Unveiling the biofilm-forming potential of wastewater-derived MDR Enterobacteriaceae on microplastic surfaces

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INTRODUCTION

Microplastics have emerged as persistent environmental pollutants and potential hotspots for antimicrobial resistance dissemination. Acting as ideal substrata for microbial attachment, they facilitate biofilm formation and create micro-niches known as plastispheres. Within these plastispheres, close microbial interactions promote horizontal gene transfer, enabling the exchange of antibiotic resistance genes (ARGs) and the emergence of multidrug-resistant (MDR) bacteria. Wastewater, enriched with diverse microbial communities and antibiotic residues, provides a conducive environment for such interactions. This study investigates the role of polyethylene terephthalate (PET) microplastics in enhancing biofilm formation by wastewater-derived MDR bacteria from the Delhi-NCR region, underscoring the contribution of microplastics to the environmental persistence and potential spread of antimicrobial resistance.

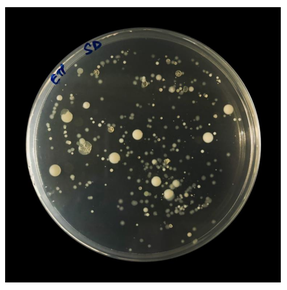
METHODOLOGY

- Wastewater samples were collected from four major open drains in Delhi NCR during February and March 2025.
- Samples were processed on Luria Agar supplemented with ertapenem (a carbapenem antibiotic) for initial screening of antibiotic-resistant isolates in accordance with Clinical and Laboratory Standards Institute (CLSI) M100-2023 (33rd edition).
- Isolates were screened against 16 antibiotics by the Kirby-Bauer disc diffusion method on Muller-Hinton Agar.
- Multiple antibiotic resistance index (MARi) was estimated to determine multidrug resistance among the initially screened resistant isolates. MARi value greater than 0.2 is considered significant.
- Crystal Violet assay was performed to evaluate the biofilm-forming potential of these isolates, based on which these were categorized as strong, moderate, weak, and non-biofilm formers.
- Moderate to strong biofilm formers were further identified by MALDI TOF- Mass Spectrometry and were selected for further study.
- Microplastics of equal proportion were obtained from polyethylene terephthalate (PET) waste bottles and were sterilized.
- Crystal violet assay was performed to evaluate the biofilm-forming potential of the selected isolates on the PET surface in three phases: early (2 days/48 hours), mid (5 days/120 hours), and late (10 days/240 hours).
- Biofilms were visualized through scanning electron microscopy.
- PCR analysis was conducted to detect various genes associated with resistance and biofilm formation.

RESULTS

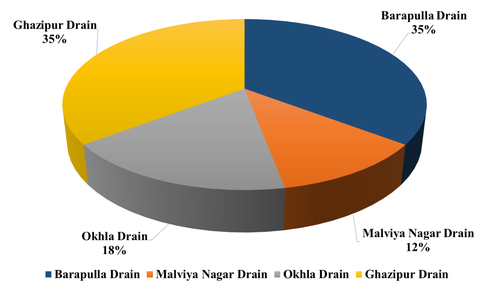
Isolation and Purification of bacterial isolates

A total number of 17 bacterial isolates were obtained on ertapenem-augmented Luria Agar.



Bacterial colonies on ertapenem-augmented media

Purification of bacterial culture



A pie chart representing the distribution of antibiotic-resistant isolates collected from different

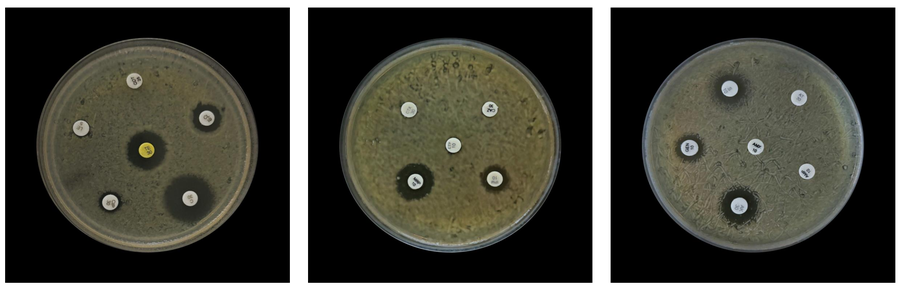
Identification of Isolates

- **Strong and moderate biofilm formers were identified via MALDI TOF mass spectrometry at Dr. Lal Path Labs, Rohini, New Delhi.**
- **All the isolates identified were members of the Enterobacteriaceae family.**

