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ARTICLE

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PREVALENCE OF BETA-LACTAMASE RESISTANCE PROFILING OF *AEROMONAS* SPP. IN THE LOWER GANGA BASIN

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This study investigates beta-lactamase resistance in Aeromonas spp. *isolated from surface water samples collected along the lower Ganga Basin. Among 33 isolates, 24 exhibited multi-drug resistance. High resistance was observed against β-lactamase inhibitors, carbapenems, and cephalosporins. Extended-spectrum β-lactamase (ESBL) production was detected in 56% of isolates, with* bla_{TEM}, bla_{SHV}, *and* bla_{CTX-M} *genes identified. Plasmid-mediated AmpC was found in 15% of isolates. These findings underscore the environmental dissemination of beta-lactamase resistance throughout this biodiversity-rich riverine system.*

Introduction

Antimicrobials have significantly contributed to improving human and animal health by effectively treating bacterial infections. However, their overuse and misuse have led to the emergence of antimicrobial resistance (AMR), a growing global issue[1]. As the global population continues to increase, the use of antimicrobials has also risen. Between 2000 and 2015, global antibiotic use increased by 65%, reaching approximately 34.8 billion defined daily doses (DDD)[2]. A critical issue is that up to 90% of an antibiotic dose may pass through the human or animal body unchanged and enter the environment through urine and feces[3]. Antibiotics and antibiotic-resistant bacteria can easily spread into natural ecosystems, especially in areas where around 1.7 billion people lack basic sanitation and untreated wastewater like used water from households, industries, or agriculture is used to irrigate crops or fish farming, affecting more than 10% of the global population[3]. This release of antibiotics and resistant microorganisms into rivers, lakes, and soil can foster the development of resistance in environmental bacteria, posing serious health risks[4].

Aeromonas spp. are a group of bacteria commonly found in aquatic environments such as rivers, lakes, and wastewater. These Gram-negative, oxidase-positive bacteria can thrive in both aerobic and anaerobic conditions. They are known to infect cold- and warm-blooded animals, including humans[3]. Due to their ability to survive in both natural environments and living hosts, *Aeromonas* spp. are considered significant from a public health perspective. Certain species of *Aeromonas* can cause diseases in fish, shellfish, and humans and are easily isolated from water samples[5]. Given their widespread presence in aquatic environments and their potential to carry antibiotic resistance genes, *Aeromonas* spp. serve as potential indicators of antimicrobial resistance in the environment. Their frequent presence in food and water sources, particularly in regions with prevalent aquaculture practices, positions them as key contributors to the spread of resistant bacteria to humans[6]. As such, some researchers have proposed using *Aeromonas* spp. as a Global One Health indicator organism[7].

In this study, we focus on water samples collected from the Lower Ganga Basin, a region experiencing increasing human impact due to pollution, wastewater

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discharge, and antibiotic misuse. The objective is to examine the presence of *Aeromonas* spp. and their resistance to beta-lactam antibiotics, with a specific focus on identifying beta-lactamase enzyme production. This antibiotic group includes a broad range of antibiotics and is commonly used. This research underscores the significance of adopting a One Health approach, which recognizes the interconnectedness of human, animal, and environmental health in monitoring and managing AMR effectively.

Materials and Methods

Sample Collection and Transportation: Water samples were collected from multiple points along the lower stretch of the River Ganga, including sites in Bhagalpur, Patna, Buxar, Nabadwip, Triveni, Balagarh, Berhampore, Jangipur, Farakka, Godakhali, Frezarganj, and Diamond Harbour. At each location, one-liter surface water samples were collected at a depth of approximately 1 meter using sterile containers. Samples were immediately placed in sterile ice boxes and transported to the laboratory for microbiological examination, including analysis of antibiotic resistance. All samples were processed on the day of collection under aseptic conditions in accordance with ISO 6887-3:2003 guidelines.

Bacterial Enrichment and Isolation: Bacterial enrichment was done according to Mohanty et al.[8] 1 mL of each water sample was inoculated into alkaline peptone water (APW; Hi-Media, Mumbai, India) and incubated at 37°C for 16–20 hours. After enrichment, to enrich bacterial populations, 1 mL of each collected water sample was transferred into alkaline peptone water (APW; Hi-Media, Mumbai, India) and incubated at 37°C for 16 to 20 hours. Post-incubation, a portion of the enriched broth was streaked onto Ampicillin Dextrin Agar with vancomycin (ADA-V; Hi-Media, Mumbai, India) and incubated again under similar temperature conditions. Colonies displaying distinct yellow pigmentation, indicative of *Aeromonas* spp., were picked for subsequent identification and characterization.

