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Antimicrobial resistance profiling of multidrug-resistant enterobacteriaceae from hospital wastewater

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Abstract

Hospital wastewater is a breeding ground for antimicrobial resistance and most treatment plants weren't designed to handle it. This research characterized the antimicrobial resistance profiles of multidrug-resistant (MDR) Enterobacteriaceae isolated from wastewater at five sampling points along the treatment chain of a 250-bed tertiary hospital in Imphal, Manipur. Between March and November 2022, 384 wastewater samples were collected biweekly and processed at the Manipur Institute of Biological Research. A total of 247 Enterobacteriaceae isolates were recovered, with *Escherichia coli* (42.1%), *Klebsiella pneumoniae* (33.2%), and *Enterobacter cloacae* (12.6%) being the most frequent species. MDR phenotypes (resistance to three or more antibiotic classes) were detected in 87.3% of raw influent isolates but only 38.1% of chlorinated effluent isolates. Extended-spectrum beta-lactamase (ESBL) production was confirmed in 63.8% of all isolates, and carbapenem resistance was found in 28.7%. PCR screening revealed blaCTX-M (71.3%), blaNDM-1 (24.6%), and blaOXA-48 (11.8%) as the dominant resistance genes. A positive correlation existed between the number of AMR genes carried and meropenem MIC values ($R^2=0.71$, $p<0.001$).

Keywords: Antimicrobial resistance, multidrug resistant, Enterobacteriaceae, hospital wastewater, AMR surveillance, ESBL, carbapenemase, resistance genes, wastewater treatment, public health

Introduction

Antimicrobial resistance kills more people each year than HIV and malaria combined. A 2019 global analysis attributed 1.27 million deaths directly to bacterial AMR, with Enterobacteriaceae responsible for a disproportionate share of the burden ^[1]. Hospital environments, where antibiotic usage is concentrated, generate wastewater that carries both resistant bacteria and the mobile genetic elements encoding resistance plasmids, integrons, and transposons that can spread resistance to environmental organisms ^[2].

Conventional wastewater treatment plants reduce bacterial loads through physical and chemical processes, but they weren't engineered to eliminate resistance genes or to prevent horizontal gene transfer ^[3]. Several investigations from urban India have found that hospital effluent contributes significantly to the AMR burden in receiving water bodies, with ESBL-producing Enterobacteriaceae detected even in treated effluent ^[4, 5]. Northeast India presents a particularly understudied context. Manipur's healthcare infrastructure has expanded in recent decades, but wastewater treatment capacity hasn't kept pace ^[6]. Understanding how resistance profiles change along the treatment chain from raw influent to final discharge is important for identifying where interventions might be most effective ^[7]. Equally important is knowing which specific resistance genes persist through treatment, since some (like blaNDM-1) encode carbapenemases that threaten last-resort antibiotics ^[8]. This research aimed to characterize the species distribution, resistance phenotypes, and resistance gene carriage of Enterobacteriaceae at sequential points in a hospital wastewater treatment system in Imphal, Manipur.

Material and Methods

Reagents and Chemicals

MacConkey agar, Mueller-Hinton agar, and brain heart infusion broth were obtained from HiMedia Laboratories (Mumbai, India). Antibiotic discs for susceptibility testing (panel of 18 antibiotics covering 8 classes) came from HiMedia and were stored at 4 °C until use. Etest strips for MIC determination of meropenem, imipenem, and colistin were purchased from

bioMerieux (through their Mumbai distributor). PCR primers targeting blaCTX-M, blaTEM, blaSHV, blaNDM-1, blaOXA-48, and blaKPC were synthesized by Eurofins Genomics India (Bangalore). DNA extraction kits (DNeasy Blood and Tissue Kit) were sourced from Qiagen (New Delhi). Taq polymerase, dNTPs, and agarose were obtained from Thermo Fisher Scientific.

Material

Wastewater samples were collected from five points: raw influent (before any treatment), post-primary sedimentation, post-secondary biological treatment, post-chlorination effluent, and an upstream control site 200 meters above the hospital discharge point on the Nambul River. Grab samples of 500 mL each were collected in sterile polypropylene bottles biweekly between March and November 2022 (total 384 samples across all sites). Samples were transported on ice to the Department of Applied Microbiology, Manipur Institute of Biological Research, Imphal, and processed within 4 hours of collection. Physico-chemical parameters (pH, temperature, dissolved oxygen, BOD, COD) were recorded at each sampling event using a YSI ProDSS multiparameter instrument

Results

Table 1: Species distribution and resistance phenotypes of enterobacteriaceae isolates (n=247)