Isolate Code	Identified bacteria
A1	*Escherichia coli*
A2	*Klebsiella pneumoniae*
B1	*Klebsiella pneumoniae*
B2	*Klebsiella pneumoniae*
C3	*Escherichia coli*
D1	*Escherichia coli*

AST Results for MARi

- **AST profiling showed resistance against all the classes of antibiotics.**
- **Isolates showed high resistance against β-lactams and fluoroquinolones.**
- **Chloramphenicol and tetracyclines exhibited susceptibility.**
- **The MARi ranged from 0.43 to 0.93 (mean= 0.76 ± 1.4), with 62.5% isolates exhibiting MARi greater than 0.80.**
- **Maximum MARi value of 0.93 was observed in 04 isolates.**



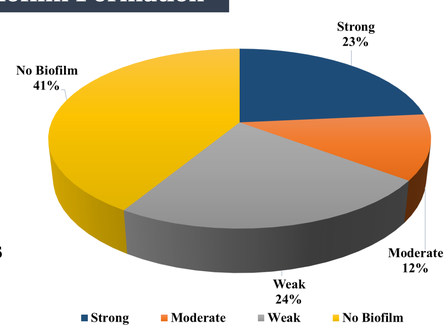
MHA plates showing zones of inhibition against different antibiotics

Isolates	β-lactams								Non β-lactams								MARi
	Penicillins		Cephalosporins		Carbapenems			Monobactams	Aminoglycosides			Fluoroquinolones		Chloramphenicol	Tetracyclines	Antifolates	
	AMX	AMP	CAZ	CTX	ETP	IPM	MRP	AT	AK	GEN	S	CIP	LE	C	TET	COT	
A1																	0.81
A2																	0.93
A3																	0.75
A4																	0.75
A5																	0.75
A6																	0.81
B1																	0.93
B2																	0.43
C1																	0.93
C2																	0.81
C3																	0.81
C4																	0.56
C5																	0.75
C6																	0.81
D1																	0.93
D2																	0.56
D3																	0.87

Resistant | Partially Resistant | Susceptible

Screening for Biofilm Formation

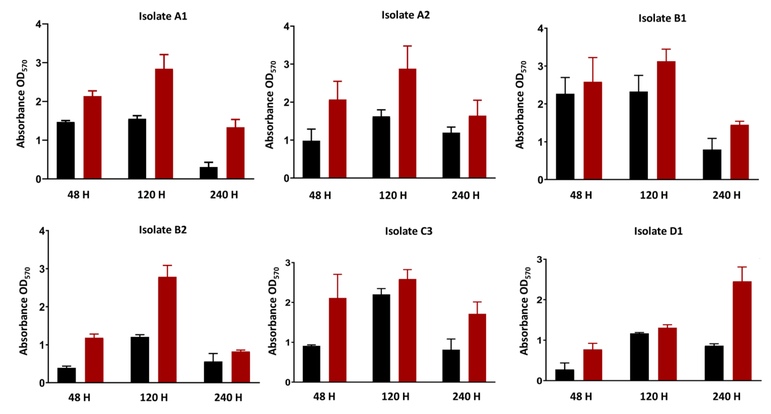
- **Biofilm formation was evaluated through the crystal violet assay, following observations were made:**
  - **Strong biofilm- 04**
  - **Moderate biofilm- 02**
  - **Weak biofilm- 04**
  - **No biofilm- 07**
- **Strong and moderate biofilm formers were selected for further study.**
- ***Klebsiella pneumoniae* ATCC® 33495 was used as the positive control.**



