



Hospital Wastewater as A Reservoir of Antimicrobial Resistance Genes: Implications for Public Health and Genomic Surveillance in The United States

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Abstract

Hospital wastewater represents a concentrated environmental reservoir of antimicrobial resistance genes (ARGs), resistant organisms, pharmaceutical residues, and mobile genetic elements. This quantitative longitudinal study examined ARG occurrence, abundance, diversity, mobility, and clinical associations across 12 hospitals in four United States census regions. Of 1,152 planned samples, 1,098 met quality requirements, producing a 95.3% inclusion rate. Samples were collected from hospital outlets, upstream community sewers, downstream locations, and municipal influent over 12 months. Quantitative polymerase chain reaction, droplet digital polymerase chain reaction, culture, and metagenomic sequencing were combined with antimicrobial-use, patient-activity, wastewater-flow, and clinical-resistance data. Hospital outlets had 1.43-fold higher total ARG abundance than upstream sites. Detection of *blaKPC* occurred in 56.5% of hospital samples and 25.2% of upstream samples, corresponding to an adjusted odds ratio of 4.12. Metagenomic analysis identified 412 ARGs across 18 resistance classes. Mean ARG richness was 176.4 genes per hospital sample compared with 132.7 upstream, while 41.7% of hospital ARGs were linked to mobility markers. A 10-defined-daily-dose increase in carbapenem use per 1,000 patient-days was associated with an 18.6% concurrent increase in wastewater *blaKPC* and a 22.4% increase after a one-month lag. Wastewater *blaKPC* was positively associated with clinical carbapenem-resistant Enterobacterales. Treatment reduced absolute ARG concentrations by 91.7%–99.8%, although viable resistant organisms declined more strongly than total detectable DNA. These findings characterized hospital wastewater as a measurable, clinically connected resistance reservoir and supported its integration with clinical microbiology, antimicrobial stewardship, and genomic surveillance.

Keywords

Antimicrobial Resistance, Hospital Wastewater, Genomic Surveillance, Resist Me, Public Health.

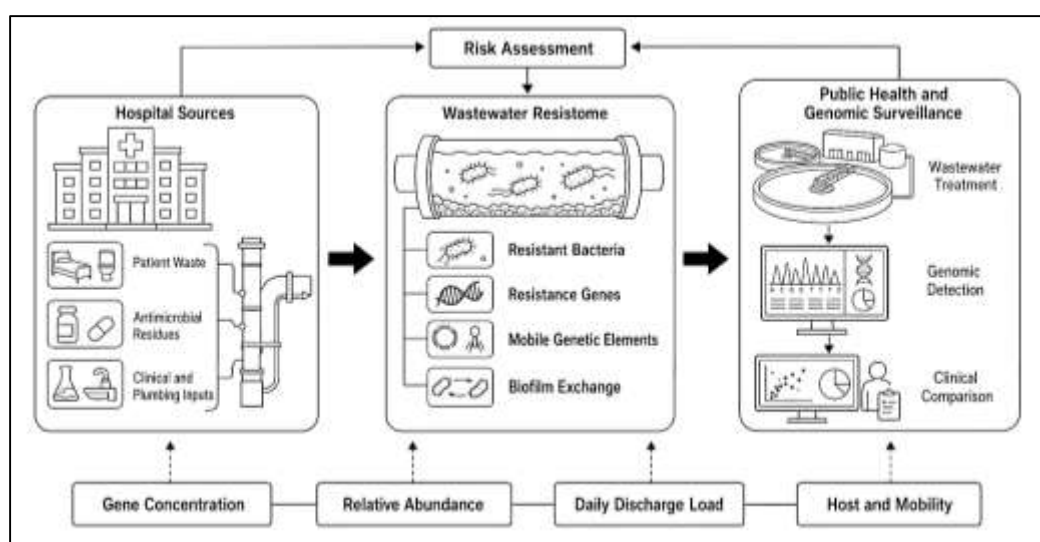
INTRODUCTION

Antimicrobial resistance is the ability of microorganisms to survive or reproduce in the presence of antimicrobial substances that would normally inhibit or destroy them. Although the term applies to bacteria, viruses, fungi, and parasites, research on hospital wastewater primarily concentrates on antibiotic-resistant bacteria and the genetic determinants responsible for their resistance (Balaban et al., 2019). Antimicrobial resistance genes are segments of genetic material that enable microorganisms to neutralize antimicrobial compounds, alter drug targets, reduce cellular permeability, expel toxic substances through efflux systems, or replace metabolic processes disrupted by treatment. The collective assortment of resistance genes within a microbial population or environmental sample is known as the resistome. A reservoir of antimicrobial resistance is an ecological setting in which resistant organisms, resistance genes, antimicrobial residues, mobile genetic elements, and suitable microbial hosts coexist. Hospital wastewater represents such a reservoir because it receives biological and chemical materials from patient rooms, intensive care units, surgical departments, laboratories, pharmacies, laundries, kitchens, and sanitation systems. Its contents may include urine, feces, blood, bodily fluids, pharmaceutical compounds, disinfectants, detergents, diagnostic reagents, pathogenic microorganisms, commensal bacteria, extracellular genetic material, and organic nutrients. These components create a chemically and biologically complex environment that differs from ordinary domestic sewage. Hospital patients often receive broad-spectrum or last-resort antimicrobial agents and may carry organisms resistant to several therapeutic classes. Wastewater discharged from healthcare facilities can consequently contain concentrated mixtures of resistant bacteria and clinically significant resistance genes (Huemer et al., 2020). The term genomic reservoir extends beyond living resistant pathogens because genetic information can remain in extracellular DNA, bacteriophages, plasmids, integrons, transposons, and gene cassettes. These genetic structures may preserve resistance determinants and facilitate their movement among bacterial populations. Hospital drains, sink traps, sewer pipes, and collection tanks also support microbial biofilms in which organisms remain in close contact for prolonged periods. Biofilms provide surfaces for bacterial attachment, protect cells from environmental stress, and create conditions that support genetic exchange. A quantitative investigation of this reservoir must distinguish gene presence from gene abundance, relative abundance, absolute concentration, daily discharge load, host association, and phenotypic resistance. Detecting a resistance gene confirms that its genetic sequence is present, but detection alone does not identify its bacterial host, biological activity, mobility, or clinical relevance (Uruén et al., 2020). Measurement in copies per wastewater volume, copies per unit of solids, copies relative to total bacterial DNA, and copies discharged per day provides separate but complementary descriptions of the resistance reservoir.