Biochemical Characterization: Presumptive *Aeromonas* colonies were subjected to a series of biochemical assays for confirmation. These included resistance to the vibriostatic agent O/129, esculin hydrolysis, catalase and oxidase tests, indole production, Methyl Red (MR) and Voges-Proskauer (VP) reactions, citrate utilization, Triple Sugar Iron (TSI) test, decarboxylase activity (lysine and ornithine), and glucose fermentation. All procedures were performed following standard methods, as outlined by Dahanayake et al (2019)[9].

Total Genomic DNA Isolation: Genomic DNA was extracted from confirmed isolates using a quick thermal lysis (boiling) technique[8]. Briefly, overnight cultures grown in nutrient broth at 37°C for 16–18 hours were centrifuged at 10,000×g for 2 minutes. The resulting pellet was resuspended in 100 µL of sterile molecular-grade water (Hi-Media, Mumbai, India) and heated at 95°C for 10 minutes to break open the cells. The lysate was rapidly cooled on ice for 5 minutes and then centrifuged at 12,000 × g for 5 minutes. The supernatant, containing crude DNA, was collected and either stored at –20°C or immediately used as a template for PCR amplification.

***Molecular Confirmation of Aeromonas* spp.:** Molecular identification of *Aeromonas* isolates was carried out using a genus-specific PCR assay targeting the 16S rRNA gene. The PCR reaction mixture (25 µL total volume) consisted of 2.5 µL of 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of both the forward and reverse primers, 1 U of Taq DNA polymerase (Hi-Media, India), and 2 µL of extracted DNA. The thermal cycling conditions involved an initial denaturation at 94°C for 5 minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 55–60°C for 30 seconds (depending on the Tm of the primers), and extension at 72°C for 1 minute, with a final extension at 72°C for 5–10 minutes. The PCR products were then separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light to verify the presence of the expected amplicon size.

Antibiotic Sensitivity Test and Detection of Beta-lactamase Resistance Pattern: *Aeromonas* spp. isolates, identified and stored on Tryptic Soy Agar (TSA) plates (Hi-Media, Mumbai, India), were transferred to test tubes containing saline solution. The turbidity of each tube was adjusted to match the McFarland 0.5 standard, and the bacterial suspensions were then evenly spread on Mueller-Hinton agar (MHA) plates (Hi-Media, Mumbai, India) using a previously sterilized cotton swab. The antimicrobial susceptibility test was performed using the disk diffusion method to measure the inhibition zone diameters. The resistance profiles were determined following the Clinical and Laboratory Standards Institute (CLSI, 2024)[10] M39-A4 guidelines. Each isolate was classified as resistant (R), intermediate (I), or susceptible (S) based on the inhibition zone sizes. A total of twenty different antimicrobials, representing eleven classes, were tested. The specific antimicrobials used are detailed in Table 1.

Detection of Beta-lactamase-Encoding Genes: Beta-lactamase genes in phenotypically resistant *Aeromonas*

Table 1: Antimicrobials Used in the Study

Antimicrobial Class	Antimicrobial	Concentration (µg)
Aminoglycosides	Gentamicin	10
	Amikacin	30
Carbapenems	Meropenem	10
	Imipenem	10
β-lactamase	Cefotaxime	30
Inhibitors	Cephalothin	30
	Ceftriaxone	30
	Cefoxitin	30
	Ceftazidime	30
	Cefepime	30
Sulphonamides	Trimethoprim-sulfamethoxazole	25
Macrolides	Erythromycin	15
Phenicols	Chloramphenicol	30
Quinolones	Nalidixic Acid	30
	Ciprofloxacin	5
Tetracyclines	Oxytetracycline	30
	Tetracycline	30
	Doxycycline	30
β-lactamase	Amoxicillin/Clavulanate	20/10
Inhibitors	Ampicillin/Sulbactam	10/10

