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Environmental Reservoirs of Resistance: Detection of MDR Bacterial Isolates from Healthcare Soil Waste

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ABSTRACT

The rising threat of antimicrobial resistance (AMR) presents significant challenges to public health, especially when resistant microorganisms emerge from environmental reservoirs such as hospital and pharmaceutical waste sites. In the present study, five soil samples collected from the vicinity of healthcare facilities were screened for microbial isolates. A total of 42 distinct bacterial and 18 fungal isolates were obtained, out of which four bacterial isolates BI4, BT3, HMR2 and TD7 were identified as multidrug-resistant (MDR), showing resistance to more than three antibiotics. Antimicrobial susceptibility testing revealed that TD7 (*Pseudomonas aeruginosa*) exhibited the highest resistance, being unresponsive to seven antibiotics, while BI4 (*P. aeruginosa*), BT3 (*Bacillus pumilus*), and HMR2 (*Cellulosimicrobium cellulans*) also demonstrated varying degrees of resistance. Notably, *C. cellulans* (HMR2), a typically non-pathogenic soil bacterium, exhibited resistance to six antibiotics, underscoring the potential role of environmental bacteria in harboring and spreading resistance traits. Biochemical testing, 16S rRNA gene sequencing, and phylogenetic analysis using MEGA 11 confirmed the identity and evolutionary relationships of these isolates. High genetic similarity to clinically relevant strains was observed, particularly among *Pseudomonas* species, suggesting possible gene exchange between environmental and pathogenic bacteria. The study emphasizes the impact of poorly managed antibiotic disposal and the role of horizontal gene transfer in accelerating resistance development. These findings underline the urgency of implementing robust waste management practices and exploring natural sources for novel antimicrobials to mitigate the growing AMR crisis. The emergence of MDR strains in environmental settings calls for integrative surveillance and preventive strategies.

1. INTRODUCTION

Antimicrobial resistance (AMR) is emerging as a critical global challenge, with infections caused by resistant microorganisms steadily increasing worldwide (Ashiru-Oredope etal., 2022). This growing threat requires coordinated international efforts to develop effective strategies to combat AMR. Numerous studies have reported a rise in diseases linked to resistant pathogens, highlighting the severity of the issue. One major contributor is the widespread use of antibiotics in hospitals and healthcare facilities, which, while intended to treat infections, has also led to the acceleration of resistance development (Frieden, 2013). Despite this, the environmental impact of antibiotic use, particularly through contaminated hospital wastewater, has received comparatively less attention. The unchecked and excessive administration of antibiotics facilitates the release of resistance genes into the environment, thereby intensifying the AMR problem.

Over the years, considerable research has been dedicated to discovering new antibiotics from natural sources, including plants and microbes. Natural products have historically played a crucial role in antibiotic discovery and are being revisited as the resistance to commonly used antibiotics continues to rise. This growing resistance underscores the urgent need for the development of innovative antimicrobial agents to replace or supplement existing treatments, which are rapidly losing their effectiveness (Sadiqi et al., 2022).

Increased hospital-acquired infections can be traced to two main factors: the presence of resistant bacteria within healthcare environments that are transmitted between patients, and the proliferation of resistant strains already present in patients' bodies, which gain dominance under the selective pressure of antibiotic therapy. The frequent and often unregulated use of antibiotics significantly contributes to the rising burden of resistance in communities, enabling the persistence and spread of resistant pathogens. Particularly problematic are infections caused by vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Mycobacterium tuberculosis*, and resistant Gram-negative

organisms. The emergence of extended-spectrum β-lactamases (ESBLs) and carbapenemases in these bacteria further complicates treatment, leading to infections that are increasingly difficult to manage and associated with higher rates of morbidity and mortality (Dahiya et al., 2012).

2. METHODOLOGY

Sample collection

A total of 5 soil samples were collected from waste disposal sites of 5 hospital sites of Hamirpur, Himachal Pradesh and transferred to Division of Microbiology, Career point university, Hamirpur (HP).