The international importance of hospital wastewater arises from the cross-border movement of resistant microorganisms and resistance genes through travel, migration, healthcare, food production, trade, sanitation networks, and connected aquatic environments. Antimicrobial resistance is not confined to the hospital or country in which it first emerges (Pachori et al., 2019). Resistant bacteria may be carried by patients receiving medical treatment abroad, international travelers, healthcare workers, animals, food products, and contaminated materials. Wastewater provides a pooled biological record of these interconnected activities because it contains microorganisms and genetic material excreted by large and diverse populations. International research involving more than 30 empirical studies has repeatedly demonstrated that sewage resistomes vary across countries according to antimicrobial consumption, sanitation coverage, healthcare access, population density, economic conditions, environmental regulation, and wastewater-treatment performance. Investigations conducted in Europe, Asia, Africa, South America, and North America have detected genes associated with resistance to β -lactams, carbapenems, tetracyclines, sulfonamides, macrolides, aminoglycosides, fluoroquinolones, glycopeptides, and colistin. Hospital wastewater has often contained higher concentrations or a broader diversity of clinically important resistance determinants than community wastewater sampled within the same geographic area. Frequently detected targets include extended-spectrum β -lactamase genes, carbapenemase genes, methicillin-resistance determinants, vancomycin-resistance genes, sulfonamide-resistance genes, tetracycline-resistance genes, and mobilized colistin-resistance genes (Laborda et al., 2022). Their occurrence is internationally significant because several confer resistance to drugs used for treating severe infections caused by multidrug-resistant organisms.

Sewage metagenomic investigations have also identified geographical clustering of resistance profiles, indicating that wastewater composition reflects national and regional differences in antimicrobial exposure, sanitation, and public health conditions. Wastewater-treatment processes can reduce bacterial biomass and the concentrations of many resistance targets, yet removal is not uniform across genes, bacterial species, treatment technologies, or seasons. Measurable quantities may remain in treated effluent, sewage sludge, biosolids, sediment, or receiving water. The degree of reduction depends on hydraulic retention, microbial treatment processes, solids separation, disinfection, temperature, organic content, and the physical state of the genetic material. Hospital effluent that enters a municipal sewer becomes diluted by domestic and commercial wastewater, but dilution does not necessarily eliminate its genetic contribution (Varela et al., 2021). A lower downstream concentration may still represent a large total quantity when the daily wastewater flow is substantial. International evidence therefore frames hospital wastewater as one component of a wider human, animal, and environmental resistance system. This global context is especially relevant to the United States because American hospitals serve internationally mobile populations, operate within highly connected healthcare networks, and generally discharge wastewater into municipal sewer systems without treatment specifically designed to remove antimicrobial resistance genes.

Figure 1: Hospital Wastewater Antimicrobial Resistance Surveillance



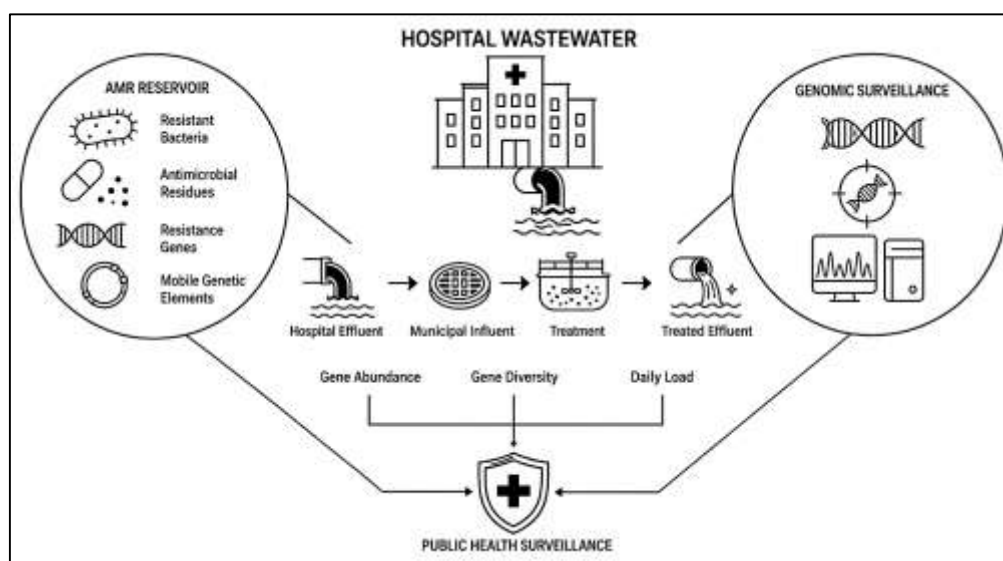
Antimicrobial resistance creates a substantial public health burden in the United States through difficult-to-treat infections, prolonged hospitalization, increased medical expenditure, treatment failure, disability, and preventable mortality (Ali et al., 2018). Conventional surveillance relies heavily on clinical cultures, antimicrobial susceptibility testing, infection reports, pharmacy records, and genomic sequencing of bacterial isolates. These methods provide essential information about diagnosed infections and the organisms recovered from individual patients. Their population coverage, however, depends on healthcare access, care-seeking behavior, clinician decisions, specimen collection, laboratory capacity, reporting practices, and the selection of patients for diagnostic testing. People carrying resistant bacteria without symptoms are rarely represented in clinical surveillance, even though they may contribute resistance genes to wastewater. Wastewater surveillance offers a complementary measurement strategy because a single sample can aggregate biological material from many individuals without requiring separate clinical examinations. Hospital wastewater provides a more localized signal than municipal sewage because it captures excretion from patients receiving specialized treatment, individuals with complex medical conditions, and persons exposed to intensive antimicrobial therapy. It may also contain organisms from healthcare workers, visitors, outpatient departments, food-service areas, laboratories, and environmental cleaning activities. This mixture allows quantitative assessment of resistance at the facility level while preserving a pooled sampling