isolates were detected through PCR using gene-specific primers targeting bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{AmpC}. Each PCR reaction was performed in a 25 µL mixture, which included 2 µL of extracted DNA, GoTaq® DNA polymerase (Promega, USA), and primers at a final concentration of 10 nM. The amplification process was carried out in an Agilent SureCycler 8800 thermal cycler, with conditions optimized for each primer pair. For the bla_{TEM} gene, amplification was performed using the forward primer (52 -CATTTCCGTGTCGCCCTTATTC-32) and reverse primer (52 -CGTTCATCCATAGTTGCCTGAC-32), resulting in an 800 bp amplicon at an annealing temperature of 64°C. The bla_{SHV} gene was amplified using the forward primer (5' -AGCCGCTTGAGCAAATTAAAC-3') and reverse primer (5' -ATCCCGCAGATAAATCACCAC-3'), producing a 713 bp product at 52°C. The bla_{CTX-M} gene was amplified with the forward primer (5' -CGCTTTGCGATGTGCAG-3') and reverse primer (5' -ACCGCGATATCGTTGGT-3'), generating a 550 bp amplicon at an annealing temperature of 50°C. For the detection of bla_{AmpC}, the forward primer (5' -CCCCGCTTATAGAGCAACAA-3') and reverse primer (5' -TCAATGGTCGACTTCACACC-3') were used to amplify a 634 bp product at 54°C. The resulting PCR products were resolved on a 1.2% agarose gel prepared in 1X TAE buffer (Hi-Media, Mumbai, India) containing 0.5 µg/mL of ethidium bromide. Electrophoresis was carried out at 80 V, using a 100 bp DNA ladder as the size marker. Gel visualization was performed using a Gel Documentation System with a UV transilluminator (GelDoc imager, Bio-Rad, USA).

Results and Discussion

Identification and Confirmation of Aeromonas Spp.: Among the 36 water samples collected from various locations along the lower stretch of the Ganga River, 33 yielded presumptive *Aeromonas* colonies, which exhibited characteristic dark yellow pigmentation on ADA-V, in accordance with the protocol established by the US Environmental Protection Agency (2001) (Fig. 1). These isolates were subjected to a comprehensive biochemical characterization to confirm their identity. All isolates demonstrated resistance to the vibriostatic agent O/129 at both 10 µg and 150 µg concentrations and tested positive for catalase and oxidase activity. Esculin hydrolysis was observed in 100% of the isolates, while indole production was noted in 85% of them. Positive reactions were recorded for the Methyl Red and Voges-Proskauer tests in 79% and 42% of the isolates, respectively. Citrate utilization was demonstrated by 91% of the isolates. The Triple Sugar Iron (TSI) test revealed an alkaline slant with an acid butt (K/A) in all cases, indicating glucose fermentation in the absence of hydrogen sulfide production. Lysine and ornithine decarboxylase activity was detected in 76% and 58% of the isolates, respectively. Furthermore, all isolates were capable of glucose fermentation, supporting their classification within the genus *Aeromonas*.

To further validate the identification, molecular confirmation was carried out through the amplification of the genus-specific 16S rRNA gene. Genomic DNA extracted using a quick-boiling method was used as a template, and PCR amplification yielded a single, specific amplicon of approximately 953 bp in all tested samples (Fig. 2), confirming their identity as *Aeromonas* spp.

These findings are consistent with prior research indicating the prevalence of *Aeromonas* spp. in aquatic ecosystems, particularly in tropical and subtropical regions[11,12]. Their presence in river systems such as the Ganga poses a public health concern, especially in the context of rising antimicrobial resistance. The confirmed

