin (10%), aztreonam (10%), tetracycline (10%), ofloxacin (10%), nitrofurantoin (10%), cefuroxime (5%) and chloramphenicol (5%) showed low antibiotic resistance rates (Table 4). Isolates were found susceptible to cefotaxime, cefepime, doripenem, tobramycin, gentamicin, levofloxacin, ciprofloxacin and erythromycin. The multiple antibiotic resistance (MAR) index for *Aeromonas* isolates ranged from 0.11 to 0.50 (Table 5). There were 21 distinct phenotypes of the *Aeromonas* isolates identified by antibiotic phenotyping utilising number codes.

Table 3. NCBI Accession numbers of 16S rRNA gene partial sequences of *Aeromonas* isolates.

Isolate code	Identified organism	NCBI Accession no
A11	<i>A. schubertii</i>	PQ305827
A12	<i>A. caviae</i>	PP990188
A315	<i>Aeromonas</i> sp.	PQ002429
A317	<i>A. hydrophila</i>	PQ002432
A41	<i>A. veronii</i>	PP990191
A43	<i>A. hydrophila</i>	PQ295860
A44	<i>A. veronii</i>	PQ002433
A47	<i>A. dhakensis</i>	PQ002427
A48	<i>A. hydrophila</i>	PQ002434
A72	<i>A. veronii</i>	PQ002428
A81	<i>A. veronii</i>	PP990193
A84	<i>A. veronii</i>	PP825363
G1	<i>A. veronii</i>	PQ243116
G2	<i>A. caviae</i>	PQ242625
G3	<i>A. veronii</i>	PQ242624
G5	<i>A. veronii</i>	PQ242623
G9	<i>Aeromonas</i> sp.	PQ243118
G15	<i>Aeromonas</i> sp.	PQ242622
K1	<i>A. veronii</i>	PQ242621
K2	<i>A. veronii</i>	PQ226006
MN4	<i>A. veronii</i>	PQ363294

Table 4: Antibigram profile of *Aeromonas* isolates

Antibiotics	No. of resistant <i>Aeromonas</i> isolates	Percentage of isolates resistant (%)
Ampicillin	19	90
Oxacillin	21	100
Methicillin	21	100
Piperacillin	4	19
Ticarcillin	12	57
Cefotaxime	0	0
Ceftazidime	3	14
Cefuroxime	1	5
Cefazolin	18	86
Cefepime	0	0
Cefoxitin	2	10
Aztreonam	2	10
Doripenem	0	0
Meropenem	8	38
Rifampicin	3	14
Tobramycin	0	0
Gentamicin	0	0
Tetracycline	2	10
Tigecycline	6	29
Norfloxacin	6	29
Levofloxacin	0	0
Ciprofloxacin	0	0
Ofloxacin	2	10
Nalidixic acid	12	57
Trimethoprim	5	24
Erythromycin	0	0
Nitrofurantoin	2	10
Chloramphenicol	1	5

Table 5: Antibiotics susceptibility test (AST) typing of *Aeromonas* species

State	<i>Aeromonas</i> Isolates	AST profile	Phenotypic typing profile	MAR index
Andhra Pradesh	A11	3331111131211211121112132111	A	0.18
	A12	3332111131211111111112311111	B	0.18
	A315	3333311131111211121111111111	C	0.21
	A317	3332311131131111111131111111	D	0.25
	A41	3333113131111311111121111111	E	0.25
	A43	3333211131111311113311111133	F	0.36
	A44	3332311131111311111111132111	G	0.25
	A47	3332211131311111111311131111	H	0.25
	A48	3331311131111311111111111111	I	0.21
	A72	3333311131111211111111111111	J	0.21
	A81	1331111121111111111111131111	K	0.11
	A84	1331111121111111111311131131	L	0.18
Gujarat	G1	333131313111131111111133111	M	0.32
	G2	333231333133133113111133111	N	0.50
	G3	333131113111121111111133111	O	0.25
	G5	333231113111122111111112111	P	0.18
	G9	3332311131111131111111131111	Q	0.25
	G15	3332311131111331113111113111	R	0.32
Karnataka	K1	333131113111131113331333111	S	0.43
	K2	3331211131111211133311231111	T	0.29
Maharashtra	MN4	3331111121111211113111132111	U	0.18

