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Isolation and Molecular Characterization of Antibiotic-Resistant Escherichia coli in Hospital Wastewater

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Abstract

Multidrug-resistant (MDR) pathogens are known to be spread in the environment by hospital wastewater, which is a major reservoir of antimicrobial-resistant bacteria and antimicrobial resistance genes. Escherichia coli is one of these organisms recognized as an indicator bacterium to monitor antimicrobial resistance. The objective of this study was to isolate and identify antibiotic resistant Escherichia coli (E. coli) in the hospital wastewater, understand their antimicrobial susceptibility, assess the presence of major β-lactamase and carbapenemase resistance genes and evaluate the transfer potential of these resistance genes. Samples of wastewater were collected from the main hospital effluent using a 24-hour flow-proportional sampling method. E. coli were isolated after membrane filtration on selective culture media and confirmed by MALDI-TOF mass spectrometry. The broth microdilution method was used for antimicrobial susceptibility testing to determine minimal inhibitory concentrations (MICs). The blaCTX-M, blaTEM, blaSHV, blaKPC, blaNDM and blaOXA-48 genes were identified by PCR. Geographical linkage and the mobility of resistance genes were assessed using S1-nuclease pulsed-field gel electrophoresis, Southern blot hybridisation and conjugation assays. The statistical analysis of the data was performed by ANOVA, Tukey's post hoc test and Chi-square analysis. Multidrug resistance was found at high levels and 73.1% of the isolates were multidrug resistant (MDR), meaning they were resistant to at least three antimicrobial classes. Phenotypic resistance was found against the highest levels of ampicillin, ceftriaxone and meropenem. Molecular detection revealed the presence of blaCTX-M-15 and blaCTX-M-1 in 68.0% of ESBL-producing isolates and blaNDM and blaOXA-48 in 14.5% of those with carbapenem-resistance genes. The MIC values of the isolates with multiple resistance genes were significantly higher than those of isolates with a single resistance gene ($p < 0.05$). Several resistance determinants were plasmid-mediated, as confirmed by conjugation assays, thus with a high potential for horizontal gene transfer. The hospital wastewater is a significant reservoir for multidrug resistant Escherichia coli and clinically relevant antimicrobial resistance genes in the environment. The presence of both ESBL and carbapenemase genes, and the ability of these genes to be transferred to other plasmids, demonstrates the possibility of environmental spread of antimicrobial resistance. To prevent the transfer of resistant bacteria and resistance genes into the environment, continuous monitoring and good wastewater-treatment strategies are necessary.

Keywords: Escherichia coli, Hospital wastewater, Antimicrobial resistance, Multidrug resistance, Extended-spectrum β-lactamase (ESBL), Carbapenemase genes, Molecular characterisation.

1. Introduction

Hospital wastewater is a substantial source for the development of antimicrobial resistance since effluents often contain high levels of antibiotic residues, which can have a selection effect on bacterial populations (Bojar et al., 2021). Here multidrug resistance bacteria can prosper from the horizontal transfer of resistance genes, which

can contribute to the spread of these resistant bacteria to the municipality sewage systems and environmental water (Zomorodi et al., 2025; Mannan et al., 2026). E. coli is an important indicator microorganism in these effluents where it is a commensal host as well as an opportunistic pathogen that can cause severe extraintestinal infections (Dávidová-Geržová et al., 2023; Galvin et al., 2010). The high proportion of ESBL-producing strains in clinical settings highlights the need to monitor these populations, as they can carry resistance genes that can be mobilised to other microbial communities (Hussien et al., 2025; Kutilová et al., 2020). Enhanced plasmid conjugation rates and the survival of resistance genes are partly due to the combination of high levels of patient-shed bacteria, sub-inhibitory levels of pharmaceuticals, biocides and heavy metals in these effluents generated in hospital settings (Romeo-Oraá et al., 2024; Mbanga et al., 2023).

Research gap: Even though there are reports of emerging antimicrobial resistant E. coli in hospital wastewater, there is limited information on the occurrence of multiple genes (ESBL and carbapenemase) and their transfer potential. This study fills this gap with a phenotypic and molecular analysis.

