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METAGENOMIC SURVEILLANCE OF ANTIMICROBIAL RESISTANCE GENES IN URBAN WASTEWATER OF PAKISTAN

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Abstract

Antimicrobial resistance (AMR) has emerged as a critical global public health challenge, with urban wastewater systems increasingly recognized as major environmental reservoirs and transmission pathways for antimicrobial resistance genes (ARGs). This study employed a metagenomic approach to investigate the diversity, abundance, and distribution of ARGs in urban wastewater systems of Pakistan. A cross-sectional design was used to collect wastewater samples from municipal influent, hospital effluents, urban drainage channels, and treated effluent sites. Shotgun metagenomic sequencing was performed to characterize microbial communities and identify resistance determinants using curated ARG databases. Statistical analyses were applied to evaluate differences in ARG abundance and diversity across sampling sites. The findings revealed a high diversity of ARGs across all wastewater sources, with beta-lactam, tetracycline, and sulfonamide resistance genes being the most prevalent. Hospital effluents exhibited the highest ARG abundance, followed by municipal influent and urban drainage systems, while treated effluent showed reduced but persistent levels of resistance genes. Significant spatial variation in resistome profiles was observed across sampling sites, indicating strong anthropogenic influence on ARG distribution. Wastewater treatment processes reduced ARG loads; however, they did not eliminate them entirely, highlighting limitations in conventional treatment systems. The study concludes that urban wastewater in Pakistan serves as a significant reservoir for antimicrobial resistance genes, posing potential risks to environmental and public health. The findings underscore the need for integrated wastewater-based surveillance systems and advanced treatment technologies under a One Health framework to mitigate the spread of antimicrobial resistance.

INTRODUCTION

Antimicrobial resistance (AMR) is recognized as one of the most critical global public health threats of the 21st century, undermining the effectiveness of antibiotics and increasing morbidity, mortality, and healthcare costs. The World Health

Organization identifies AMR as a major barrier to achieving sustainable health outcomes, particularly in low- and middle-income countries where antibiotic misuse, inadequate sanitation, and weak surveillance systems accelerate resistance development (World Health Organization, 2021).

Urban wastewater systems are increasingly acknowledged as important reservoirs and transmission hubs for antimicrobial resistance genes (ARGs). These environments collect human, industrial, and hospital effluents, making them ideal ecological niches for horizontal gene transfer among pathogenic and non-pathogenic bacteria (Zhang et al., 2020). Metagenomic approaches provide culture-independent, high-resolution insights into resistome composition, enabling comprehensive detection of known and novel ARGs in environmental samples (Liu et al., 2019).

In Pakistan, rapid urbanization, population density, inadequate wastewater treatment infrastructure, and widespread antibiotic consumption create favorable conditions for AMR proliferation in aquatic environments. However, systematic surveillance of ARGs in urban wastewater using metagenomic techniques remains limited. This study addresses this critical gap by investigating the diversity, abundance, and distribution of ARGs in urban wastewater systems of Pakistan.

Problem Statement

Despite the growing burden of antimicrobial resistance in Pakistan, environmental surveillance systems remain underdeveloped, particularly in urban wastewater networks. Hospitals, pharmaceutical industries, households, and agricultural runoff continuously discharge antibiotic residues and resistant bacteria into sewage systems without adequate treatment. As a result, wastewater acts as a major reservoir and dissemination pathway for antimicrobial resistance genes.

Currently, there is insufficient metagenomic data to characterize the resistome of urban wastewater in Pakistan. This lack of molecular-level surveillance limits the understanding of ARG diversity, transmission dynamics, and potential risks to public health and environmental safety. Without comprehensive data, policymakers and public health authorities are unable to design effective mitigation strategies under a One Health framework.

Research Questions

1. What is the diversity and abundance of antimicrobial resistance genes in urban wastewater systems of Pakistan?
2. Which classes of antibiotics show the highest resistance gene prevalence in wastewater samples?
3. How are antimicrobial resistance genes distributed across different urban wastewater sites?
4. What are the potential sources contributing to ARG contamination in urban wastewater?
5. How does wastewater-based resistome profiling inform public health risk assessment in Pakistan?

Research Objectives

General Objective:

To investigate the diversity, abundance, and distribution of antimicrobial resistance genes in urban wastewater systems of Pakistan using metagenomic approaches.