framework (Rather et al., 2021). The United States has extensive experience with wastewater monitoring for infectious agents, demonstrating that sewage can be collected systematically, analyzed across multiple laboratories, and interpreted alongside epidemiological data. Applying this infrastructure to antimicrobial resistance requires additional analytical caution because a resistance gene can occur in a harmless commensal organism, a clinically important pathogen, a nonviable cell, or free genetic material. The presence of a gene therefore cannot be treated as equivalent to a confirmed resistant infection. National wastewater measurements have identified regional differences in resistance-gene concentrations and considerable variability among individual targets. Genes associated with tetracycline and common β -lactam resistance may appear broadly distributed, whereas rare carbapenemase or colistin-resistance determinants may display stronger geographical variation. Hospital presence, nursing-home density, international travel connections, urbanization, housing conditions, healthcare access, and demographic characteristics can also correspond with variation in wastewater resistance profiles. Such associations are not direct evidence of causation, but they demonstrate that resistance abundance reflects a combination of clinical, social, demographic, and infrastructural conditions (Christaki et al., 2020). Hospital-specific sampling can strengthen geographic attribution by narrowing the population contributing to a sample. Quantitative comparisons among hospital outlets, upstream sewer locations, municipal influent, and treated effluent can clarify how the concentration, diversity, and composition of resistance genes change as healthcare wastewater moves through the broader sanitation system.

Hospitals may contribute concentrated antimicrobial resistance signals because they combine intensive drug exposure with high densities of patients carrying susceptible, resistant, or multidrug-resistant organisms. Antimicrobial compounds and their metabolites enter wastewater through patient excretion, discarded liquid medication, laboratory procedures, cleaning activities, and routine clinical operations (Serwecińska, 2020). Their concentrations depend on prescribing patterns, drug absorption, human metabolism, disposal practices, wastewater volume, sewer retention time, chemical degradation, and dilution. Low concentrations of antimicrobial compounds can still influence microbial communities when susceptible organisms experience greater growth inhibition than resistant organisms. Disinfectants, metals, detergents, and other chemical stressors may also participate in co-selection when resistance to different substances is controlled by linked genes or shared cellular mechanisms. Hospital wastewater therefore contains both resistance determinants and environmental pressures capable of altering the composition of microbial populations. Plumbing infrastructure adds another dimension to this reservoir. Sinks, drains, toilets, traps, pipes, and storage tanks contain wet surfaces where bacteria form biofilms. These biofilms retain organisms, nutrients, DNA, and chemical residues while providing close physical contact among microbial cells. Genetic material may move through conjugation, transformation, or bacteriophage-mediated transfer. Conjugation transfers plasmids or related mobile elements directly between bacterial cells, transformation occurs when bacteria acquire free DNA from their surroundings, and transduction involves gene movement through bacteriophages. Integrons and transposons can capture or rearrange gene cassettes, allowing several resistance determinants to become linked within the same genetic structure. The discharge of hospital wastewater into municipal sewers connects this localized environment with larger microbial communities (Vidovic & Vidovic, 2020). Mixing introduces hospital-associated organisms and genes into domestic wastewater while exposing them to additional bacterial hosts, organic matter, pharmaceuticals, and environmental contaminants. Quantitative evaluation must account for both concentration and flow because concentration indicates the amount of a target in a defined sample volume, whereas mass load estimates the total number of gene copies released over a specified period. A hospital with moderately concentrated wastewater and a large discharge volume may release more resistance material than a smaller facility with a higher measured concentration. Bed occupancy, patient-days, case complexity, intensive-care activity, antimicrobial-defined daily doses, wastewater production, temperature, pH, suspended solids, and fecal strength can influence observed measurements. Repeated sampling is necessary because hospital activities vary by hour, weekday, season, ward occupancy, and patterns of antimicrobial administration (Boolchandani et al., 2019). Composite samples collected across an entire day generally represent facility discharge more reliably than isolated grab samples. Matched samples from hospital effluent and surrounding municipal sites

allow resistance measurements to be interpreted within their immediate environmental background.