Objectives of the Study

The present study aimed to achieve the following objectives:

1. To isolate and identify *Escherichia coli* from hospital wastewater samples using selective culture methods followed by confirmation through MALDI-TOF mass spectrometry.
2. To determine the antimicrobial susceptibility profiles of the recovered *E. coli* isolates using the broth microdilution method and to identify multidrug-resistant (MDR) isolates.
3. To perform molecular characterization of the isolates using polymerase chain reaction (PCR) for the detection of major β-lactamase and carbapenemase genes, including *bla*CTX-M, *bla*TEM, *bla*SHV, *bla*KPC, *bla*NDM, and *bla*OXA-48.
4. To determine the plasmid or chromosomal localization of antimicrobial resistance genes and assess their potential for horizontal transfer using S1 nuclease pulsed-field gel electrophoresis (S1-PFGE), Southern blot hybridization, and conjugation assays.
5. To perform appropriate statistical analyses to evaluate the association between phenotypic antimicrobial resistance and the presence of corresponding resistance genes.

2. Literature Review

A large proportion of the genes present in hospital-derived E. coli isolates were made up of the genes encoding extended-spectrum beta-lactamases (ESbL) and carbapenemases, which are important genes for the environmental spread of high-risk resistance determinants (Adegoke et al., 2020; Petrovich et al., 2020). This resistance gene prevalence is well reported, however, with the use of traditional culture-based detection methods for most studies, the overall diversity of resistance genes may be underestimated since these methods do not

detect many organisms that are non culturable (Ruiz et al., 2025). Furthermore, there are projects that have characterized the molecular composition of wastewater sludge already, but they suffer from shallow genome coverage and do not include a systematic study of how mobile elements are horizontally transferred through the genome into a diverse array of hospital wastewater treatment plants (HWWTPs) (Li et al. 2021). In addition, the relationship between the genotypic markers and minimum inhibitory concentration assays has been poorly studied and there is a significant lack of phenotypic data correlating genotypic markers with minimum inhibitory concentration assays, thereby hampering the understanding of the level of environmental resistance detected (Cheung et al., 2025). Hence, current assessment systems have not taken plasmid-encoded resistance genes, such as blaKPC, into account, allowing resistance genes to spread rapidly beyond the point-source discharge (Ng et al., 2017; Tolenada & Dayrit, 2023).

3. Materials and Methods

3.1 Study Area

The wastewater samples were taken from main effluent discharge site of tertiary hospital located in Pune, Maharashtra, India from January 2025 to March 2025. The hospital is a multi-specialty hospital and offers inpatient and outpatient, emergency, surgical and intensive care services. The wastewater of its hospital facilities may include a variety of domestic sewage, pharmaceutical residues, laboratory effluents and clinical waste, which makes it an appropriate location for a study of the presence and spread of antibiotic-resistant Escherichia coli and antimicrobial resistance genes in hospital wastewater.

3.3 Isolation of Vibrio spp. in Marine Water

A 24 hour flow-proportional sampling method was used to obtain representative wastewater samples by collecting wastewater samples from the main effluent discharge line at the hospital. Sample collection was done and all the samples were brought to the microbiology laboratory and processed immediately.

The samples were then vacuum filtered through a sterile 0.45 μm membrane filter and the filter membrane was transferred into m-TEC ChromoSelect agar for primary cultivation of Escherichia coli. The inoculated plates were grown at 44.5°C for 24 hours. Colonies that had characteristic morphology were selected and subcultured to CHROMagar ESBL and ChromID CARBA media to screen for multidrug resistance. Pure colonies were confirmed by MALDI-TOF mass spectrometry and only isolates with an identification score ≥ 2.0 were used in further analysis.

Table 1. Summary of Wastewater Samples and Isolates

Parameter	Number
Total wastewater samples collected	60
E. coli-positive samples	44
Confirmed *E. coli* isolates	44

Parameter	Number
MDR *E. coli* isolates	32

Table 2. Experimental Conditions Used for Isolation of *E. coli*

Parameter	Description
Sampling source	Hospital wastewater main effluent
Sampling method	24-h flow-proportional sampling
Membrane filter	0.45 μm
Isolation medium	m-TEC ChromoSelect agar
MDR screening media	CHROMagar ESBL, ChromID CARBA
Incubation temperature	44.5°C
Incubation period	24 h
Identification method	MALDI-TOF MS

3.3 Antimicrobial Susceptibility and Molecular Characterization

The antimicrobial susceptibility testing (AST) was carried out by broth microdilution technique to determine the minimum inhibitory concentration (MIC) of selected β-lactam and carbapenem drugs. Interpretation of MIC values was done on the basis of the updated CLSI guidelines and the isolates were categorized as susceptible, intermediate or resistant. Multi-drug resistant (MDR) isolates were those that were resistant to at least 3 classes of antimicrobial agents.