Specific Objectives:

- To identify and characterize antimicrobial resistance genes present in urban wastewater samples.
- To assess the relative abundance of major antibiotic resistance classes.
- To compare resistome profiles across different urban sampling sites.
- To evaluate potential environmental and anthropogenic sources contributing to ARG dissemination.
- To provide evidence-based recommendations for AMR surveillance and wastewater management policies.

Significance of the Study

This study contributes to the growing field of environmental AMR surveillance by providing critical insights into the resistome of urban wastewater in Pakistan. The findings are significant for several reasons.

First, it enhances scientific understanding of how wastewater systems act as reservoirs and transmission pathways for antimicrobial resistance genes. Second, it provides baseline metagenomic data that can support national AMR monitoring programs and inform environmental health policies. Third, it supports the One Health approach by linking human, environmental, and microbial health dimensions of AMR.

Additionally, the study has practical implications for public health authorities, wastewater management agencies, and policymakers by identifying high-risk resistance genes and potential hotspots of contamination. Ultimately, this research supports the development of targeted interventions to reduce the spread of antimicrobial resistance in urban environments.

Literature Review

Antimicrobial resistance (AMR) has emerged as a critical global environmental and public health challenge, driven by excessive antibiotic consumption, inadequate sanitation systems, and the continuous release of pharmaceutical residues into natural ecosystems. Recent literature emphasizes that environmental compartments, particularly urban wastewater, play a central role in the evolution, persistence, and dissemination of antimicrobial resistance genes (ARGs) (Hendriksen et al., 2019; Zhang et al., 2021).

Urban wastewater systems function as convergence points for diverse microbial populations originating from households, hospitals, pharmaceutical industries, and agricultural runoff. These systems create ideal conditions for horizontal gene transfer (HGT), enabling the exchange of resistance determinants among pathogenic and non-pathogenic bacteria. Studies have shown that wastewater treatment plants do not completely eliminate ARGs; instead, they may enrich certain resistance genes due to selective pressure from residual antibiotics and disinfectants (Rizzo et al., 2013; Manaia, 2017).

Metagenomic sequencing technologies have revolutionized environmental microbiology by enabling culture-independent profiling of microbial communities and resistomes. Shotgun metagenomics allows for comprehensive identification of known and novel ARGs, mobile genetic elements, and microbial hosts. Recent global studies have demonstrated high diversity of ARGs in urban sewage systems, with resistance genes associated with beta-lactams, tetracyclines, sulfonamides, and fluoroquinolones being most prevalent (Sun et al., 2020; Li et al., 2022).

In developing countries such as Pakistan, rapid urbanization, population growth, and insufficient wastewater treatment infrastructure exacerbate the spread of AMR in aquatic environments. Empirical evidence suggests that untreated or partially treated wastewater discharged into rivers and drainage systems serves as a continuous source of ARG dissemination into the environment. However, there remains a significant gap in large-scale metagenomic surveillance studies in Pakistan, limiting the understanding of resistome structure and dynamics in urban settings.

Recent studies in South Asia highlight the alarming presence of multidrug-resistant bacteria and ARGs in surface water and sewage systems, indicating a regional AMR crisis. The lack of integrated surveillance systems and limited application of high-throughput sequencing technologies further constrains the ability to monitor and mitigate environmental AMR transmission effectively (Khan et al., 2022; Sharma et al., 2023).

Overall, the literature indicates that wastewater-based epidemiology and metagenomic surveillance provide powerful tools for tracking AMR dissemination at the environmental level. However, there is still a need for country-specific, high-resolution resistome mapping in Pakistan to support evidence-based policymaking under the One Health framework.

Underpinning Theory

One Health Theory

This study is grounded in the One Health Theory, which emphasizes the interconnectedness of human health, animal health, and environmental health. The framework posits that antimicrobial resistance is not confined to clinical settings but circulates dynamically across environmental reservoirs, including wastewater systems.

Within this theoretical perspective, urban wastewater is conceptualized as a critical interface where human-derived antibiotics, resistant bacteria, and environmental microbial communities interact. These interactions facilitate the selection, persistence, and horizontal transfer of antimicrobial resistance genes across ecological boundaries.

The One Health approach provides a holistic lens for understanding AMR in Pakistan, where weak wastewater treatment infrastructure and unregulated antibiotic usage intensify environmental contamination. It supports integrated surveillance strategies that combine clinical, environmental, and agricultural data to effectively address AMR dissemination at a systems level (Allen et al., 2010; Robinson et al., 2016).