Figure 2: Hospital Wastewater Resist Me Monitoring Framework



The selection of laboratory methods determines which components of the hospital wastewater resistome can be measured and how confidently the results can be interpreted. Quantitative polymerase chain reaction provides sensitive measurement of predefined resistance genes and is suitable for analyzing large numbers of samples at comparatively low cost ([Pontes et al., 2022](#)). It can quantify priority targets such as genes encoding extended-spectrum β -lactamases, carbapenemases, methicillin resistance, vancomycin resistance, tetracycline resistance, sulfonamide resistance, and mobilized colistin resistance. Droplet digital polymerase chain reaction divides a sample into thousands of small reactions and estimates absolute target concentration from the number of positive and negative partitions. This method can improve precision for low-abundance genes and reduce reliance on external standard curves. Targeted molecular assays remain limited to genes selected before analysis and cannot independently reveal unknown resistance determinants, bacterial hosts, or the genetic structures surrounding a target. Shotgun metagenomic sequencing addresses these restrictions by analyzing the total DNA within a wastewater sample. It can characterize bacterial communities, resistance-gene families, virulence factors, metabolic functions, and mobile genetic elements within a common dataset ([Subedi et al., 2018](#)). Its results depend on sequencing depth, nucleic-acid extraction, read quality, reference-database coverage, alignment criteria, and normalization procedures. Short sequencing reads may detect a resistance gene without showing whether it is located on a chromosome, plasmid, integron, bacteriophage, or extracellular DNA fragment. Long-read sequencing produces larger genomic fragments that can improve the linkage of resistance genes to bacterial hosts and mobile elements. Hybrid analysis combining short and long reads can provide both sequence accuracy and structural resolution. Genome-resolved metagenomics may reconstruct partial or nearly complete genomes from mixed-community data, permitting investigation of organisms carrying multiple resistance determinants. Culture-based analysis provides another essential measurement because it confirms the presence of viable organisms and permits antimicrobial susceptibility testing. Culture methods capture only organisms capable of growing under the selected laboratory conditions and may underrepresent slow-growing, injured, rare, or unculturable bacteria. Culture enrichment increases detection of low-abundance resistant organisms but changes the original community composition by favoring organisms suited to the enrichment medium. Meta transcriptomic analysis can identify expressed genes, although gene expression does not alone establish phenotypic resistance or successful transmission. Combining targeted quantification, metagenomic sequencing, long-read analysis, bacterial culture, and susceptibility testing provides a more complete assessment of abundance,

diversity, viability, host identity, and genetic mobility ([Makabenta et al., 2021](#)). Standardized collection, preservation, extraction, quality control, and reporting procedures are required for valid comparisons among U.S. hospitals. Negative controls, recovery controls, detection limits, inhibition testing, duplicate analysis, and transparent treatment of nondetectable values reduce the risk that laboratory variation is mistaken for biological variation.

Genomic surveillance is the systematic collection and analysis of genetic information to monitor microorganisms, resistance determinants, transmission patterns, and changes in population composition. In hospital wastewater, genomic surveillance can be conducted at the level of individual resistance genes, gene families, bacterial species, strain lineages, plasmids, integrins, or entire microbial communities ([Bowler et al., 2020](#)). Targeted genomic surveillance determines whether selected high-priority resistance determinants are present and how their quantities vary across locations or sampling dates. Resistive-wide surveillance measures a broader collection of resistance genes and compares their relative abundance, richness, diversity, and co-occurrence. Genome-resolved analysis attempts to connect resistance determinants with the organisms carrying them, while genetic-context analysis examines whether genes are associated with plasmids, insertion sequences, transposons, integrins, or chromosomes. These distinctions matter because identical resistance genes can appear in unrelated bacterial species and move between organisms through mobile genetic elements. A carbapenem's gene detected in wastewater may originate from a clinically important pathogen, a harmless intestinal bacterium, an environmental organism, or extracellular DNA. Gene detection alone cannot establish an outbreak or identify transmission between patients. Greater interpretive confidence arises when wastewater sequences are examined alongside clinical isolates, antimicrobial-susceptibility profiles, patient census data, antimicrobial consumption, infection-control records, and hospital epidemiology. Whole-genome sequencing of clinical isolates can determine genetic relationships among resistant organisms recovered from patients ([Urban-Chmiel et al., 2022](#)). Comparing those genomes with assembled wastewater sequences may identify shared resistance genes, plasmids, or strain-associated markers. Analytical interpretation must consider differences in timing because patient cultures, antimicrobial exposure, colonization, and wastewater excretion do not occur simultaneously. Plumbing layout and sewer retention may also delay or mix facility signals. Statistical models can account for repeated observations, hospital-level clustering, variable wastewater flow, and differences among resistance targets. Mixed-effects regression can separate within-hospital changes from between-hospital variation, while count-based models can accommodate sparse or over dispersed gene measurements. Multivariate analysis can compare the overall resistive structure among facilities, and source-tracking methods can estimate the proportional contribution of hospital wastewater to downstream municipal samples. Data governance is also relevant because hospital-level results may affect facility reputation or be misunderstood as direct measures of patient safety ([Kaprou et al., 2021](#)). Pooled wastewater normally contains no direct patient identifiers, but highly localized sampling can represent small groups. Reporting frameworks must distinguish an environmental genomic signal from a confirmed clinical event. Quantitative thresholds, reproducibility across samples, evidence of viable hosts, genetic similarity to clinical isolates, and consistency with facility epidemiology provide separate levels of evidence within a hospital wastewater surveillance system.