Species	n (%)	MDR (%)	ESBL (%)	Carbapenem resistant (%)	Colistin resistant (%)
<i>E. coli</i>	104 (42.1)	78.8	68.3	22.1	3.8
<i>K. pneumoniae</i>	82 (33.2)	84.1	71.9	38.4	7.3
<i>E. cloacae</i>	31 (12.6)	74.2	48.4	29.0	0.0
<i>C. freundii</i>	18 (7.3)	66.7	44.4	16.7	0.0
Others	12 (4.8)	58.3	33.3	8.3	0.0
Total	247	78.9	63.8	28.7	4.0

Klebsiella pneumoniae showed the highest rates of both ESBL production (71.9%) and carbapenem resistance (38.4%) among the identified species (Table 1). Colistin resistance, while uncommon overall at 4.0%, was

Methods

Samples were serially diluted and plated on MacConkey agar supplemented with cefotaxime (2 µg/mL) for selective isolation of resistant Enterobacteriaceae. Colonies were identified to species level using the VITEK 2 automated system (bioMerieux) and confirmed by 16S rRNA gene sequencing for ambiguous identifications. Antimicrobial susceptibility testing followed CLSI 2022 guidelines using the disc diffusion method. MIC values for carbapenems and colistin were determined by Etest. ESBL production was confirmed by the combined disc test (cefotaxime ± clavulanate). Carbapenemase production was screened using the modified carbapenem inactivation method (mCIM). Genomic DNA was extracted from all MDR isolates and screened by multiplex PCR for six beta-lactamase genes. All experiments had the approval of the Institutional Biosafety Committee (IBC/MIBR/2022-06, dated February 2022). Data were analyzed using chi-square tests and Spearman rank correlation in SPSS v26.

concentrated in *K. pneumoniae* isolates a worrying finding given that colistin serves as a last-resort treatment for carbapenem-resistant infections.

Table 2: Resistance Gene Prevalence across Sampling Points

Gene	Raw influent	Post- primary	Post- secondary	Post- chlor.	Upstream control
blaCTX-M	84.3%	76.8%	61.2%	48.7%	18.4%
blaTEM	67.1%	58.9%	42.6%	31.4%	12.7%
blaSHV	38.6%	31.2%	24.8%	17.3%	6.2%
blaNDM-1	31.4%	27.8%	19.4%	14.2%	4.1%
blaOXA-48	17.1%	13.6%	9.3%	6.8%	1.4%
blaKPC	4.3%	3.2%	1.6%	0.0%	0.0%

Treatment reduced but did not eliminate resistance genes (Table 2). blaCTX-M persisted most stubbornly, with nearly half of post-chlorination isolates still carrying it. The

BlaNDM-1 gene was detected even after chlorination (14.2%), suggesting that some carbapenemase-producing organisms survive the full treatment chain.

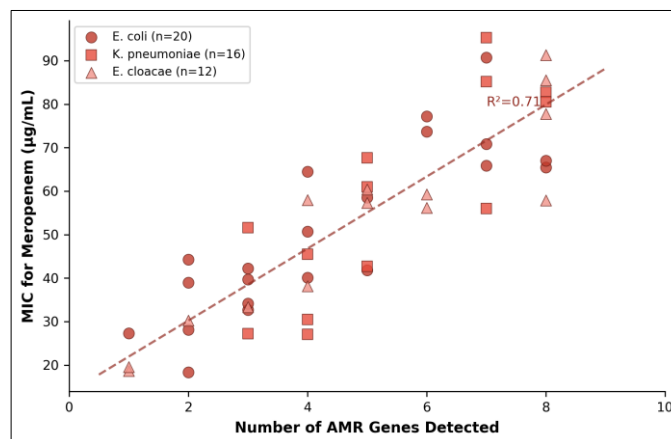


Fig 1: Correlation between number of AMR genes detected per isolate and meropenem MIC values. Each point represents one isolate, colored by species. The dashed line shows the linear regression fit ($R^2=0.71$, $p<0.001$)