Isolation of bacterial and fungal strains

The microbial strains in the soil samples were isolated using 10 fold serial dilution as described by Emami et al. Soil suspension was made by using 1g of soil in 9ml of sterile distilled water and mixed thoroughly. This soil suspension was further serially diluted from 10^{-1} to 10^{-8} dilutions. 100µl soil suspension was pipette out and spreaded with the help of spreader on the nutrient agar plate for isolation of bacterial strains and on Potato dextrose agar for fungal isolates. The nutrient agar petriplates were then incubated at 37ºC for 24 hours, potato dextrose agar plates were incubated at 25ºC for 24-72 hrs. Pertiplates which shows uniform and distinct colony were selected. The bacterial and fungal colonies which show different morphological features were picked up and pure cultured further on agar plates. Pure cultures were confirmed through gram staining and stored at 4ºC for further analysis.

Screening of Multi Drug Resistant (MDR) isolates

Standard agar disk diffusion technique given by CSLI guidelines was used to determine the antibiotic sensitivity patterns of isolates isolates on Muller-Hinton agar plates (Clinical and laboratory standards Institute, 2012). 9 broad spectrum antibiotics, Amoxicillin-clavulanic acid 30 mg (AMC30), Ampicillin 10mg (AMP10), Azithromycin 30mg (AZM30), Ciprofloxacin 10mg (CIP10), Clindamycin 10mg (CD10), Kanamicin 30mg, Penicillin 2mg (P2), Streptomycin 10mg (S10), Tetracycline 30mg (TE30) were used for antibiotic sensitivity test to isolate multi-drug resistant bacteria. While Terbinafine 30mg, Itraconazole 30mg, Econazole 10mg, Ketoconazole 10mg, voriconazole 10mg and Fluconazole 25mg were used to check the antibiotic sensitivity of the fungal isolates. Pure bacterial isolates were uniformly spread on Mueller-Hinton agar plates, followed by placement of antibiotic discs using sterile forceps. The plates were then incubated at 37°C for 24 hours. After incubation, zones of inhibition around the antibiotic discs were examined and their diameters measured in millimetres using a Himedia zone measuring scale. Isolates exhibiting resistance to more than three antibiotics were classified as multidrug-resistant (MDR) strains and chosen for further investigation.

Biochemical characterization of isolated MDR bacteria

To identify the isolated multidrug-resistant (MDR) bacterial strains, a series of biochemical tests including Methy red, voges-proskauer, citrate utilization, indole, oxidase, catalase and lactose fermentation were carried out following the methodology described by Talaiekhozani (2013). These tests help determine specific enzymatic functions and metabolic characteristics essential for the accurate identification of bacterial species. All bacteriological media required for these analyses were sourced from HiMedia Lab. Lmt, India.

Molecular identification of MDR isolates

Molecular identification of isolated MDR strains was carried out by using 16s rRna gene sequencing, outsourced from Green Array Genomic Research & Solutions of Accurate Diagnostic Private Limited, Pune, Maharastra, India.

Extraction of genomic DNA

Genomic DNA from the bacterial isolates was extracted using a commercially available DNA extraction kit provided by Biopro. The isolated DNA was then dissolved in TE buffer to be used for subsequent PCR amplification.

PCR Amplification of 16S rRNA gene

The amplification of the 16S rRNA gene was carried out in a 24 µL reaction mixture containing 20 ng of genomic DNA. Universal primers F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-TACCTTGTTACGACTT-3′) were employed for the PCR amplification process.

Qualitative analysis and sequencing

The amplified 16S rRNA product was electrophoresed on a 1.5% agarose gel prepared in 1.0% TBE (Tris-borate-EDTA) buffer. The resulting PCR product was purified using a commercially available purification kit from Biopro. Sequencing was then performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems ABI 3500 DNA sequencer.

Identification and phylogenetic tree

BLAST analysis of the 16S rRNA gene sequences from the four isolates was performed using the NCBI database. Sequences with the highest similarity and percentage identity were selected to assess genetic diversity and evolutionary relationships. This analysis was conducted using the Molecular Evolutionary Genetics Analysis software version 11 (MEGA 11), and a phylogenetic tree was generated employing the neighbor-joining method.