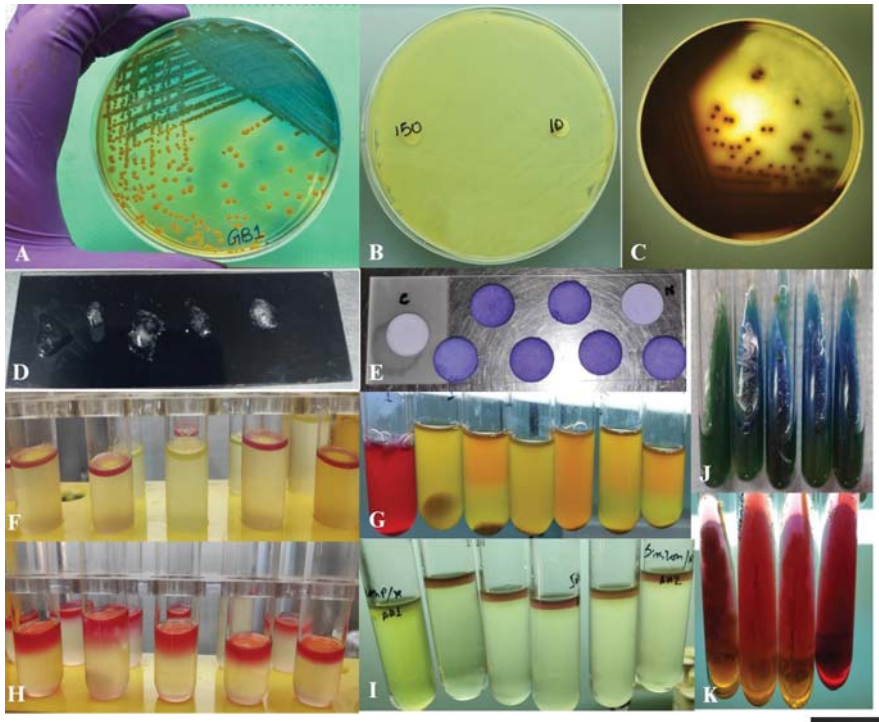

Fig. 1: The cultural and biochemical characterization of *Aeromonas* spp. A. presumptive *Aeromonas* colonies exhibiting distinctive yellow pigmentation on ADA-V. B. The vibriostatic agent (O/129) resistance test, confirmed the isolates' resistance. C. Positive esculin hydrolysis is indicated by the blackening of the medium. D. Catalase activity is confirmed by bubble formation upon the addition of hydrogen peroxide. E. Positive oxidase test with purple color development. F. Indole production with a red ring formation after the addition of Kovac's reagent. G. Represents the decarboxylase test for lysine and ornithine, confirming enzymatic activity. H and I. Methyl Red (MR) and Voges-Proskauer (VP) test, indicating mixed acid and acetoin production, respectively. J. Citrate utilization, is evident by growth and color change. K. The TSI slant test, exhibited an alkaline slant and acid butt (K/A), indicating glucose fermentation without hydrogen sulfide production.

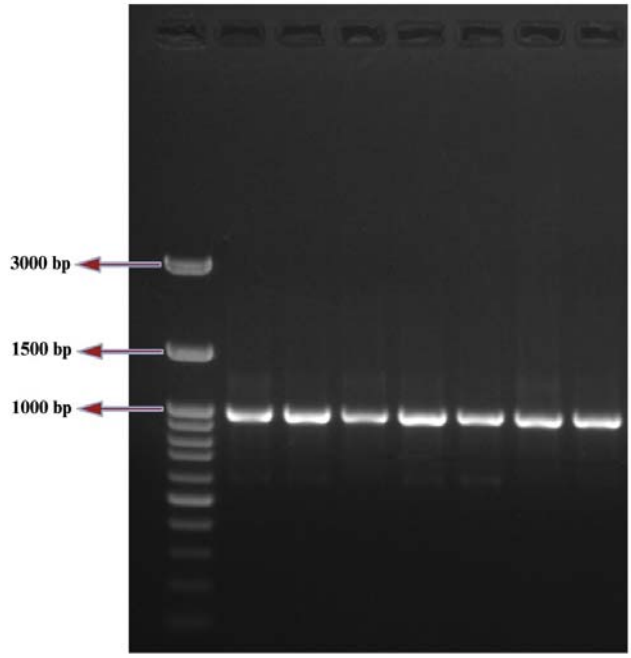

Fig. 2: Agarose gel electrophoresis showing genus-specific amplification of *Aeromonas* spp. using 16S rRNA-targeted primers. A distinct amplicon of approximately 953 base pairs (bp) was observed in lanes 2 to 7, confirming the presence of *Aeromonas* spp. Lane 1: 100 bp DNA ladder (molecular size marker); Lanes 2–7: representative *Aeromonas* isolates showing genus-specific PCR product at 953 bp.