The quantitative research problem centers on the limited understanding of how hospital wastewater resistance profiles correspond with antimicrobial use, clinical resistance, facility characteristics, and downstream municipal wastewater in the United States. Existing evidence is distributed across studies using different countries, sampling matrices, hospital types, laboratory protocols, resistance targets, and analytical units ([Combarros-Fuertes et al., 2020](#)). Many investigations rely on one hospital, a short sampling interval, grab samples, or a restricted set of genes. Some report only whether a gene was detected, while others calculate relative abundance without determining absolute concentration or daily discharge load. Municipal surveillance provides valuable estimates for large sewer sheds but cannot isolate hospital contributions from households, nursing homes, businesses, industrial facilities, animal-related sources, and environmental background contamination. A multisite quantitative study can address this measurement gap by defining resistance-gene abundance, diversity, and mobility as primary outcomes. Absolute abundance may be expressed as gene copies per milliliter of wastewater or per gram of wastewater solids. Relative abundance may be calculated by normalizing resistance-

gene copies to the 16S rRNA gene, total DNA, bacterial cell counts, or human fecal markers. Daily load may be estimated by multiplying gene concentration by measured wastewater flow. Resistive richness can represent the number of distinct genes or gene families in each sample, while diversity indices can describe how sequencing reads are distributed across resistance categories. Mobility can be measured through the proportion of resistance genes located near integrases, transposases, insertion sequences, plasmid markers, or other mobile genetic features (Osman et al., 2018). Independent variables may include hospital-bed capacity, occupied-bed days, patient-days, intensive-care activity, antimicrobial-defined daily doses, clinical detection rates of resistant organisms, wastewater temperature, pH, suspended solids, organic content, and facility discharge volume. Matched sampling at hospital outlets, upstream community locations, downstream sewer points, municipal-treatment influent, and treated effluent permits spatial comparison across the wastewater pathway. Repeated measurements allow the study to separate short-term fluctuations from persistent differences among hospitals. The principal hypotheses can test whether hospital effluent contains higher concentrations, relative abundances, daily loads, and diversity of clinically important resistance genes than matched community wastewater; whether antimicrobial consumption corresponds with resistance genes affecting related drug classes; whether wastewater gene measurements are associated with clinical detection rates of resistant organisms; and whether hospital discharge explains measurable variation in downstream municipal resistors (Torres-Barceló, 2018). Prespecified statistical models, effect sizes, confidence intervals, multiple-comparison corrections, standardized detection limits, and sensitivity analyses using different normalization methods provide the quantitative structure needed to examine these relationships within the U.S. healthcare and wastewater environment.

The primary objective of this quantitative study is to evaluate hospital wastewater as a reservoir of antimicrobial resistance genes and determine its relationship with public health indicators and genomic surveillance measurements in the United States. Specifically, the study seeks to quantify the absolute concentration, relative abundance, diversity, and daily discharge load of clinically important resistance genes in wastewater collected from selected U.S. hospitals. The analysis focuses on genetic determinants associated with resistance to major antimicrobial classes, including β -lactams, carbapenems, tetracyclines, sulfonamides, macrolides, aminoglycosides, glycopeptides, methicillin, and colistin. A further objective is to compare resistance-gene profiles in hospital effluent with those observed at upstream community locations, downstream sewer points, municipal wastewater-treatment influent, and treated effluent to estimate the measurable contribution of healthcare facilities to broader wastewater epistomes. The study also aims to examine associations between wastewater resistance measurements and hospital-level characteristics, including bed capacity, patient-days, intensive-care occupancy, antimicrobial consumption, clinical detection rates of multidrug-resistant organisms, wastewater flow, and selected physicochemical conditions. Through targeted molecular quantification and genomic sequencing, the research intends to determine the occurrence of resistance genes, identify their probable bacterial hosts, characterize their association with plasmids, integrins, transposons, and other mobile genetic elements, and evaluate their co-occurrence with virulence determinants. Another objective is to assess whether temporal changes in wastewater resistance-gene abundance correspond with variations in clinical antimicrobial-resistance records and facility antimicrobial use during matched sampling periods. The investigation additionally seeks to measure differences in resistance-gene concentration and resistive composition across hospitals, geographic regions, seasons, and wastewater-treatment stages. Statistical modeling is used to identify factors that explain within-hospital and between-hospital variation while controlling for wastewater volume, fecal strength, bacterial biomass, temperature, pH, suspended solids, and sampling frequency. The study also evaluates the sensitivity, consistency, and quantitative agreement of polymerase chain reaction, droplet digital polymerase chain reaction, culture-based testing, and metagenomic sequencing for detecting priority resistance targets in complex hospital wastewater samples. By integrating environmental measurements, hospital antimicrobial-use data, clinical resistance records, wastewater characteristics, and genomic information, the research establishes a structured quantitative basis for examining how hospital wastewater reflects the occurrence, concentration, distribution, host association, and mobility of antimicrobial resistance genes within the United States healthcare and sanitation environment.