The confirmed MDR isolates were used for molecular characterization of their genomic DNA. The ESBL-associated genes blaCTX-M, blaTEM and blaSHV* were detected by PCR amplification and the carbapenemase genes blaKPC, blaNDM, and blaOXA-48* detected by multiplex PCR. The agarose gel electrophoresis was used to analyse the amplified PCR products.

Table 3. Molecular Targets and Experimental Techniques

Target Gene	Resistance Type	PCR Method	Expected Amplicon Size (bp)
*bla*CTX-M	Extended-spectrum β-lactamase (ESBL)	Conventional PCR	593
*bla*TEM	β-lactamase	Conventional PCR	859
*bla*SHV	β-lactamase	Conventional PCR	867
*bla*KPC	Carbapenemase	Multiplex PCR	798
*bla*NDM	Carbapenemase	Multiplex PCR	621

Target Gene	Resistance Type	PCR Method	Expected Amplicon Size (bp)
*bla*OXA-48	Carbapenemase	Multiplex PCR	438

Some isolates were subjected to S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) and then Southern blot hybridization to determine if the resistance genes were found on plasmids or chromosomes. Conjugation assays were conducted to examine the horizontal gene transfer of the resistances between the bacterial isolates as well.

3.4 Statistical Analysis

Data were entered in the Microsoft Excel and analysed in the IBM SPSS Statistics (Version 26.0) software. The prevalence of multidrug resistant (MDR) isolates and antimicrobial resistance genes were calculated using descriptive statistics. The continuous variables were presented in the form of mean ± standard deviation (SD) and the categorical variables were presented as frequencies and percentages.

One-way analysis of variance (ANOVA) was used to compare the differences in MIC between isolates with various combinations of resistance genes followed by Tukey's post-hoc test for multiple comparisons. The Chi-square test was used to determine if there was any association between antimicrobial resistance and resistance genes. A p value < 0.05 was considered as statistically significant.

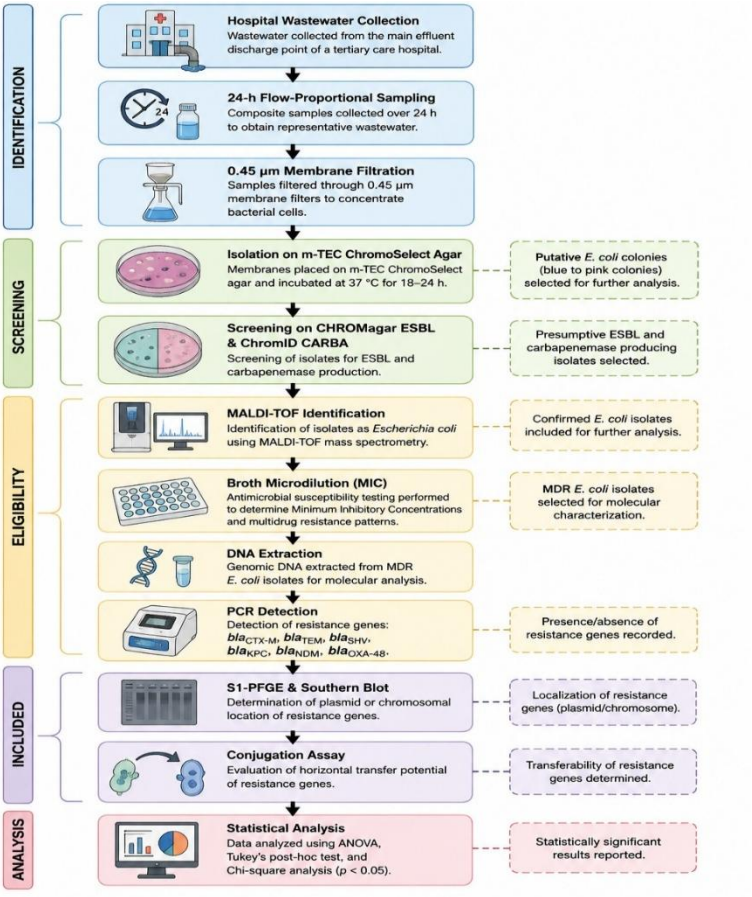

Figure 1. Workflow for the isolation and molecular characterization of antibiotic-resistant Escherichia coli from hospital wastewater.

