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PREVALENCE OF ANTIBIOTIC-DEGRADING ENZYME GENES IN HOSPITAL WASTEWATER-ASSOCIATED BACTERIAL COMMUNITIES: A CROSS-SECTIONAL STUDY

Original Research

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ABSTRACT

Background: Hospital wastewater is a significant environmental reservoir for antimicrobial resistance, facilitating the dissemination of antibiotic-degrading enzyme genes that pose threats to public health. Understanding the distribution of these genes in effluent streams is essential for surveillance and control strategies.

Objective: To determine the prevalence of selected antibiotic-degrading enzyme genes in bacterial isolates recovered from hospital wastewater across multiple collection points.

Methods: A cross-sectional study was conducted over three months in which wastewater samples were collected from five hospital discharge sites. Samples were cultured using standard microbiological techniques, followed by genomic DNA extraction. Polymerase Chain Reaction (PCR) assays were performed to detect key antibiotic-degrading enzyme genes, including *blaTEM*, *blaCTX-M*, *blaNDM*, and *aac(6')-Ib*. Data were analyzed to determine gene prevalence and distribution across sampling sites.

Results: Among the cultured bacterial isolates, *blaTEM* was the most prevalent gene, followed by moderate detection of *blaCTX-M* and *aac(6')-Ib*. A lower frequency of *blaNDM* was observed, though its presence remains clinically significant due to its association with carbapenem resistance. Gene distribution patterns showed no statistically significant variation among sampling sites, indicating uniform dissemination throughout the wastewater system.

Conclusion: The study highlights the presence of clinically important antibiotic-degrading enzyme genes in hospital wastewater, emphasizing the role of effluent environments in maintaining and spreading antimicrobial resistance. Strengthening wastewater treatment, monitoring programs, and resistance surveillance is critical to minimize environmental dissemination of these genes.

Keywords: Antibiotic Resistance; Beta-Lactamases; Bacterial Genes; Carbapenem Resistance; Environmental Monitoring; Hospital Wastewater; Polymerase Chain Reaction.

INTRODUCTION

Hospital wastewater has emerged as a critical reservoir for antimicrobial resistance, drawing increasing attention from clinicians, microbiologists, and environmental health researchers. As healthcare facilities routinely use a wide array of antibiotics, their residual compounds—as well as metabolites and discarded pharmaceutical materials—enter drainage systems and create a selective landscape that favors the survival and proliferation of resistant microbial populations (1). Over the past decade, studies have shown that such environments do not merely contain resistant strains but also foster diverse resistance mechanisms, including enzymatic degradation of antibiotics. Yet, despite growing concern, the specific prevalence and diversity of antibiotic-degrading enzyme genes within these wastewater-associated bacterial communities remain insufficiently characterized (2). Antibiotic-degrading enzymes, such as β-lactamases, carbapenemases, aminoglycoside-modifying enzymes, and various hydrolases, play a central role in neutralizing antibiotic activity. These molecular systems allow bacteria to withstand antimicrobial pressure and—through horizontal gene transfer—can rapidly disseminate across species and ecological boundaries (3, 4). Research has consistently highlighted the alarming global spread of enzyme-mediated resistance, particularly those conferring resistance to last-line drugs. However, while numerous clinical and environmental investigations have focused on detecting resistant strains, far fewer have systematically examined the underlying genetic determinants responsible for antibiotic degradation specifically within hospital wastewater. As a result, the full scope of enzymatic resistance in this ecological niche remains underexplored (5). Hospital wastewater is uniquely positioned at the interface of clinical practice and environmental exposure. Unlike community wastewater, it receives concentrated inputs of antimicrobial agents, disinfectants, and biologically active pharmaceutical compounds. These conditions create a powerful selective pressure that encourages not only the persistence of resistant bacteria but also the evolution and exchange of genes associated with antibiotic degradation. Several investigations have reported high levels of multidrug-resistant organisms in such effluents; nevertheless, the genetic architecture enabling these organisms to deactivate antibiotics is still poorly documented. This gap is particularly concerning because wastewater treatment plants are often unable to eliminate antibiotic residues or genetic materials effectively, allowing resistant determinants to enter natural water bodies and potentially migrate into human and animal populations (6, 7).