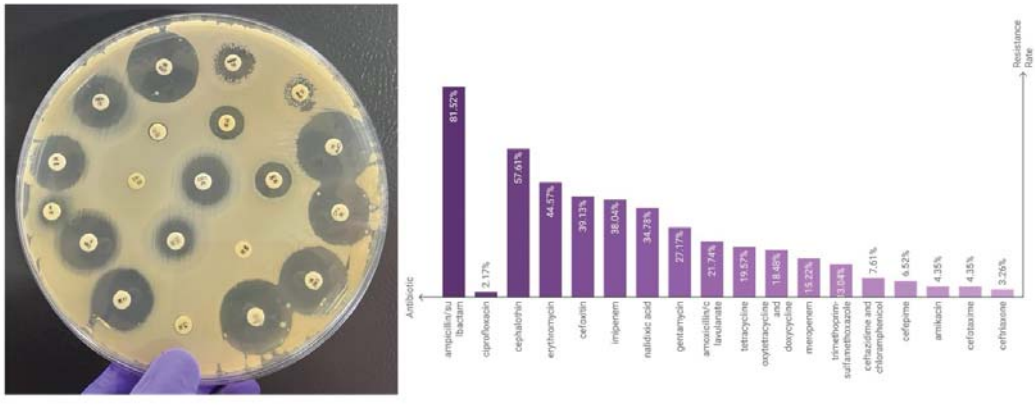

Fig. 3: The in vitro antibiotic susceptibility profile of *Aeromonas* spp. isolates recovered from surface water samples in the lower Ganga basin. The figure illustrates the percentage of isolates exhibiting resistance to various classes of antibiotics.

biochemical and molecular identification of *Aeromonas* spp. from these water bodies highlights their adaptability and persistence in diverse environments. Given their capacity to harbor and disseminate antimicrobial resistance genes, these results emphasize the need for continuous surveillance and molecular-level monitoring in freshwater ecosystems[13].

Susceptibility of the Aeromonas* spp. *to Different Antibiotics: Out of the 33 *Aeromonas* spp. isolates recovered from the lower Ganga River basin, 24 (approximately 73%) exhibited multidrug resistance (MDR), defined as resistance to at least three different classes of antibiotics. Antimicrobial susceptibility testing revealed the highest resistance to β-lactam/β-lactamase inhibitor combinations, with 81% of isolates resistant to ampicillin/sulbactam (A/S). Resistance to cefoxitin (CX) was observed in 39.13% of isolates, followed by imipenem (IMP), a last-resort carbapenem antibiotic, with 34% resistance. Among third- and fourth-generation cephalosporins, resistance was lower, with 7.61% of isolates resistant to ceftazidime (CAZ), 6.52% to cefepime (CPM), and 3.26% to ceftriaxone (CTR). Resistance to non-β-lactam antibiotics was comparatively low, with 19% of isolates resistant to tetracycline and only 7.61% to chloramphenicol. These findings align with previous reports documenting extensive MDR in environmental *Aeromonas* spp., especially in areas burdened by human activities and lacking proper wastewater treatment infrastructure[12]. Tiwari et al. (2023) and Moradi et al. (2025)[13,14] emphasized the growing global trend of MDR *Aeromonas* strains, often linked to aquatic systems polluted by effluents from hospitals, agriculture, and households[13]. Similarly, Salgueiro et al. (2021)[15] identified comparable resistance patterns in *Enterobacteriaceae* and *Aeromonas* spp. recovered from aquatic food sources[15]. These resistance trends illustrate the selective pressure induced by antibiotic misuse in clinical and agricultural settings and affirm the role of aquatic ecosystems as both reservoirs and transmission pathways for antimicrobial resistance genes. The dense human population and anthropogenic burden along the lower Ganga stretch create a conducive environment for the proliferation and persistence of MDR bacteria.

Beta-lactamase Genes Harboured by Confirmed Aeromonas* spp.*: Extended-spectrum β-lactamases represent a diverse group of enzymes that can hydrolyze a broad spectrum of β-lactam antibiotics, including penicillins, third-generation cephalosporins, and aztreonam, thereby compromising their therapeutic efficacy[16]. These enzymes are typically encoded by genes such as bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, which are frequently plasmid-mediated, enabling horizontal gene transfer among bacterial populations[16]. The emergence and dissemination of ESBL-producing bacteria in the environment, especially in aquatic ecosystems that receive untreated or partially treated effluents, pose serious public health threats[12,14,17]. In this study, molecular characterization of ESBL-producing isolates revealed the presence of multiple β-lactamase encoding genes (Figure 4). The bla_{TEM} gene was the most prevalent, detected in 94% (n = 17) of ESBL-positive isolates, indicating its dominant presence in environmental *Aeromonas* strains. The bla_{SHV} gene was found in 39% (n = 7), while the clinically significant bla_{CTX-M} group was present in 27% (n = 5) of the isolates. The detection of ESBL-producing *Aeromonas* spp. in the lower Ganga River is concerning, as resistance mechanisms of this nature are increasingly documented across global environmental matrices[18]. Previous studies have reported the prevalence of ESBL-producing bacteria in environmental and clinical settings ranging from 33% to 91%[19]. The predominance of bla_{TEM}, followed by bla_{SHV} and bla_{CTX-M}, is consistent with earlier findings from environmental and foodborne *Aeromonas* isolates, where bla_{TEM} has been frequently linked to multidrug resistance in aquatic ecosystems[19].