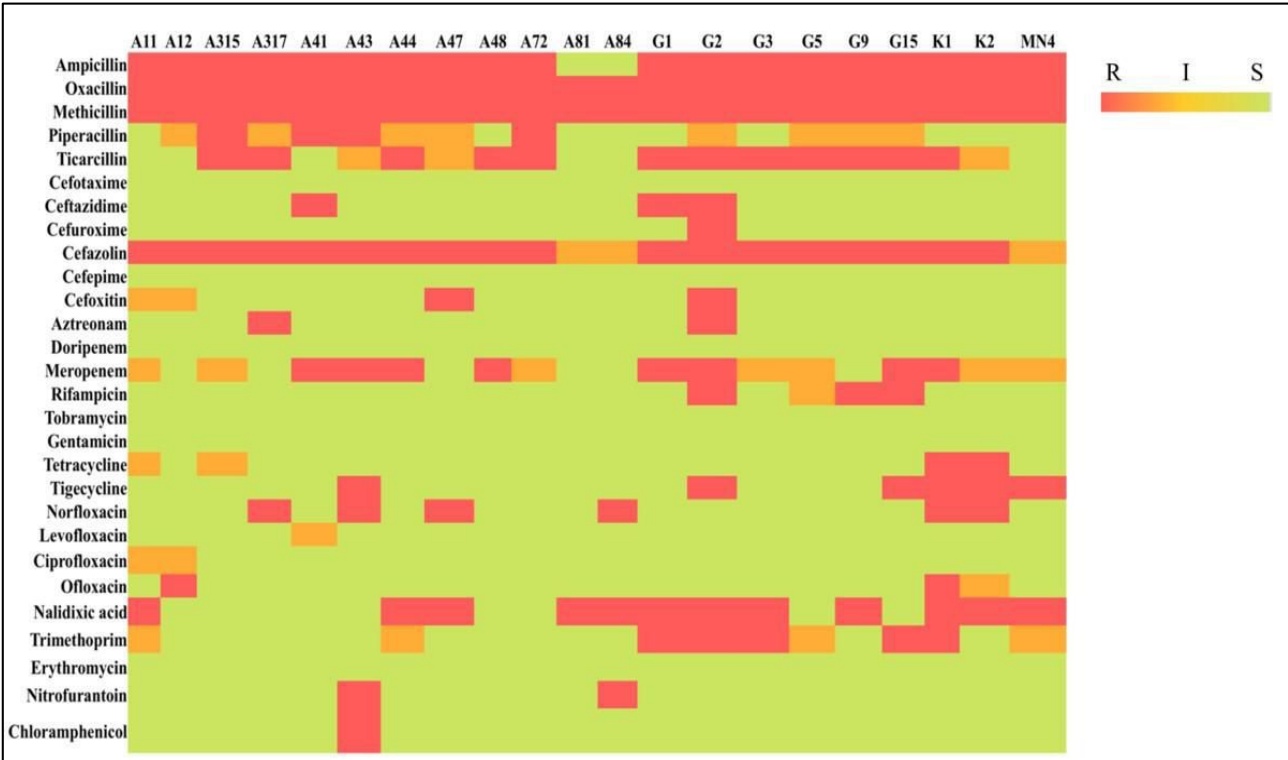


Fig 1: Heatmap representation of antibiogram results of *Aeromonas* species

Discussion

Aeromonads are widely distributed bacterial species in aquatic environments, posing a significant threat to both environmental and human health due to their high adaptability and growing antibiotic resistance (Pinto *et al.*, 2025) [35]. The present study investigated the antibiotic resistance patterns of *Aeromonas* species isolated from freshwater-farmed Asian seabass. *Aeromonads* have been reported to cause a disease known as aeromoniasis in Asian seabass, characterized by hemorrhagic lesions, fin erosion and significant mortality (Kathirkaman *et al.*, 2018) [22]. Antibiotic sensitivity profiling of all *Aeromonas* isolates revealed complete resistance to oxacillin and methicillin (100%), along with varying degrees of resistance to

ampicillin (90%), cefazolin (86%), ticarcillin (57%), meropenem (38%), piperacillin (19%), ceftazidime (14%), cefoxitin (10%), aztreonam (10%) and cefuroxime (5%). Similar resistance patterns of *Aeromonas* spp. isolated from freshwater cultured fish species i.e., walking catfish, tilapia, striped catfish, African catfish, rohu, catla, etc., against β -lactam antibiotics were recorded by several authors (Azzam-Sayuti *et al.*, 2021; Mursalim *et al.*, 2022; Lavanya *et al.*, 2023; Kumar and Rathore, 2024; Adah *et al.*, 2024; Nokhwal *et al.*, 2025) [5, 30, 26, 24, 1, 33]. High resistance rates to β -lactam antibiotics (First generation) are attributed to presence of genes that encode β -lactamases and metallo- β -lactamases (MBLs) (Bello-López *et al.*, 2019; Nasser *et al.*, 2022) [6, 31]. However, the second and third generation

antibiotics such as cefoxitin, cefuroxime and ceftazidime showed lower resistance in *Aeromonas* spp. in the study. Though the usage of these antibiotics are rare in the commercial aquaculture systems, >5% resistance may be due to the transfer of AMR genes/integrations via the existing barter system among the microbial communities present in the aquatic environment. Furthermore, all *Aeromonas* isolates were sensitive to the third-and fourth-generation cephalosporins, such as cefotaxime and cefepime. These cephalosporins are reported to resist hydrolysis of the pathogen by plasmid or chromosomally mediated beta-lactamases and they are weak inducers of group 1 beta-lactamases (Hardefeldt and Prescott, 2024) [19].