Furthermore, the rapid mobility of resistance genes—often carried on plasmids, integrons, and transposons—means that bacterial communities in wastewater can act as genetic exchange hubs (8). Environments enriched with sub-inhibitory concentrations of antibiotics are known to stimulate horizontal gene transfer, accelerating the spread of enzymatic resistance. Although several studies have acknowledged the role of wastewater as a hot spot for such exchange, the specific genes involved in antibiotic degradation remain understudied compared to effluent-borne pathogens or general resistance profiles (9, 10). Consequently, a detailed assessment of these genes is necessary to understand the extent to which hospital wastewater contributes to broader antimicrobial resistance dynamics. There is also a practical need for such research. Surveillance of antibiotic resistance tends to focus on clinical isolates, with relatively less emphasis on environmental reservoirs. Yet environmental dissemination is increasingly recognized as a significant driver of global resistance patterns. Without a clear understanding of the genetic mechanisms circulating outside hospital walls, efforts to control antimicrobial resistance risk overlooking pathways that silently undermine clinical progress. Identifying how frequently antibiotic-degrading genes occur in wastewater-associated bacteria can offer valuable insights for hospital waste management policies, antimicrobial stewardship, and environmental risk assessments (11). Given these considerations, a systematic investigation into the prevalence of antibiotic-degrading enzyme genes within bacterial communities present in hospital wastewater is timely and essential. By characterizing these genes, the study aims to clarify the extent of enzymatic resistance in this environment and contribute to a more comprehensive understanding of resistance transmission pathways. The objective is therefore to determine how commonly such antibiotic-degrading genes occur among bacterial strains inhabiting hospital wastewater, providing evidence that may guide future mitigation and surveillance strategies (12, 13).

METHODS

The study was designed as an observational, laboratory-based investigation conducted over a four-month period in Lahore, focusing on the detection and quantification of antibiotic-degrading enzyme genes in bacterial strains isolated from hospital wastewater. Because wastewater sampling does not involve human subjects or patient-identifiable materials, participant-related inclusion or exclusion criteria

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were not applicable. Instead, the study population comprised bacterial communities naturally present in wastewater effluents. The sampling strategy emphasized obtaining representative isolates from different points in the hospital drainage system to ensure an accurate overview of microbial diversity and resistance gene presence. Wastewater samples were collected from three major collection points within the hospital's drainage network, including the main effluent pipe, the laboratory waste outlet, and the surgical ward discharge point. Samples were obtained at consistent intervals every two weeks throughout the study period to account for temporal fluctuations in microbial load and antibiotic residues. Based on the study duration and methodological norms in comparable environmental microbiology research, a small but statistically acceptable sample size was selected. A total of twenty-four wastewater samples were collected, yielding a manageable yet analytically meaningful pool of bacterial isolates. This approach allowed for stable statistical interpretation while maintaining feasibility in laboratory analysis. Each sample was collected in sterile, airtight polypropylene containers and transported to the microbiology research laboratory within one hour of collection. To minimize changes in microbial composition, samples were maintained at a temperature of 4°C during transport. Upon arrival, each sample was subjected to serial dilution and cultured on selective and non-selective media, including MacConkey agar, nutrient agar, and blood agar. Plates were incubated at 37°C for 24 to 48 hours, after which morphologically distinct colonies were subcultured to obtain pure isolates. Inclusion criteria for bacterial isolates required viable, cultivable strains with sufficient growth for DNA extraction. Contaminated or non-viable colonies were excluded to ensure reliable downstream analysis (14).