AmpC β-lactamases, another important class of enzymes, confer resistance by hydrolyzing a broad spectrum of β-lactam antibiotics including cephamycins and are generally not inhibited by β-lactamase inhibitors[20]. These enzymes may be chromosomally encoded or plasmid-mediated and contribute significantly to β-lactam resistance dissemination in both clinical and environmental settings[20]. In the present study, plasmid-mediated AmpC β-lactamase production was detected in 15% (n = 5) of the *Aeromonas* isolates (Figure 4). This finding supports earlier research reporting the detection of AmpC genes in environmental *Aeromonas* strains. For instance, Voolaid et al. reported FOX-type AmpC genes in *Aeromonas* spp. isolated from freshwater systems in Estonia[21], while Piotrowska and Popowska observed a range of AmpC variants, including novel FOX genes, in *Aeromonas* spp. from urban wastewater treatment facilities[22], highlighting the role of such environments as reservoirs for antibiotic resistance genes.

Co-occurrence of beta-lactamase resistance genes: The co-occurrence pattern of β-lactamase resistance genes among the *Aeromonas* spp. isolates is illustrated in the Venn diagram (Figure 5). A total of 33 *Aeromonas* spp. isolates from the lower Ganga River basin were screened for the presence of â-lactamase resistance genes, namely bla_{TEM}, bla_{CTX-M}, bla_{SHV}, and $_{AmpC}$. Among these, the most

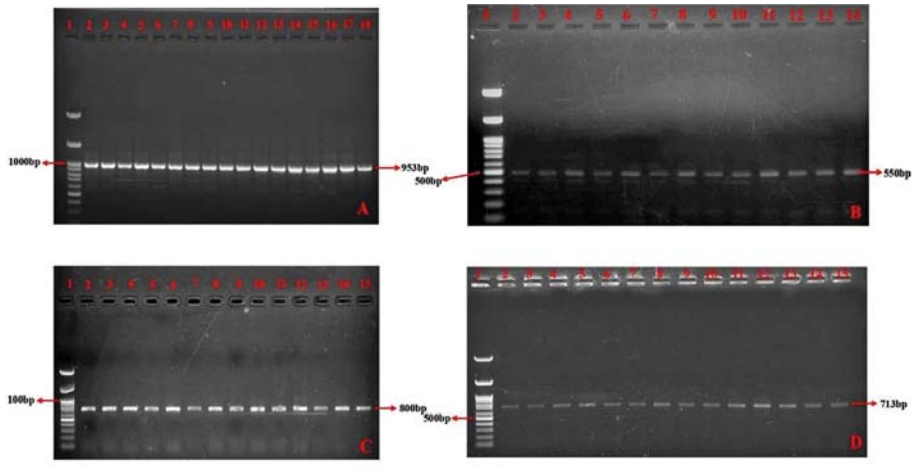

Fig. 4: the agarose gel electrophoresis of PCR-amplified products for molecular confirmation and resistance gene detection in *Aeromonas* spp. isolates. A. lanes 2–18 represent confirmed *Aeromonas* isolates with amplification of the 16S rRNA gene at 953 bp. B. amplification of the bla_{CTX-M} gene at 550 bp, C. bla_{TEM} gene amplified at 800 bp. And D. represents the amplification of the bla_{SHV} gene at 713 bp. First lane in each gel corresponds to the 100 bp DNA ladder used as a molecular size marker.