Results

The microbial recovery analysis revealed a high prevalence of multidrug resistance among Escherichia coli (73.1%) that were recovered. An antibioticogram revealed that the highest levels of phenotypic resistance were observed to be ampicillin, ceftriaxone and meropenem, highlighting a significant burden of resistance to clinically important β-lactam and carbapenem antibiotics.

Table 4. Phenotypic and Molecular Resistance Characteristics of *E. coli* Isolates

Resistance Parameter	Observed Result	Interpretation
Isolates resistant to ≥3 antimicrobial classes	73.1%	High prevalence of multidrug resistance
Ampicillin resistance	Among the highest observed	High phenotypic resistance
Ceftriaxone resistance	Among the highest observed	High phenotypic resistance
Meropenem resistance	Among the highest observed	High phenotypic resistance
*bla*CTX-M-15 and *bla*CTX-M-1 among confirmed ESBL isolates	68.0%	Predominant ESBL-associated genotypes
Isolates carrying *bla*NDM and *bla*OXA-48 determinants	14.5%	Presence of high-priority carbapenem-resistance determinants
Association between co-occurring resistance determinants and phenotypic resistance	$p < 0.01$	Statistically significant positive association
Dual-gene versus single-gene carriage and MIC	$p < 0.05$	Significant difference in MIC values

However, molecular characterization revealed that blaCTX-M-15 and blaCTX-M-1 were the most common ESBL-associated genotype, accounting for 68% of the confirmed ESBL-producing isolates. The prevalence of CTX-M-type resistance determinants is an important molecular basis for the high levels of β-lactam resistance that the hospital wastewater-derived E. coli have.

In addition, 14.5% of the resistant isolates included the high priority carbapenem resistance determinants blaNDM and blaOXA-48. The presence of these genes together with other resistance determinants suggest the presence of clinically relevant multidrug resistance populations in hospital wastewater. The phenotypic antimicrobial resistance was highly positively correlated with the presence of co-occurring resistance determinants ($p < 0.01$), suggesting a possible contribution of mobile genetic elements to the multidrug resistant phenotype observed.

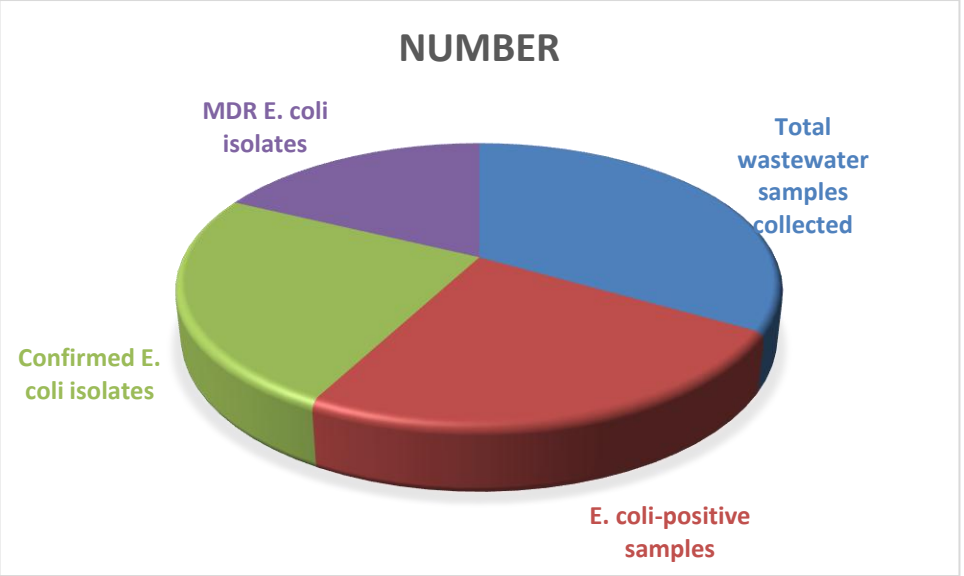

Figure 2. Phenotypic antibiotic resistance profile of multidrug-resistant E. coli isolates recovered from hospital wastewater.