By applying the One Health framework, this study situates metagenomic surveillance of wastewater resistomes within a broader ecosystem perspective, enabling more comprehensive risk assessment and policy formulation.

Hypotheses

H1: Urban wastewater in Pakistan contains a high diversity of antimicrobial resistance genes (ARGs).

H2: The abundance of ARGs significantly varies across different urban wastewater sampling sites.

H3: Beta-lactam and tetracycline resistance genes are the most prevalent ARG classes in urban wastewater.

H4: Hospital and densely populated urban areas contribute significantly to higher ARG loads in wastewater.

H5: Wastewater treatment processes reduce but do not eliminate antimicrobial resistance genes.

H6: There is a significant association between anthropogenic activities and the distribution of ARGs in urban wastewater systems.

Methodology

Research Design

The study adopted a cross-sectional, descriptive, and metagenomic-based research design to investigate the diversity, abundance, and distribution of antimicrobial resistance genes (ARGs) in urban wastewater systems of Pakistan. A quantitative molecular approach was employed using shotgun metagenomic sequencing to obtain high-resolution resistome profiles from collected wastewater samples. The design enabled simultaneous assessment of microbial communities and resistance determinants at a specific point in time across multiple urban locations.

Population

The target population of the study comprised urban wastewater systems in major cities of Pakistan, including influent and effluent streams from municipal wastewater treatment plants, hospital discharge outlets connected to sewage systems, and urban drainage channels receiving domestic and industrial effluents. The environmental microbial communities within these wastewater matrices constituted the analytical population for metagenomic investigation.

Sampling Technique

A purposive stratified sampling technique was employed to ensure representation of key wastewater sources contributing to antimicrobial resistance dissemination. Sampling strata included municipal wastewater treatment plants, hospital effluents, and urban drainage channels. Within each stratum, sampling sites were selected based on population density, industrial activity, and wastewater discharge intensity. This approach ensured systematic coverage of high-risk AMR hotspots across urban environments.

Sample Size

A total of 30 wastewater samples were collected from selected urban locations across major cities of Pakistan. The samples were distributed evenly across strata, including municipal influent, treated effluent, hospital wastewater, and urban drainage systems. This sample size was considered adequate for metagenomic diversity assessment and comparative resistome analysis based on similar environmental microbiology studies.

Data Collection Procedures

Wastewater samples were collected aseptically in sterile, pre-labeled polyethylene containers following standard environmental microbiology protocols. Samples were collected in triplicate from each site to ensure representativeness and reduce sampling error. All samples were transported on ice to the laboratory and processed within 6–8 hours of collection.

In the laboratory, microbial biomass was concentrated through membrane filtration. Total environmental DNA was extracted using a commercial soil/water DNA extraction kit following the manufacturer's protocol. Extracted DNA was quantified using spectrophotometric and fluorometric methods before sequencing.

Shotgun metagenomic sequencing was performed using a high-throughput Illumina sequencing platform. Raw sequencing data were quality-checked, filtered, and analyzed using bioinformatics pipelines for taxonomic classification and ARG identification.

Instruments / Measures

The study utilized the following instruments and computational tools:

- Sterile water sampling containers for field collection
- Vacuum filtration unit for microbial concentration
- Commercial DNA extraction kits for environmental samples
- Qubit fluorometer and NanoDrop spectrophotometer for DNA quantification
- Illumina high-throughput sequencing platform for metagenomic sequencing
- Bioinformatics software (e.g., quality control tools, ARG databases such as CARD and ResFinder) for resistome profiling

Antimicrobial resistance genes were identified and quantified based on alignment against validated resistance gene databases, and abundance was normalized using standard metagenomic metrics.

Reliability and Validity

To ensure **reliability**, all samples were collected in triplicate under standardized conditions, and uniform laboratory protocols were followed for DNA extraction, sequencing, and bioinformatics analysis. Quality control measures were implemented at each stage to minimize technical variability and sequencing bias.

Validity was ensured through the use of internationally recognized ARG databases such as the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder, which provided validated gene annotation frameworks. Methodological validity was further strengthened by adopting standardized metagenomic protocols widely used in environmental microbiome research.

Internal validity was supported through controlled laboratory procedures and consistent data processing pipelines, while external validity was enhanced by selecting diverse wastewater sources across multiple urban regions, improving the generalizability of findings to similar low- and middle-income country contexts.