LITERATURE REVIEW

Hospital wastewater has emerged as an important environmental reservoir of antimicrobial resistance genes because it contains concentrated mixtures of resistant microorganisms, antimicrobial residues, disinfectants, mobile genetic elements, human waste, and organic materials generated through healthcare activities. Patients receiving antimicrobial treatment may excrete unmetabolized drugs, active metabolites, resistant bacteria, and genetic determinants into hospital sanitation systems (Chandler, 2019; Debasish, 2021). Additional inputs arise from clinical laboratories, pharmacies, operating rooms, intensive care units, isolation wards, laundries, kitchens, and environmental cleaning procedures. These inputs create wastewater conditions in which resistance genes can persist, accumulate, and interact with diverse bacterial communities. Genes conferring resistance to β -lactams, carbapenems, tetracyclines, sulfonamides, macrolides, aminoglycosides, glycopeptides, methicillin, and colistin have been identified in hospital-associated wastewater environments. Clinically important determinants include blaKPC, blaNDM, blaVIM, blaOXA-48-like, blaCTX-M, mecA, vanA, mcr-1, sul1, tetW, and ermB. Their occurrence becomes particularly important when they are carried by plasmids, integrons, transposons, insertion sequences, or bacteriophages that facilitate genetic movement among bacterial hosts. A quantitative examination of hospital wastewater requires more than reporting whether individual genes are present or absent. The literature must differentiate absolute concentration, relative abundance, daily mass load, detection frequency, resistome richness, genomic diversity, host association, phenotypic resistance, and mobility potential. Absolute concentration may be measured as gene copies per milliliter of wastewater or per gram of wastewater solids, whereas relative abundance may be expressed as resistance-gene copies per 16S rRNA gene copy. Daily load incorporates wastewater flow and provides an estimate of the total number of gene copies discharged from a hospital during a defined period. Sequencing-based measures can quantify resistance-gene richness, diversity, read abundance, co-occurrence, and linkage with mobile genetic elements. These outcomes may vary according to hospital size, occupied-bed days, patient case mix, intensive-care activity, antimicrobial consumption, wastewater flow, sampling frequency, season, temperature, pH, suspended solids, organic content, and treatment stage (Abdur & Iftekhhar, 2021; Pieri et al., 2020). Comparisons among hospital outlets, community sewers, municipal influent, treated effluent, and receiving waters are necessary to determine whether healthcare facilities contribute measurable increments to broader wastewater resistomes. The literature review therefore examines the biological, clinical, environmental, methodological, and surveillance dimensions of hospital wastewater antimicrobial resistance within the United States. It evaluates evidence concerning the sources and persistence of resistance genes, patterns of horizontal gene transfer, associations with antimicrobial use and clinical resistance, and the effectiveness of wastewater-treatment processes. It also examines quantitative detection methods, including quantitative polymerase chain reaction, droplet digital polymerase chain reaction, bacterial culture, antimicrobial susceptibility testing, short-read metagenomics, long-read sequencing, and genome-resolved analysis. Particular attention is given to differences in sampling design, normalization procedures, detection limits, genomic databases, and statistical modeling because these methodological choices influence reported resistance levels. The review further considers how hospital wastewater data can be integrated with clinical isolates, antimicrobial-consumption records, infection-control information, and municipal wastewater measurements (Debasish, 2022; Lambert et al., 2019). This organization establishes the conceptual and empirical foundation for measuring hospital wastewater as a resistance reservoir and evaluating its relationship with public health and genomic surveillance indicators across U.S. healthcare and sanitation systems.

Conceptual Foundations of Antimicrobial Resistance

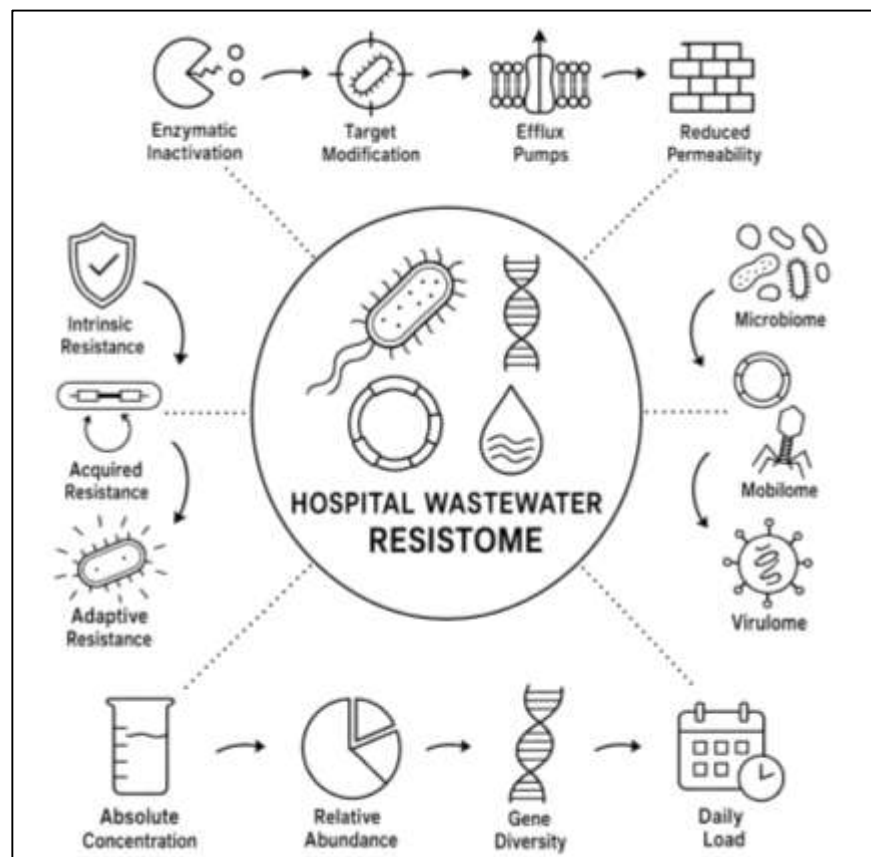
Antimicrobial resistance is a biological condition in which a microorganism survives, grows, or reproduces in the presence of an antimicrobial concentration that would normally inhibit or eliminate a susceptible member of the same species. Although the term applies to bacteria, viruses, fungi, and parasites, wastewater research largely concentrates on bacterial resistance to antibiotics because resistant bacteria and their genetic determinants circulate extensively through human excreta, healthcare facilities, sewer networks, and wastewater-treatment systems (Legido-Quigley et al., 2018; Ashfaq & Mohammad, 2022). At the genetic level, antimicrobial resistance reflects the presence,