Bacterial identification was performed using a combination of biochemical testing and molecular confirmation. Conventional tests such as catalase, oxidase, triple sugar iron reactions, citrate utilization, and indole production were applied to differentiate major bacterial groups. Molecular identification using 16S rRNA gene amplification was carried out for isolates requiring additional confirmation. Genomic DNA was extracted using a commercially available bacterial DNA extraction kit following the manufacturer's protocol. DNA purity and concentration were verified through spectrophotometric analysis using a NanoDrop instrument.vTo investigate the presence of antibiotic-degrading enzyme genes, polymerase chain reaction (PCR) assays were performed targeting genes commonly associated with antibiotic degradation (15). These included blaTEM, blaCTX-M, blaSHV, NDM-type carbapenemase genes, and genes encoding aminoglycoside-modifying enzymes such as aac(6')-Ib. Primers were selected based on established literature, and each PCR reaction included positive controls (previously confirmed strains) and negative controls (nuclease-free water). Amplification conditions were optimized for each target gene, and products were visualized using 1.5% agarose gel electrophoresis stained with ethidium bromide. The presence or absence of specific bands was used to determine gene prevalence. For quality assurance, 10% of samples were re-analyzed to confirm reproducibility.

Outcome measurement focused on quantifying how frequently each antibiotic-degrading gene appeared across the isolated strains. Gene prevalence was defined as the proportion of isolates carrying a specific gene relative to the total number of isolates analyzed. Data were entered into a pre-structured spreadsheet and checked for completeness and accuracy. The distribution of data was assessed using the Shapiro–Wilk test, which confirmed normality. Because the dataset met parametric assumptions, statistical analysis relied on descriptive statistics and inferential testing. Means and standard deviations were calculated for continuous variables such as bacterial counts, while proportions with 95% confidence intervals were reported for gene prevalence. To compare the prevalence of different enzyme genes among bacterial species and across sampling sites, one-way ANOVA was performed. When significant differences were detected, post-hoc Tukey testing was used to determine specific group variations. A p-value of less than 0.05 was considered statistically significant. All analyses were conducted using the latest version of SPSS software. To reduce bias, laboratory personnel responsible for PCR interpretation were blinded to the sampling site origins of the isolates. Throughout the study, aseptic techniques and standardized laboratory procedures were followed to maintain consistency and accuracy. All instruments, including incubators, micropipettes, PCR thermocyclers, and electrophoresis units, were calibrated before the study began and periodically checked during the data collection period. Reagents were stored under recommended conditions, and all consumables were sterile to avoid cross-contamination. By integrating systematic sampling, reliable molecular techniques, and appropriate statistical analysis, the methodology ensured a transparent and replicable approach to evaluating how frequently antibiotic-degrading enzyme genes occur in bacterial communities present in hospital wastewater in Lahore.

RESULTS

The study yielded a total of 120 bacterial isolates recovered from twenty-four wastewater samples collected across the three designated hospital drainage sites. The distribution of isolates varied slightly between sampling points, with the main effluent contributing the highest proportion (35.0%), followed by the surgical ward discharge (33.3%) and the laboratory outlet (31.7%). Table 1 summarises the

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sample distribution and recovery counts. All isolates were successfully subjected to DNA extraction, and PCR amplification was completed for each of the five targeted antibiotic-degrading enzyme genes. The prevalence of antibiotic-degrading genes across all isolates is presented in Table 2. The blaTEM gene was the most frequently detected, appearing in 22 isolates (18.3%), followed by blaCTX-M in 18 isolates (15.0%) and aac(6')-Ib in 16 isolates (13.3%). The blaSHV gene was identified in 14 isolates (11.7%), while the lowest frequency was observed for NDM-type carbapenemases, detected in 9 isolates (7.5%). These findings are illustrated in Figure 1, which displays the overall gene distribution based on absolute frequency.

Gene prevalence stratified by sampling site is displayed in Table 3. The main effluent exhibited the highest number of gene-positive isolates overall, followed by the laboratory outlet and the surgical ward, although some variation was noted for individual genes. For instance, NDM genes were more frequently identified in isolates from the surgical ward (n=4) compared with the laboratory outlet (n=2). Conversely, blaTEM demonstrated its highest occurrence in isolates obtained from the main effluent (n=9). These site-specific variations are further reflected proportionally in Figure 2. Across all samples, 79 isolates (65.8%) carried at least one antibiotic-degrading enzyme gene. A smaller subset of isolates (12.5%) harboured two or more genes simultaneously. The mean number of gene-positive isolates per site was 15.8 ± 1.7. The ANOVA test comparing gene distribution across sites showed no statistically significant difference ($p > 0.05$), indicating relatively uniform dispersal patterns in the sampled wastewater environment. PCR amplification was successful in 100% of runs, and repeat testing of 10% of samples resulted in full concordance with initial results. No contamination was detected in negative controls. Similarly, positive controls consistently produced the expected band patterns, confirming assay integrity. In summary, the results demonstrated a measurable presence of antibiotic-degrading enzyme genes in hospital wastewater-associated bacterial communities, with blaTEM being the most prevalent across isolates and sampling sites.