3. RESULTS AND DISCUSSION

Five soil samples were collected from nearby soil associated with five hospitals. From these samples, a total of 42 distinct pure bacterial isolates were recovered (as shown in Table 1).

Table 1. Isolation of MDR bacteria from different soil samples.

Sr. No.	Soil samples	No of pure isolates	No. of MDR isolates
1	BI	7	1 (BI4)
2	BR	7	0
3	BT	10	1 (BT3)
4	HMR	10	1 (HMR2)
5	TD	8	1 (TD7)
Total no. of isolates		**42**	**4**

All isolates were subjected to antimicrobial testing, and among them, four BI4, BT3, HMR2, and TD7 exhibited resistance to more than three antibiotics (Fig. 1). These were identified as multidrug-resistant (MDR) strains and selected for further detailed analysis (Fig. 2).

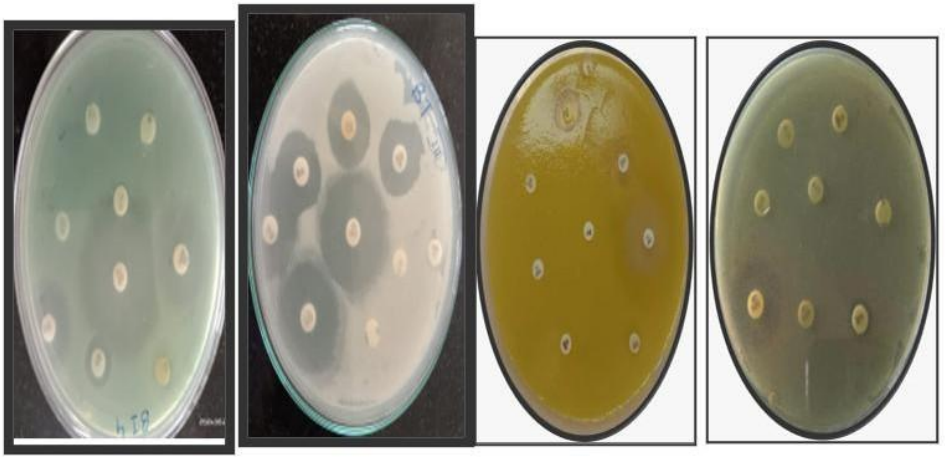

Fig. 1. Antimicrobial assay of isolate (a) BI4, (b) BT3, (c) HMR2 and (d) TD7. Size of zone of inhibition around standard antibiotics represents the sensitive/ resistant nature of the isolates.

The isolate BI4 exhibited resistance to four antibiotics: Clindamycin (10 mg), Ampicillin (10 mg), Tetracycline (30 mg), and Amoxicillin-clavulanic acid (30 mg).

BT3 was found to be resistant to three antibiotics, namely Amoxicillin-clavulanic acid (30 mg), Penicillin (2 mg), and Ampicillin (10 mg).

The HMR2 isolate demonstrated resistance to six antibiotics, including Kanamycin (30 mg), Ampicillin (10 mg), Amoxicillin-clavulanic acid (30 mg), Penicillin (2 mg), Streptomycin (10 mg), and Ciprofloxacin (10 mg).

Among all, TD7 emerged as the most resistant strain, showing resistance to seven antibiotics: Clindamycin (10 mg), Ampicillin (10 mg), Penicillin (2 mg), Streptomycin (10 mg), Tetracycline (30 mg), Amoxicillin-clavulanic acid (30 mg), and Ciprofloxacin (10 mg).