modification, acquisition, or expression of hereditary determinants that protect microorganisms from antimicrobial activity. These determinants may be encoded within bacterial chromosomes or located on mobile genetic elements such as plasmids, integrons, transposons, insertion sequences, and bacteriophages. At the cellular level, resistance is expressed through physiological processes that reduce antimicrobial entry, remove drugs from the cell, modify drug targets, destroy antimicrobial molecules, or replace the metabolic functions disrupted by treatment. At the clinical level, resistance is generally identified when a bacterial isolate grows at antimicrobial concentrations exceeding established susceptibility breakpoints. This classification assists laboratories in categorizing isolates as susceptible, intermediate, or resistant and supports the selection of appropriate treatment. At the environmental level, antimicrobial resistance encompasses resistant organisms, resistance genes, antimicrobial residues, mobile genetic elements, and ecological interactions occurring in wastewater, soil, surface water, sediment, sewage sludge, and built plumbing systems (Mattar et al., 2020). Environmental resistance is broader than clinical resistance because resistance genes may occur in nonpathogenic, commensal, unculturable, or nonviable organisms that are not represented in routine patient testing. The literature further distinguishes intrinsic, acquired, and adaptive resistance. Intrinsic resistance is a stable characteristic naturally shared by most members of a bacterial species and may result from the absence of a drug target, low membrane permeability, constitutive efflux activity, or naturally occurring enzymes. Acquired resistance develops when a previously susceptible bacterium obtains a resistance determinant through mutation or horizontal gene transfer. Adaptive resistance is a reversible decrease in antimicrobial susceptibility caused by environmental conditions such as drug exposure, nutrient limitation, oxidative stress, temperature changes, pH variation, or biofilm growth. These categories can interact because an intrinsically resistant bacterium may acquire additional resistance genes, while environmental stress may temporarily increase the expression of chromosomal or acquired mechanisms (Marvasi et al., 2021; Sheak & Risha, 2022). This multilevel understanding is essential in hospital wastewater research because each sample may contain clinical pathogens, intestinal commensals, environmental microorganisms, extracellular DNA, and mobile resistance determinants with different degrees of clinical and ecological relevance.

Antimicrobial resistance must be differentiated from tolerance, persistence, and treatment failure because these concepts describe related but biologically distinct conditions. Resistance usually involves an increase in the antimicrobial concentration required to inhibit bacterial growth, whereas tolerance describes the ability of a bacterial population to survive temporary exposure to a lethal antimicrobial concentration without necessarily increasing the concentration required to prevent growth (Frid-Nielsen et al., 2019; Rashedul & Kaium, 2022). Tolerant bacteria may survive longer because of slowed metabolism, reduced cellular activity, stress responses, or delayed antimicrobial killing. Persistence is a population-level condition in which a small subpopulation of genetically similar cells enters a temporary physiological state that permits survival during antimicrobial exposure. After the antimicrobial is removed, persistent cells can resume growth and produce descendants that remain susceptible according to conventional laboratory testing. Treatment failure is a clinical outcome and cannot be treated as direct evidence of microbial resistance. Therapy may fail because of incorrect drug selection, insufficient dosage, poor adherence, inadequate penetration into the infection site, delayed source control, immune suppression, drug interactions, biofilm formation, tolerance, or persistence. A resistant organism may still be controlled through appropriate antimicrobial combinations, dosage adjustments, drainage, or removal of an infected device, while a susceptible organism may remain difficult to eliminate under unfavorable clinical conditions (Jit et al., 2020; Chowdhury & Tonay, 2022). The major bacterial resistance mechanisms further clarify these distinctions. Enzymatic antimicrobial inactivation occurs when bacteria produce enzymes that destroy or chemically modify antimicrobial compounds. β -lactamases break down β -lactam drugs, while aminoglycoside-modifying enzymes alter aminoglycoside molecules and reduce their ability to bind bacterial targets. Target modification occurs when bacteria change penicillin-binding proteins, ribosomal structures, DNA-processing enzymes, or metabolic enzymes so that antimicrobial compounds can no longer bind effectively. Efflux-pump activation removes antimicrobial molecules from bacterial cells and may provide resistance to several unrelated drug classes. Reduced membrane permeability restricts antimicrobial entry through changes in porins, membrane composition, cell-envelope structures, or transport pathways. Metabolic pathway

substitution enables bacteria to bypass an inhibited biochemical reaction by using an alternative enzyme or pathway. Biofilm-associated protection combines restricted antimicrobial penetration, slow cellular growth, nutrient gradients, stress responses, altered gene expression, high cell density, and the accumulation of extracellular material. Biofilms also create conditions that support bacterial tolerance, persistence, and horizontal gene transfer (Majumder et al., 2020; Nishat & Kaniz, 2022). These processes are particularly important in hospital sinks, drains, sewer pipes, wastewater tanks, and other continuously wet environments. A single bacterial strain may use several mechanisms simultaneously, producing multidrug resistance through the combined effects of enzymatic inactivation, target alteration, efflux activity, and reduced permeability. Hospital wastewater analysis must therefore avoid treating gene detection, phenotypic resistance, bacterial survival, and clinical treatment failure as interchangeable measurements.