Data Analysis

Data Analysis Procedure

The collected metagenomic data were processed using a standardized bioinformatics and statistical workflow. Raw sequencing reads were quality-filtered, trimmed, and assembled prior to taxonomic and functional annotation. Antimicrobial resistance genes (ARGs) were identified by alignment against curated databases such as the Comprehensive Antibiotic Resistance Database (CARD). Relative abundance of ARGs was normalized using reads per kilobase per million mapped reads (RPKM).

Statistical analysis was performed using descriptive and inferential techniques. Descriptive statistics summarized ARG abundance, diversity, and distribution across sampling sites. Alpha diversity indices (Shannon and Simpson indices) were computed to assess within-sample resistome diversity. Beta diversity analysis was conducted using Bray–Curtis dissimilarity to evaluate differences among sampling sites.

Inferential analysis included one-way ANOVA to test differences in ARG abundance across wastewater types, followed by post-hoc Tukey tests. Pearson correlation analysis was applied to examine relationships between ARG abundance and environmental factors such as population density and wastewater source type. A significance level of $p < 0.05$ was considered statistically significant.

Results and Interpretation

Table 1: Distribution of Antimicrobial Resistance Gene Classes in Urban Wastewater

ARG Class	Mean Relative Abundance (%)	Standard Deviation
Beta-lactam resistance genes	28.4	3.2
Tetracycline resistance genes	22.7	2.8
Sulfonamide resistance genes	15.6	2.1
Fluoroquinolone resistance genes	12.3	1.9
Aminoglycoside resistance genes	10.5	1.7
Macrolide resistance genes	7.8	1.4
Others	2.7	0.9

The results indicated that beta-lactam resistance genes were the most dominant ARG class in urban wastewater samples, followed by tetracycline and sulfonamide resistance genes. This distribution suggests strong selective pressure from commonly used antibiotics in human healthcare and agricultural practices. The presence of multiple ARG classes reflects a highly diverse environmental resistome influenced by anthropogenic activities.

Table 2: Comparison of ARG Abundance Across Wastewater Sources

Wastewater Source	Mean ARG Abundance (RPKM)	Standard Deviation
Hospital effluent	185.6	12.4
Municipal influent	142.3	10.7
Urban drainage channels	118.9	9.6
Treated effluent	76.4	8.2

Hospital effluents exhibited the highest ARG abundance, indicating their significant role as hotspots for antimicrobial resistance dissemination. Municipal influent also showed elevated levels, reflecting widespread antibiotic usage in the community. Although wastewater treatment reduced ARG abundance, treated effluent still contained measurable resistance genes, suggesting incomplete removal during conventional treatment processes.

Table 3: Alpha Diversity Indices of Resistome Across Sampling Sites

Site Type	Shannon Index	Simpson Index
Hospital effluent	4.12	0.91
Municipal influent	3.85	0.88
Urban drainage	3.47	0.84
Treated effluent	2.96	0.79

The highest resistome diversity was observed in hospital wastewater, indicating a complex mixture of resistance determinants. Lower diversity in treated effluent suggests partial reduction of microbial and genetic diversity through treatment processes. However, the persistence of moderate diversity post-treatment indicates that conventional wastewater systems are insufficient for complete ARG elimination.

Inferential Analysis

One-way ANOVA revealed statistically significant differences in ARG abundance across wastewater sources ($p < 0.05$). Post-hoc analysis confirmed that hospital effluent had significantly higher ARG levels compared to all other sources. Pearson correlation analysis showed a strong positive relationship between population density and ARG abundance ($r = 0.78$), indicating that densely populated urban regions contribute substantially to environmental resistome load.

The findings demonstrate that urban wastewater systems in Pakistan serve as critical reservoirs and transmission pathways for antimicrobial resistance genes. The dominance of beta-lactam and tetracycline resistance genes reflects widespread antibiotic usage patterns in both clinical and community settings. Hospital wastewater was identified as the primary hotspot for ARG dissemination, highlighting the need for targeted wastewater management strategies in healthcare facilities.

Although wastewater treatment processes reduced ARG abundance, they did not eliminate resistance genes entirely, indicating that conventional treatment technologies are insufficient for controlling environmental AMR spread. The strong association between population density and ARG abundance further emphasizes the role of anthropogenic pressure in shaping environmental resistomes.

Overall, the results underscore the urgent need for integrated wastewater surveillance systems and advanced treatment technologies in Pakistan to mitigate the spread of antimicrobial resistance under a One Health framework.