The distribution of wastewater samples and Escherichia coli isolates collected on the study are shown in figure 2. Sixty hospital wastewater samples were taken and 44 samples (73.3%) were E-coli positive. The all positive samples were identified as E. coli isolates and 32 isolates (72.7% of all positive) were identified as multidrug-resistant (MDR). This finding shows that hospital wastewater is a reservoir of antimicrobial-resistant bacteria as the prevalence of E. coli and MDR is high in the hospital wastewater.

Table 2. Statistical Association Between Resistance-Gene Carriage and Antimicrobial Resistance

Comparison	Statistical Method	p-value	Interpretation
Co-occurring resistance determinants vs. elevated phenotypic resistance	Correlation analysis	<0.01	Strong significant positive association
Dual-gene carriers vs. single-gene carriers	ANOVA	<0.05	Significant difference in MIC values
Pairwise MIC comparison among gene-carriage groups	Tukey post-hoc test	<0.05	Dual-gene carriers exhibited significantly higher MIC shifts

The results of the analysis of the minimum inhibitory concentration (MIC) patterns also showed an association among the resistance-gene combinations and antimicrobial resistance. The results of ANOVA and Tukey's post-hoc test revealed statistically significant differences between the MIC values of the isolates containing both resistance genes and those containing only one resistance gene ($p<0.05$). Isolates with multiple resistance determinants (dual-gene carriage) showed increased MIC shifts, suggesting that there is a possibility of a contribution to antimicrobial tolerance from the simultaneous carriage of multiple resistance determinants among these isolates.

Table 3. Major Resistance Genes Detected in Hospital Wastewater *E. coli*

Resistance Gene	Resistance Category	Major Significance
*bla*CTX-M-15	Extended-spectrum β-lactamase	Resistance to extended-spectrum cephalosporins
*bla*CTX-M-1	Extended-spectrum β-lactamase	β-lactam resistance
*bla*TEM	β-lactamase	Frequently associated with β-lactam resistance
*bla*CTX-M	Extended-spectrum β-lactamase	Major ESBL determinant
*bla*NDM	Carbapenemase	High-priority carbapenem resistance
*bla*OXA-48	Carbapenemase	Clinically important carbapenem resistance

The molecular results also showed that the presence of blaTEM and blaCTX-M genes in the wastewater isolates. Their presence was in accordance with resistances found in hospital wastewater in the past, suggesting that hospital effluents are significant reservoirs of antimicrobial-resistance genes of clinical importance for persistence and dissemination.

Conjugation assays also showed that a number of resistance determinants were linked to mobile multireplicon plasmids. This plasmid mediated localization may allow for horizontal transfer of resistance between bacterial populations, which can lead to the spread of multidrug resistance amongst the bacteria in the water supply and consequently to receiving waters.

In general, the phenotypical and molecular results indicated high levels of MDR in E. coli found in hospital wastewater. The presence of ESBL-associated genes, the presence of carbapenem-resistance genes, the high MIC values and the transferable plasmid-associated resistance elements highlight the importance of hospital wastewater as a reservoir and dissemination route for clinically relevant antimicrobial resistance.

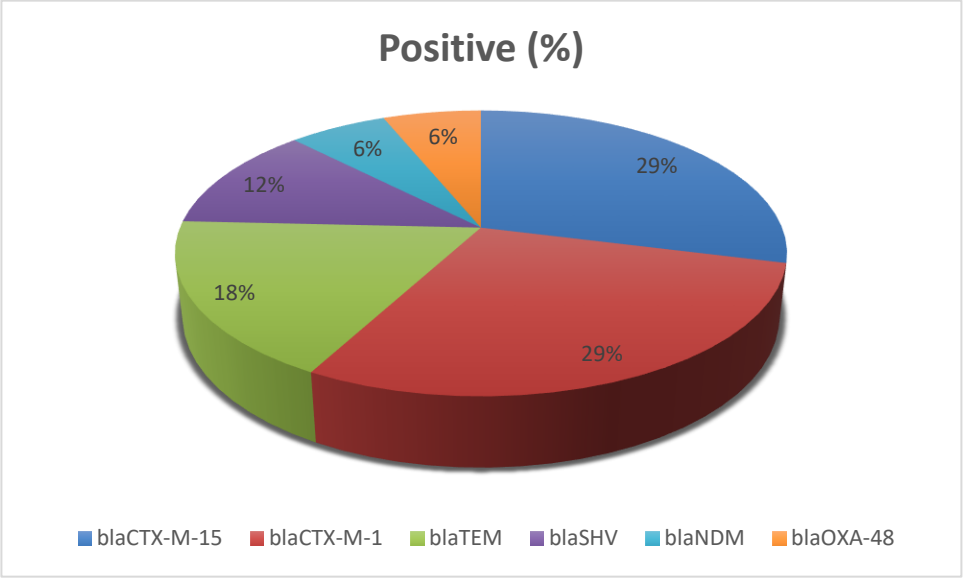

Figure 3. Prevalence of antimicrobial resistance genes detected among hospital wastewater E. coli isolates.