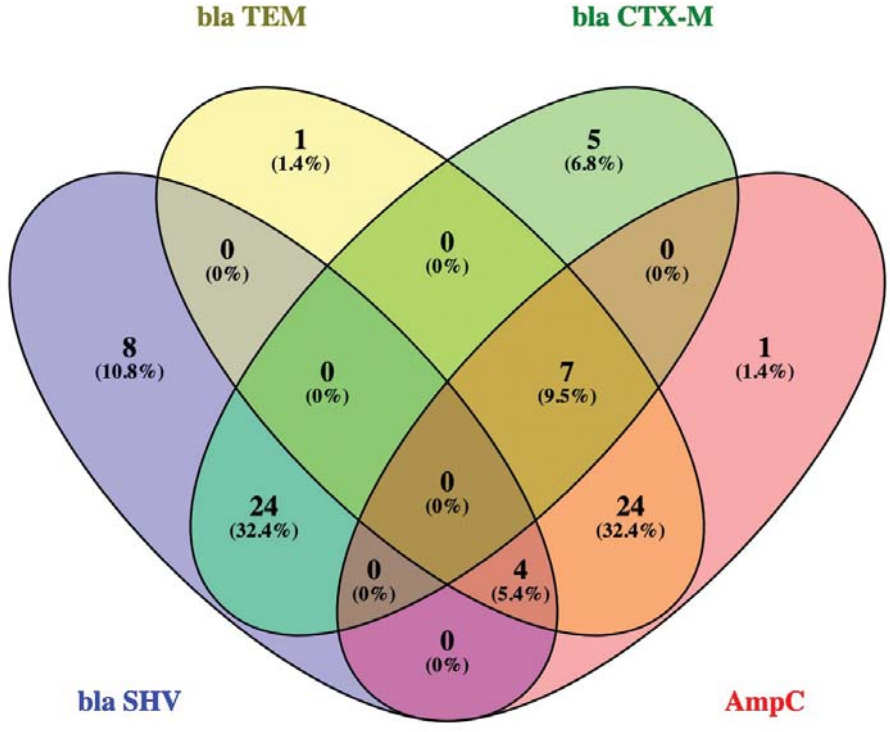

Fig. 5: Venn diagram illustrating the co-occurrence of β-lactamase resistance genes bla TEM, bla SHV, bla CTX-M, and AmpC among *Aeromonas* spp. isolated from the lower Ganga River basin. The overlapping areas represent isolates harboring combinations of two or more resistance genes, while the non-overlapping sections indicate isolates carrying only a single gene.

prevalent gene was bla_{SHV}, detected alone in 8 isolates (10.8%) and in various combinations with other genes. The co-occurrence of bla_{SHV} with bla_{TEM} was observed in 24 isolates (32.4%), and an equal number (24; 32.4%) also harbored both bla_{SHV} and AmpC. Additionally, 7 isolates (9.5%) carried a triple combination of bla_{CTX-M}, bla_{SHV}, and bla_{TEM}, while 4 isolates (5.4%) carried bla_{SHV}, bla_{TEM}, and $_{AmpC}$. Only 1 isolate (1.4%) carried blaTEM alone, 5 isolates (6.8%) carried bla_{CTX-M} alone, and 1 isolate (1.4%) harbored only $_{AmpC}$. Notably, no isolates were found to carry dual combinations of bla_{TEM} and $_{AmpC}$ or bla_{CTX-M} and $_{AmpC}$, indicating that $_{AmpC}$ was consistently associated with bla_{SHV} in this dataset. Previous studies have shown that *Escherichia coli* isolates harboring bla_{CTX-M} may still be susceptible to cefoxitin, although resistance due to porin loss has been reported, especially in *Klebsiella pneumoniae* and *E. coli* strains[23]. In our study, 32.4% of the isolates co-harbored bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, a finding that mirrors reported by Hossain and Heo, who detected similar gene combinations in *Aeromonas* spp. from aquaculture settings[23].

Conclusion

Natural water systems play a pivotal role in the environmental mobilization and persistence of antimicrobial resistance. Rivers, in particular, act as convergence points for diverse microbial communities and resistance genes, facilitating the exchange of genetic material among environmental and pathogenic bacteria. The detection of β-lactamase-producing *Aeromonas* spp. in these waters highlights their potential to contaminate food chains, aquaculture systems, and drinking water sources, thereby posing direct threats to human and animal health. These findings underscore the urgent need for an integrated One Health approach that recognizes the interconnectedness of environmental, animal, and human health. Monitoring resistance in environmental reservoirs—especially within

aquatic ecosystems must become a core component of national and global antimicrobial resistance surveillance programs.

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