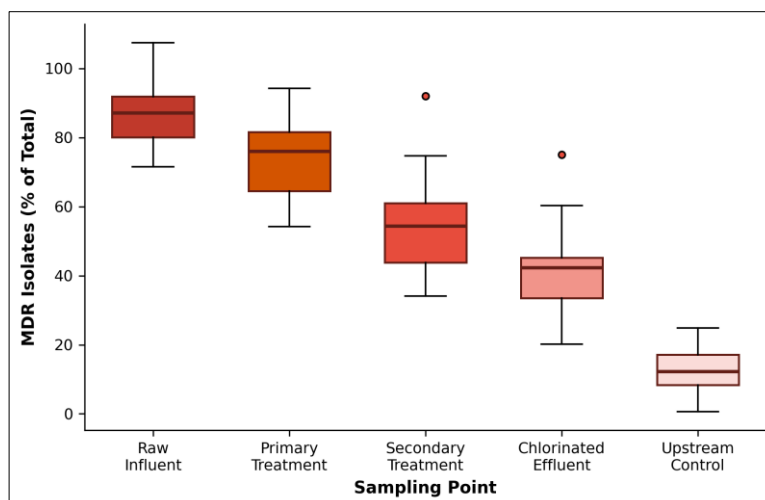


Fig 2: Distribution of multidrug-resistant isolates (% of total) across five sequential sampling points in the hospital wastewater treatment system. Box plots show median, interquartile range, and individual outliers from biweekly sampling events

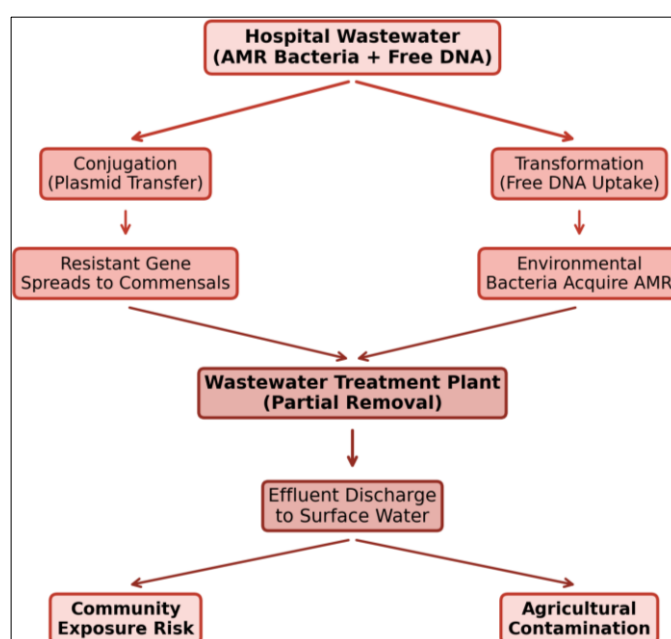


Fig 3: Schematic representation of AMR gene transfer pathways from hospital wastewater to environmental and community exposure routes.

Discussion

The high prevalence of MDR Enterobacteriaceae in hospital wastewater from Imphal mirrors patterns reported from larger Indian cities like Delhi and Chennai^[4, 9] but this is among the first systematic characterizations from northeast India. The 87.3% MDR rate in raw influent is alarming, though not unexpected for a tertiary care facility with active ICU and surgical wards where broad-spectrum antibiotics are prescribed routinely^[10]

What stands out is how incompletely the treatment system removes resistance. Chlorination reduced MDR prevalence from 87.3% to 38.1% a meaningful drop, but it means that more than a third of surviving isolates in the final effluent are still multidrug resistant. Other research groups in India have reported similar persistence after conventional treatment^[5, 11]. The detection of blaNDM-1 in 14.2% of post-chlorination isolates is particularly concerning, since NDM-1-producing bacteria have caused untreatable infections in healthcare settings globally and the gene spreads efficiently on mobile genetic elements^[12].

The strong correlation between resistance gene count and carbapenem MIC ($R^2=0.71$) suggests additive or synergistic

interactions among co-carried resistance determinants. Isolates carrying four or more resistance genes consistently showed MIC values above the CLSI breakpoint for resistance. This co-carriage pattern was most pronounced in *K. pneumoniae*, which also showed the highest rates of carbapenem and colistin resistance a combination that severely limits therapeutic options^[13, 14].

Conclusion

Hospital wastewater in Imphal harbors a high burden of multidrug-resistant Enterobacteriaceae, with ESBL-producing and carbapenem-resistant strains persisting through conventional treatment processes. The blaCTX-M and blaNDM-1 genes were detected even in chlorinated effluent, confirming that standard wastewater treatment is insufficient to prevent the environmental dissemination of clinically important resistance genes. *Klebsiella pneumoniae* emerged as the most resistant species across all metrics, including colistin resistance at 7.3%. A clear gradient of decreasing resistance prevalence from influent to effluent demonstrates that treatment does reduce the AMR burden, but not enough to protect downstream water users. These

findings argue for upgrading hospital wastewater treatment systems in northeast India particularly through adding advanced treatment steps such as UV disinfection, ozonation, or membrane filtration to reduce the release of resistant organisms and their genetic determinants into the environment.

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