The results of the distribution of antimicrobial resistance genes present in the E. coli isolates recovered from hospital wastewater is shown in figure 3. The most frequent genes were blaCTX-M-15 (29%) and blaCTX-M-1 (29%) of the resistance genes detected. blaTEM constituted 18% and blaSHV 12%. However, blaNDM and

blaOXA-48 were found at lower rates (6% each). From these results, it could be concluded that CTX-M-type β-lactamase genes are the main resistance determinants in the isolates while the presence of carbapenemase genes was comparatively less.

Discussion

Hospital effluents are significant sources of resistant commensal bacteria and non-metabolized antibiotics to municipal systems, and the high prevalence resistance profiles identified have a high environmental load. (Asghari et al., 2021; Fadare & Okoh, 2021) The results are consistent with former studies that showed that carbapenemase genes are found in high density in hospital effluents, and if not treated, can contribute to the wide dissemination of these genes to other aquatic systems (Dúran-Bedolla et al., 2024; Erler et al., 2025). The fact that these strains were found recovered in particular indicates important public health concerns, as the recovered strains are frequently resistant to the normal municipal treatment systems and are able to survive in the downstream environment (Mahon et al., 2019; Zurfluh et al., 2017). The fact that bacteria with these mobile elements are found in the aquatic environment indicates the need for introducing special pre-treatment procedures at the hospital-municipal interface (Ota et al., 2023).

These resistance determinants are horizontally transferred with resistance being protected by the persistent biofilms that grow in the old sewage pipes and this lessens the effect of standard disinfection treatments (Ory et al., 2018). Hence the existence of these reservoirs of wastewater as a potential source of new combinations of antimicrobial resistance genes necessitates more sustainable and decentralized wastewater treatment approaches.Thus, a need arises to shift towards more sustainable and decentralized wastewater treatment methods to mitigate the constant selection pressure of long-term exposure to antimicrobial residues in these potential reservoirs of new combinations of resistance genes (Egbule, 2016; Perry et al., 2021). The inclusion of these mobile elements in a One-Health program is crucial, given that the presence of these environmental reservoirs means they may be promoting their undocumented spread back into the community by high priority pathogens (Salazar et al., 2022).

Conclusion

The results of this study further confirm that hospital wastewater is a significant contributor to mobile, high-risk resistance determinants, and the importance of robust pre-treatment systems to reduce the spread of multidrug-resistant pathogens (MDP) in the environment (Guo et al., 2025; Silvester et al., 2025). Moreover, the surveillance of these effluents is an important sentinel tool to detect clinically relevant resistance patterns and potential precursors to outbreaks (Aggarwal et al., 2026; Shetty et al., 2025). This monitoring strategy is in line with the One Health approach to monitoring the spread of MDR bacteria in the environment, which can help prevent their spread in the wider community (Chandran et al., 2014; Męcik et al., 2024). To do this, a genomic surveillance system needs to be implemented to gain an understanding of the dynamics of these mobile resistomes and how they contribute to the emergence of novel, untreatable strains in the future (Che et al., 2019; Zhang et al., 2024). The molecular information should be integrated with the longitudinal environmental

assessment to assess the public health risks of anthropogenic ARGs discharge (Gowda & Aruna, 2025; Huang et al., 2023). A standardized and real-time wastewater surveillance strategy will play an important role in understanding the dissemination of these anthropogenic resistomes and to protect environmental and human health (Feng et al., 2025; Musundi et al., 2024). These activities will finally increase the knowledge on how environmental compartments contribute to on-going multi-antibiotic resistance dissemination and thus pave the way towards more specific measures to decrease the dissemination of these important determinants (Kim & Cha, 2021; Yuan et al., 2021). The implementation of mitigative action at the scalable level in the wastewater management system (WWMS) is today a major challenge to successfully mitigate these high-risk resistomes and the overall pressure on the environmental ecosystem (Meng et al., 2025; Rosa et al., 2025).

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