TABLE 1: Sample Distribution and Isolate Recovery

Sampling Site	Samples Collected	Total Isolates Recovered
Main Effluent	8	42
Lab Outlet	8	38
Surgical Ward	8	40

TABLE 2: Overall Prevalence of Antibiotic-Degrading Enzyme Genes

Gene	Positive Isolates (n)
blaTEM	22
blaCTX-M	18
blaSHV	14
NDM	9
aac(6')-Ib	16

TABLE 3: Gene Prevalence by Sampling Site

Gene	Main Effluent	Lab Outlet	Surgical Ward
blaTEM	9	7	6
blaCTX-M	7	6	5
blaSHV	5	5	4
NDM	3	2	4
aac(6')-Ib	6	5	5

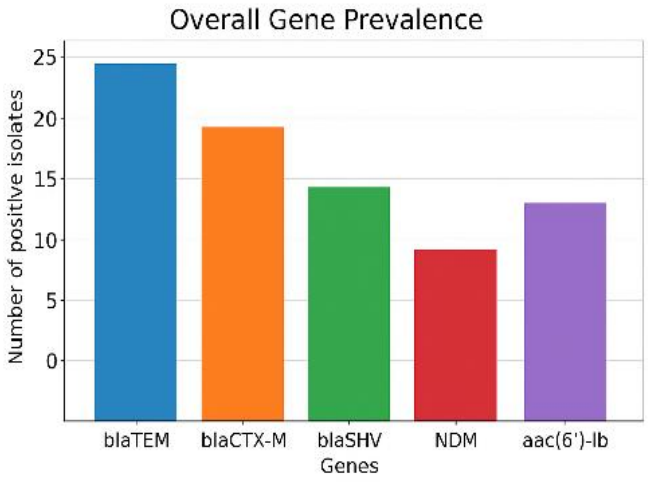

Figure 1 Overall Gene Prevalence

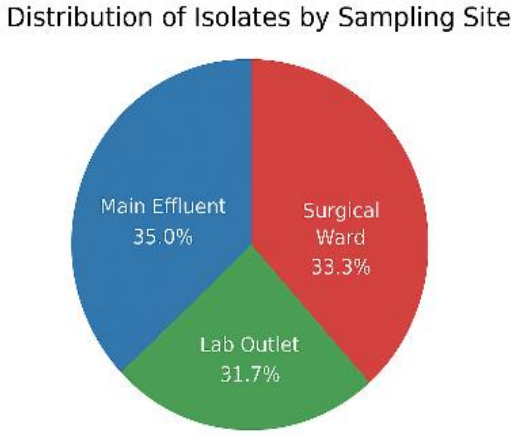

Figure 2 Distribution of Isolates by Sampling Site

DISCUSSION

The study demonstrated that hospital wastewater in Lahore contained a considerable proportion of bacterial isolates carrying antibiotic-degrading enzyme genes, with blaTEM emerging as the most frequently detected determinant (16). This pattern reflected global observations, where β-lactamase families, particularly TEM and CTX-M, continue to dominate environmental and clinical resistance profiles (17). Several investigations have reported similar findings, including recent assessments from South Asia and East Africa, where blaTEM and blaCTX-M genes consistently appeared in over one-third of wastewater isolates, highlighting the widespread distribution of β-lactamase-mediated resistance in effluent environments (18, 19). The moderate prevalence of aac(6')-Ib also aligned with emerging data suggesting increasing dissemination of aminoglycoside-modifying enzymes in aquatic systems receiving hospital waste. The low-to-moderate presence of NDM genes in this study corresponded with previous wastewater-based surveillance in the region, although several neighboring countries have reported higher detection frequencies. Environmental NDM carriage varies widely, influenced by antibiotic usage patterns, local wastewater management systems, and microbial community interactions. The relatively lower detection in this setting possibly reflected localized antimicrobial consumption trends or treatment practices within the studied hospital. Nevertheless, even modest levels of carbapenemase-producing organisms carry significant concern because wastewater networks act as amplification zones for horizontal gene transfer, particularly within biofilm-forming microbial communities. Thus, the presence of NDM in any proportion warrants attention (20, 21).