Nalidixic acid, one of the first-generation quinolone antibiotics discovered in the year 1962 and introduced to the clinical practice from 1967 in human medicine. Due to the emergence of AMR and food safety issues, this antibiotic is banned in commercial aquaculture globally by several national and international agencies. In the present study, high resistance rate to nalidixic acid (57%) was observed and our results are in consistent with findings from multiple AMR research investigations (Dhanapala *et al.*, 2021; Deng *et al.*, 2023; Nokhwal *et al.*, 2025) [15, 14, 33]. This suggests that resistant bacterial populations are still persisting in the aquaculture environment against this quinolone. It is also reported that resistance genes can be maintained even without active antibiotic use, especially through horizontal gene transfer. Norfloxacin (29%) and ofloxacin (10%) i.e., second generation quinolones also showed moderate resistance against *Aeromonas* isolates. In general, *Aeromonas* species are found to be resistant to these quinolones due to the presence of plasmid-mediated quinolone resistance genes and chromosomal mutations in drug target genes (Kosikowska *et al.*, 2022) [23].

Furthermore, resistance to tetracycline class antibiotics, including tigecycline (29%) and tetracycline (10%), was also evident from the current study. Resistance against this antibiotic class mainly occurs due to the acquisition of tetracycline resistance genes which encode for efflux pump or ribosomal protection (Hossain *et al.*, 2019) [20]. In this study, some isolates displayed resistance to nitrofurantoin (10%) and chloramphenicol (10%), which are banned in veterinary and aquaculture. Although low, the presence of resistant strains to banned antimicrobials suggests possible environmental persistence of resistance genes, cross-resistance mechanisms or historical selective pressure of these antibiotics (Nguyen *et al.*, 2014) [32]. In the study, all *Aeromonas* isolates were found to be susceptible to doripenem, tobramycin, gentamicin, levofloxacin, ciprofloxacin, cefotaxime, cefepime and erythromycin. Several AMR studies have reported similar susceptibility patterns in *Aeromonas* isolated from freshwater aquaculture systems (Azzam-Sayuti *et al.*, 2021; Mazumder *et al.*, 2021; Algamal *et al.*, 2022; Kumar and Rathore, 2024; Adah *et al.*, 2024) [5, 29, 3, 24, 1]. These findings suggest that these antibiotics can still be effective for treating *Aeromonas* infections in commercial aquaculture practices.

Multiple Antibiotic Resistance index analysis provides a quantitative value that estimates the extent to which a bacterial isolate is resistant to multiple antibiotics. In general, MAR values >0.2 typically suggests that the bacteria originated from high-risk sources where antibiotics are often used heavily (e.g., intensive aquaculture, hospitals, wastewater) while low MAR index (<0.2) indicates bacteria

from environment with less antibiotic exposure. In the current study, the MAR index of *Aeromonas* ranged from 0.11 to 0.50, which is comparatively lower than values reported in other freshwater culture systems. For instance, study in freshwater farms of Haryana recorded MAR indices between 0.07 and 0.73, with 29.9% of *Aeromonas* isolates showing MAR index values above 0.2 (Nokhwal *et al.*, 2025) [33]. Similarly, studies from Andhra Pradesh reported MAR index values consistently above 0.2, ranging from 0.33 to 0.78 (Lavanya *et al.*, 2021, 2023) [25, 26], while isolates from Uttar Pradesh showed values between 0.39 and 0.56 (Kumar and Rathore, 2024) [24].

A high proportion of *Aeromonas* isolates (71%) exhibited MAR index values >0.2, indicating exposure to high-risk environments with frequent antibiotic application (Dhanapala *et al.*, 2021) [15], and highlighting the potential emergence of antimicrobial resistance in freshwater-farmed Asian seabass culture systems.

Conclusion

This study provides critical insights into the antibiotic resistance profiles of *Aeromonas* species isolated from freshwater-farmed Asian seabass. Antibiotic resistance can arise from both intrinsic and acquired resistance mechanisms resulting from antibiotic application or contamination of water source with AMR pathogens at the farm-level. The widespread resistance poses potential health risks not only to aquatic animals but also to humans through possible transmission and environmental dissemination of resistance genes. By cataloguing the resistance profiles of these pathogens, it provides a baseline data for predicting and understanding the patterns of antimicrobial resistance in freshwater aquaculture systems. This study also highlights the need for continued surveillance, targeted monitoring and gene-level investigations for better management of antibiotic resistance in aquatic environment.

Acknowledgements

The present study was financially supported by Department of Biotechnology, Government of India (Project No. BT/PR47267/AAQ/3/1060/2022). The authors acknowledge the Director/Vice chancellor of ICAR-Central Institute of Fisheries Education, Mumbai for providing facilities for this research.

Conflicts of Interest

The authors declare no conflict of interest.

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