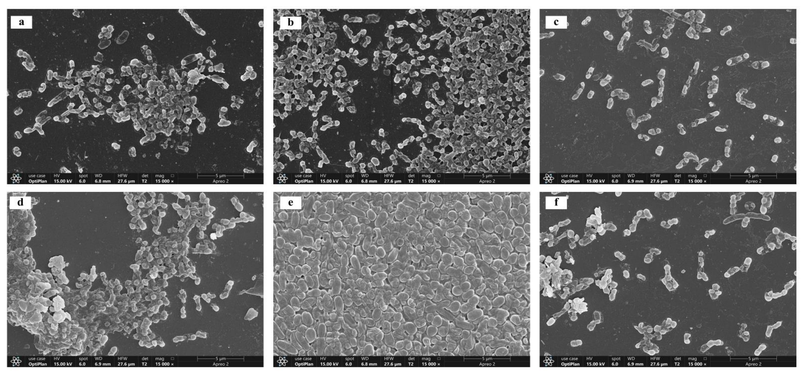
A pie chart representing the results of biofilm assay

Biofilm Assay with Microplastics

- **In the presence of microplastics, biofilm formation was high in all the isolates at three different phases: early, mid, and late.**
- ***Klebsiella pneumoniae* showed colonization in the early phase.**
- ***Escherichia coli* showed a varied colonization pattern.**



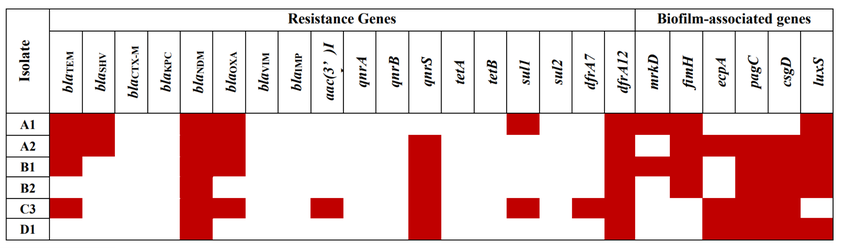
Biofilm formation in the presence and absence of microplastic at different stages

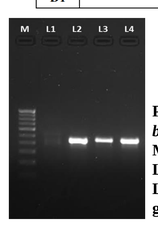


Biofilm visualization on microplastic surface through scanning electron microscopy (a-c) *Klebsiella pneumoniae* ATCC® 33495 (positive control) in early, mid, and late phases, respectively; (d-f) Isolate A1 (*Escherichia coli*) in early, mid, and late phases, respectively.

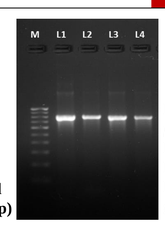
PCR-based amplification of resistance and biofilm-associated genes

- **Among resistance genes- bla_{NDM}, *dfr*A12, *qnr*S, bla_{OXA}, and bla_{TEM}. Isolate C3 carried the maximum number of resistance genes.**
- **All the isolates carried at least one fimbrae gene (*mrk*D, *fim*H, and *ecp*A), 5 out of 6 isolates carried EPS production genes (*pag*C and *csg*D), and 5 isolates carried quorum-sensing gene *lux*S.**

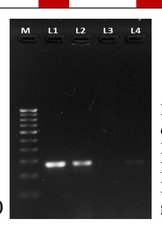




PCR analysis for bla_{OXA} gene. M: 100bp DNA Ladder; L1 to L4: Amplified gene product (438bp)



PCR analysis for *fim*H gene. M: 100bp DNA Ladder; L1 to L4: Amplified gene product (817bp)



PCR analysis for *csg*D gene. M:100bp DNA Ladder; L1 to L4: Amplified gene product (292bp)

CONCLUSIONS

- A significant prevalence of multidrug resistance has been identified in aquatic environments.
- Wastewater-derived MDR bacteria actively colonize the microplastic surface.
- Microplastics serve as a potent substratum, enhancing biofilm formation upto two-fold.
- Resistance determinants have been identified among studied isolates, suggesting the potential for transfer to susceptible bacteria in the environment through horizontal gene transfer within the plastispheres.

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DECLARATION

Shayan Ahmed declares no conflict of interest. All the data put forwarded is original research work carried out by the authors at Microbiology Research Laboratory, Jamia Millia Islamia, New Delhi.

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