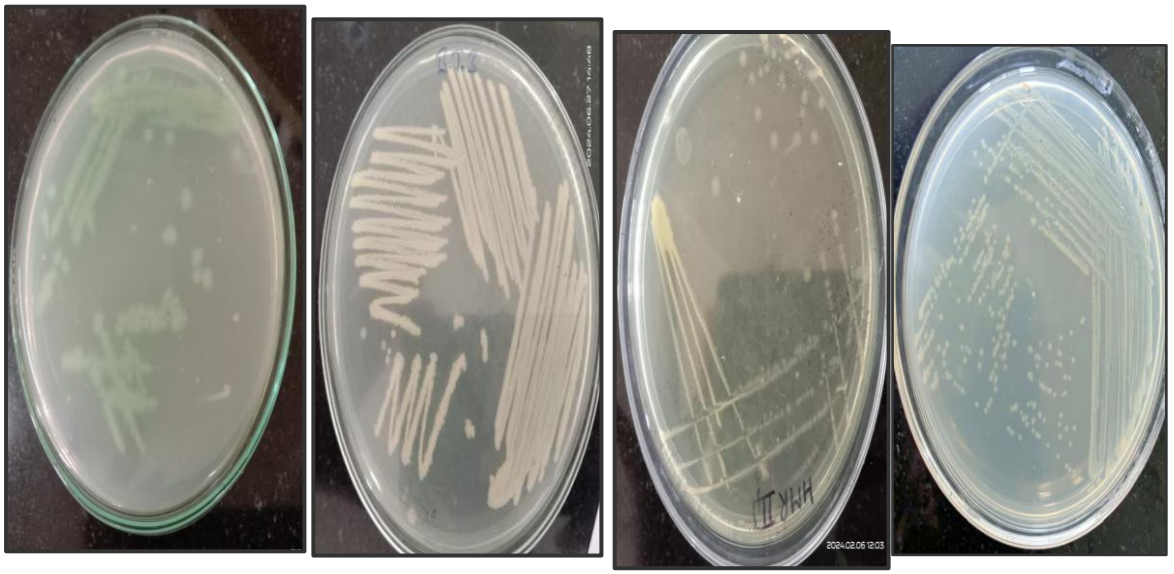

Fig.2. Pure culture of isolate (a) BI4 (*P. aeruginosa*), (b) BT3 (*B. pumilus*), (c) HMR2 (*C. cellulans*) and (d) TD7 (*P. aeruginosa*) on nutrient agar plates

18 fungal isolates were isolated from the five soil samples as shown in table 2. None of the fungal isolates was found to be MDR.

Table 2. Isolation of fungal isolates from different soil samples.

Sr. No.	**Soil samples**	**No of pure isolates**	**No. of MDR isolates**
1	BI	2	0
2	BR	3	0
3	BT	4	0
4	HMR	4	0
5	TD	5	0
Total no. of isolates		**18**	**0**

A series of biochemical tests were conducted to identify the four MDR bacterial isolates (as shown in Table 3). Their identities were further confirmed through 16S rRNA gene sequencing. The combined analysis of morphological features, biochemical test results, and molecular data revealed that isolates BI4, BT3, HMR2, and TD7 corresponded to *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Cellulosimicrobium cellulans*, and *Pseudomonas aeruginosa*, respectively.

Table 3. Microscopic and Biochemical test results of MDR isolates.

Microscopic and Biochemical tests	**MDR isolates**			
	BI4	**BT3**	**HMR2**	**TD7**
Gram's staining	Gram negative Rod	Gram positive Rod	Gram positive Rod	Gram negative Rod
Indole	-	-	+	-
Methyl Red	-	-	+	-
Voges-Proskauer	-	-	-	-
Citrate	+	+	+	+