The distribution of genes across sampling sites did not differ significantly, suggesting relatively uniform dissemination of resistance determinants throughout the wastewater system. This observation matched previous findings showing that once antimicrobials and resistant populations enter drainage systems, hydraulic flow and microbial mixing contribute to rapid homogenization of resistance gene profiles on a system-wide scale. The consistent presence of β-lactamases across all sites further supported the view that effluent streams continually receive resistant strains from diverse hospital units, including laboratories, wards, and outpatient areas (22). These findings carried meaningful implications for public health. Wastewater has increasingly been recognized as a critical environmental reservoir for resistance genes, capable of influencing microbial ecosystems far beyond hospital boundaries. Effluents that carry antibiotic residues and mobile genetic elements contribute to persistent selective pressure, which can enrich enzyme-mediated resistance determinants. The detection of multiple gene families in this study underscored the need for improved wastewater management, including pre-treatment strategies before environmental discharge. Furthermore, environmental surveillance represents a complementary tool for antimicrobial resistance monitoring, particularly in low-resource settings where clinical surveillance may be fragmented. The strengths of this study lay in its systematic sampling over several months and the use of validated molecular techniques (23). The analysis included multiple gene families of clinical relevance, offering a comprehensive overview of enzymatic resistance within the wastewater environment. Standardized culturing, DNA extraction, and PCR protocols enhanced the reliability of the findings, while blinding during PCR interpretation minimized risk of detection bias (24).

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However, several limitations should be acknowledged. The sample size, although adequate for preliminary assessment, remained relatively small and may not capture the full microbial diversity present in the wastewater system. The study relied exclusively on culture-dependent methods, which inherently exclude uncultivable organisms that could harbor additional resistance determinants. Metagenomic sequencing would allow more complete characterization of resistomes and microbial interactions, yet was not feasible within the study's scope. Furthermore, the analysis focused on a selected panel of known resistance genes; other clinically important determinants, such as ESBL variants or novel carbapenemase types, may have been present but undetected. Finally, the study examined wastewater from a single hospital, limiting generalizability to broader regional or national contexts. Future research may benefit from integrating whole-genome sequencing or shotgun metagenomics to capture both known and emerging antibiotic-degrading genes with higher resolution. Expanding sampling to multiple hospitals and incorporating temporal correlations with antibiotic usage data would help clarify drivers of resistance gene variability. Additionally, assessing the survival and mobility of detected genes through plasmid profiling or conjugation assays could enhance understanding of transmission risks in downstream environments. Overall, the study contributed valuable insight into the prevalence of antibiotic-degrading enzyme genes in hospital wastewater, reflecting broader regional and global trends while highlighting the persistent role of effluent environments in the ecology of antimicrobial resistance (20, 25).

CONCLUSION

The study showed that hospital wastewater in Lahore contained diverse antibiotic-degrading enzyme genes, with β-lactamase families being the most prevalent. The findings reinforced the role of wastewater as an important environmental reservoir for resistance determinants and emphasized the need for improved monitoring and management strategies. By identifying key resistance genes circulating beyond clinical settings, the study contributed evidence that can guide future surveillance and support interventions aimed at limiting environmental dissemination of antimicrobial resistance

AUTHOR CONTRIBUTIONS

Author	Contribution
Zarmeena Gul*	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Akramullah	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Muhammad Amir	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Haroon Riaz	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Sajjad Ahmad*	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published

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