Discussion

The present study demonstrated that urban wastewater systems in Pakistan function as significant reservoirs of antimicrobial resistance genes (ARGs), with marked variation across different wastewater sources. The predominance of beta-lactam and tetracycline resistance genes aligns with global findings that these antibiotic classes are among the most widely consumed and environmentally persistent (Hendriksen et al., 2019; Zhang et al., 2021). The high abundance of ARGs in hospital effluents further confirms the role of healthcare facilities as critical hotspots for antimicrobial resistance dissemination due to intensive antibiotic usage and high bacterial load.

Municipal wastewater also exhibited substantial ARG presence, indicating widespread community-level antibiotic consumption and improper disposal practices. Although wastewater treatment processes reduced ARG abundance, their incomplete removal highlights limitations in conventional treatment technologies, particularly in low- and middle-income countries. This finding is consistent with previous studies reporting that standard biological treatment systems are insufficient to eliminate genetic determinants of resistance, and may even facilitate selective enrichment under certain conditions (Rizzo et al., 2013; Manaia, 2017).

The observed positive correlation between population density and ARG abundance underscores the influence of anthropogenic pressure on environmental resistomes. Urbanization, inadequate sanitation infrastructure, and unregulated antibiotic usage collectively contribute to the persistence and dissemination of resistance genes in aquatic ecosystems. These findings reinforce the need for integrated environmental surveillance systems to monitor AMR at the community level.

Conclusion

This study concluded that urban wastewater in Pakistan is heavily contaminated with diverse antimicrobial resistance genes, with hospital effluents representing the most significant source. Wastewater treatment processes were found to reduce but not eliminate ARGs, indicating persistent environmental risks. The study confirmed that anthropogenic factors, particularly population density and healthcare waste discharge, significantly influence the distribution of resistance genes. Overall, wastewater systems serve as critical environmental reservoirs facilitating the spread of antimicrobial resistance under current infrastructural and regulatory conditions.

Implications of the Study

The findings have important implications for public health, environmental management, and policy development. From a public health perspective, the persistence of ARGs in treated and untreated wastewater poses a continuous risk of exposure to resistant pathogens through water reuse, irrigation, and environmental contamination. Environmentally, wastewater systems act as mixing zones where resistance genes can proliferate and transfer across microbial communities.

Policy-wise, the study supports the integration of wastewater-based epidemiology into national AMR surveillance frameworks. It also highlights the necessity of adopting the One Health approach, linking human health, environmental monitoring, and antimicrobial stewardship programs. Furthermore, the results provide empirical evidence for upgrading wastewater treatment infrastructure to include advanced tertiary or membrane-based technologies capable of reducing ARG loads.

Recommendations

It is recommended that national environmental and public health authorities establish a structured wastewater-based AMR surveillance system across major urban centers in Pakistan. Hospitals should be required to implement pre-treatment systems for wastewater before discharge into municipal networks.

Advanced treatment technologies such as membrane bioreactors, ozonation, ultraviolet disinfection, and advanced oxidation processes should be considered to enhance ARG removal efficiency. Strict regulation and monitoring of antibiotic usage in both clinical and agricultural sectors should also be enforced to reduce environmental loading.

Capacity building in molecular microbiology and metagenomics should be strengthened in local research institutions to support continuous monitoring and data-driven policymaking. Public awareness campaigns should be introduced to promote responsible antibiotic use and proper waste disposal practices.

Limitations and Future Directions

The study was limited by its cross-sectional design, which captured resistome profiles at a single time point and did not account for seasonal or temporal variations in ARG distribution. The sample size, although adequate for exploratory metagenomic analysis, may not fully represent all urban regions of Pakistan.

Another limitation was the reliance on short-read sequencing, which may restrict the ability to fully resolve mobile genetic elements and plasmid-associated resistance mechanisms. Additionally, the study did not directly measure antibiotic concentrations in wastewater, which could have strengthened causal interpretations.

Future research should incorporate longitudinal sampling to assess temporal dynamics of ARG dissemination. Integration of chemical analysis of antibiotic residues with metagenomic profiling would provide a more comprehensive understanding of selective pressures. Advanced sequencing approaches such as long-read metagenomics and metatranscriptomics should also be utilized to better characterize functional resistance mechanisms and gene mobility.

Expanding surveillance to rural-urban gradients and industrial zones would further enhance national-level understanding of environmental antimicrobial resistance patterns in Pakistan.

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