Catalase	+	+	+	+
Oxidase	+	+	-	+
Lactose fermentation	_	-	+	_

To evaluate the genetic variation and evolutionary relationships among the identified MDR bacterial isolates, their 16S rRNA gene sequences were compared with closely related sequences from the NCBI database using BLAST analysis. The sequences with the highest similarity were selected for further phylogenetic assessment. Multiple sequence alignment was performed to identify conserved and divergent regions among the isolates and reference strains. Using MEGA 11 software, a phylogenetic tree was constructed employing the neighbor-joining method along with bootstrap analysis to ensure statistical reliability. This tree illustrated the evolutionary distances and clustering patterns of the isolates. The analysis confirmed the molecular identification of the strains and highlighted their genetic relatedness to known species (Fig. 3). In the present study, two of the four isolates, BI4 and TD7, were identified as members of the *Pseudomonas* genus. BLAST analysis of their 16S rRNA gene sequences revealed that both isolates showed 100% similarity with the top ten *Pseudomonas aeruginosa* strains. Additionally, isolate HMR2 demonstrated 99.93% similarity with *Cellulosimicrobium cellulans* strains, and BT3 showed a 99.3% match with *Bacillus pumilus* strains. The highly resistant *Pseudomonas* genus is likely to carry multiple virulence factors that contribute to its disease-causing ability, such as adhesins, toxins, and specialized secretion systems (Mikkelsen et al., 2011). *P. aeruginosa* resistant to antibiotics is a well-known pathogen in healthcare environments, recognized for its ability to persist through mechanisms such as biofilm development, active efflux pump systems, and quick adaptation via acquisition of resistance genes (Kunz et al., 2022). An unusual multidrug-resistant isolate, *Cellulosimicrobium cellulans* (HMR2), was recovered from a hospital waste disposal soil sample (HMR). Members of the *Cellulosimicrobium* genus are Gram-positive, filamentous bacteria typically found in environments such as soil and sewage. These species are generally considered non-pathogenic and are known for their capabilities in heavy metal tolerance or reduction, oil degradation, and production of various enzymes (Bhati et al., 2019; Bertel-Sevilla et al., 2019; Naeem et al., 2013; Ferracini-Santos and Sato, 2009).

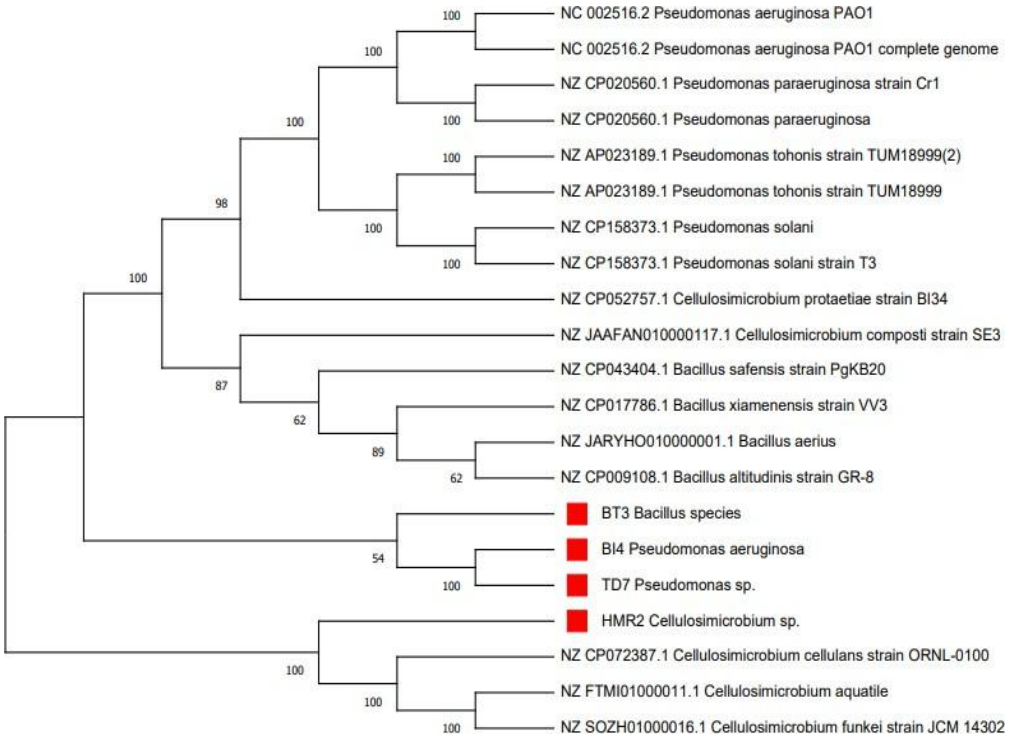

Fig. 3 Phylogenetic tree analysis of 16S rRNA gene of isolates *P. aeruginosa* (BI4), *B. pumilus* (BT3), *C. cellulans* (HMR2) and *P. aeruginosa* (TD7) using neighbor-joining method.

All four bacterial isolates demonstrated resistance to more than three antibiotics, confirming their classification as MDR strains. Among them, isolate **TD7** (*P. aeruginosa*) was the most resistant, showing complete resistance to **seven antibiotics**: Clindamycin (10 mg), Ampicillin (10 mg), Penicillin (2 mg), Streptomycin (10 mg), Tetracycline (30 mg), Amoxicillin-

clavulanic acid (30 mg), and Ciprofloxacin (10 mg). Similarly, **BI4**, also identified as *P. aeruginosa*, exhibited resistance to **four antibiotics**, namely Clindamycin, Ampicillin, Tetracycline, and Amoxicillin-clavulanic acid. The high resistance levels in *Pseudomonas aeruginosa* observed in this study are consistent with previous findings by Mahmud et al. (2024) and Aggarwal et al. (2015), who reported comparable multidrug resistance patterns in *Pseudomonas* strains isolated from clinical and industrial settings.

The isolate **BT3**, identified as *B. pumilus*, showed resistance to **three antibiotics**: Amoxicillin-clavulanic acid, Penicillin, and Ampicillin. In contrast, **HMR2** (*C. cellulans*) was resistant to **six antibiotics**; Kanamycin, Ampicillin, Amoxicillin-clavulanic acid, Penicillin, Streptomycin, and Ciprofloxacin. The ability of *C. cellulans* to resist multiple antibiotics could be linked to its environmental adaptability and previously reported resilience to toxic substances including heavy metals and pollutants (Bhati et al., 2019; Naeem et al., 2013).

Notably, in the current study, **most isolates were resistant to common antibiotics such as Amoxicillin, Ampicillin,** indicating a shared resistance mechanism across different genera. Similar resistance profiles were observed by Naik and Deepak (2016), who reported MDR strains of *E. coli*, *Pseudomonas spp.*, *Enterobacter spp.*, and *Staphylococcus aureus* from pharmaceutical effluents, showing high resistance to antibiotics including amoxicillin, cefotaxime, and penicillin.

The widespread presence of MDR bacteria in environmental sources such as hospital and pharmaceutical waste sites highlights the serious risk posed by improper disposal of antibiotics and waste materials. Studies by Egbenyah et al. (2021) and Al-Bahry et al. (2014) have shown that such sites serve as reservoirs for resistant bacteria including *Bacillus spp.*, *Klebsiella spp.*, *Pseudomonas spp.*, and *Escherichia coli*, all of which contribute to the dissemination of resistance genes in the environment.

Environmental exposure to sub-lethal concentrations of antibiotics and residual contaminants creates a selection pressure that enhances the horizontal transfer of resistance genes, facilitating the development of new MDR strains (Michaelis & Grohmann, 2023). This mechanism not only compromises the efficacy of existing treatments but also promotes the emergence of highly resilient pathogens in both clinical and non-clinical ecosystems.

4. CONCLUSION

Microorganisms that do not directly cause infections can still play a major role in spreading antibiotic resistance to harmful bacteria, posing a serious threat to public health. Antibiotics and resistant microbes often enter the environment, especially through waste from hospitals and pharmaceutical industries, worsening the problem. These non-pathogenic microbes serve as reservoirs for resistance genes and can transfer them to disease-causing bacteria through horizontal gene transfer, making infections harder to treat. Poor disposal practices contribute to environmental contamination, allowing resistant bacteria to thrive and evolve in natural settings like water, soil, and crops. This not only increases the risk of multidrug-resistant infections but also threatens ecosystem and human health. To combat this, strict waste management systems, advanced treatment methods, and strong regulatory frameworks must be implemented to limit the environmental spread of resistance genes.

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Conflict of interest

There is no conflict of interest between the authors, all authors contributed directly to the article.

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