Figure 3: Hospital Wastewater Antimicrobial Resistance Framework



The wastewater resistome is the complete collection of antimicrobial resistance genes and resistance-associated genetic determinants present within a wastewater microbial community. It includes genes carried by pathogenic, commensal, opportunistic, and environmental microorganisms, along with resistance sequences found in extracellular DNA and bacteriophages. The resistome concept expands antimicrobial resistance analysis beyond organisms that can be cultivated under routine laboratory conditions because many wastewater microorganisms cannot be recovered through standard culture procedures (Shaiful et al., 2022; Wee et al., 2020). It also recognizes that environmental genes with limited immediate clinical significance may serve as evolutionary sources from which clinically important resistance mechanisms develop. The resistome is closely connected to the microbiome, mobilome, and virulome. The microbiome comprises the microorganisms, their genetic material, and their ecological relationships within a defined environment. In wastewater, it includes bacteria, fungi, viruses, protozoa, bacteriophages, and microbial genetic material originating from humans, animals, healthcare systems, households, industries, soil, and water. The mobilome consists of mobile or

potentially mobile genetic elements, including plasmids, integrons, transposons, insertion sequences, integrative conjugative elements, and bacteriophages. These elements facilitate the movement of resistance genes within and between bacterial species. The virulome represents genes associated with pathogenicity, toxin production, adhesion, host invasion, immune evasion, nutrient acquisition, and other functions that contribute to disease. These genomic components may overlap when the same plasmid or mobile element carries resistance and virulence determinants, allowing antimicrobial survival and pathogenic properties to circulate together (Mohammad, 2023; Birgand et al., 2018). Wastewater resistomes contain clinically relevant genes associated with difficult-to-treat infections, commensal genes carried by normal intestinal bacteria, and environmental genes originating from microorganisms adapted to water, sediments, soil, wastewater infrastructure, and plumbing biofilms. Clinically important determinants include genes associated with resistance to extended-spectrum β -lactams, carbapenems, methicillin, vancomycin, aminoglycosides, fluoroquinolones, tetracyclines, sulfonamides, macrolides, and colistin. Commensal bacteria may retain and exchange these genes without directly causing disease, while environmental bacteria maintain extensive natural reservoirs of resistance functions. Hospital wastewater is a distinctive component of this genetic network because it receives microorganisms from patients undergoing intensive antimicrobial treatment, resistant organisms from clinical environments, pharmaceutical residues, disinfectants, bodily waste, and laboratory materials. Its resistome is therefore shaped by antimicrobial consumption, patient characteristics, clinical activity, microbial ecology, wastewater flow, chemical exposure, plumbing conditions, and genetic mobility (Kaprou et al., 2021; Chowdhury, 2023). Differences between hospital and community wastewater may occur in resistance-gene concentration, genetic diversity, bacterial composition, and the prevalence of mobile elements, although the magnitude of those differences varies among healthcare facilities and geographic settings.

The composition of the wastewater resistome can be classified according to the location, mobility, prevalence, persistence, and biological origin of its resistance determinants. Intracellular antimicrobial resistance genes are located within intact bacterial cells and may occur on chromosomes or mobile genetic elements. Their detection may indicate carriage by living cells, damaged cells, recently inactivated bacteria, or organisms that cannot be cultivated under standard laboratory conditions. Extracellular resistance genes occur outside intact cells following bacterial lysis, active DNA release, biofilm disruption, or incomplete degradation of genetic material (Hannah & Baekkeskov, 2020; Badrul & Mominul, 2023). Extracellular DNA can remain protected through attachment to suspended solids, organic particles, minerals, sewage sludge, and biofilm matrices. This protection may allow resistance sequences to persist after the original bacterial host has died. Chromosomal determinants are generally inherited during bacterial replication and may represent intrinsic characteristics or acquired genes integrated into the chromosome. Mobile resistance determinants are carried by plasmids, integrons, transposons, insertion sequences, bacteriophages, and related structures that facilitate horizontal transfer. Class 1 integrons are especially important because they can capture and arrange gene cassettes and frequently occur with multiple resistance determinants in human-affected environments. The core wastewater resistome consists of genes consistently detected across most samples, locations, or sampling periods. It commonly includes resistance determinants related to tetracyclines, sulfonamides, macrolides, aminoglycosides, and multidrug efflux. The accessory resistome consists of genes detected intermittently, at low abundance, or within restricted geographic or institutional locations. This component may include uncommon carbapenemase, colistin-resistance, and other clinically significant genes (Charoenboon et al., 2019; Zahidul, 2023). Quantitative characterization can report absolute resistance-gene concentration as gene copies per milliliter of liquid wastewater or per gram of wastewater solids. Relative abundance can be reported by comparing resistance-gene copies with total bacterial markers such as the 16S rRNA gene. Sequencing analysis may describe normalized resistance-gene reads, detection frequency, the number of unique genes or gene families, and the proportional representation of different resistance classes. Resistome richness refers to the number of distinct resistance determinants identified in a sample, while Shannon and Simpson diversity indices describe both the number of resistance determinants and the distribution of their abundances. Each measurement represents a different feature of the reservoir. Absolute concentration is influenced by wastewater dilution and flow, relative abundance is affected by bacterial-community composition, and

sequencing results depend on DNA extraction, sequencing depth, database coverage, and gene-annotation criteria (Ashrafuzzaman, 2024; Khan et al., 2020). A rigorous hospital wastewater assessment therefore combines absolute concentration, relative abundance, daily discharge load, detection frequency, richness, diversity, host identification, and linkage with mobile genetic elements.

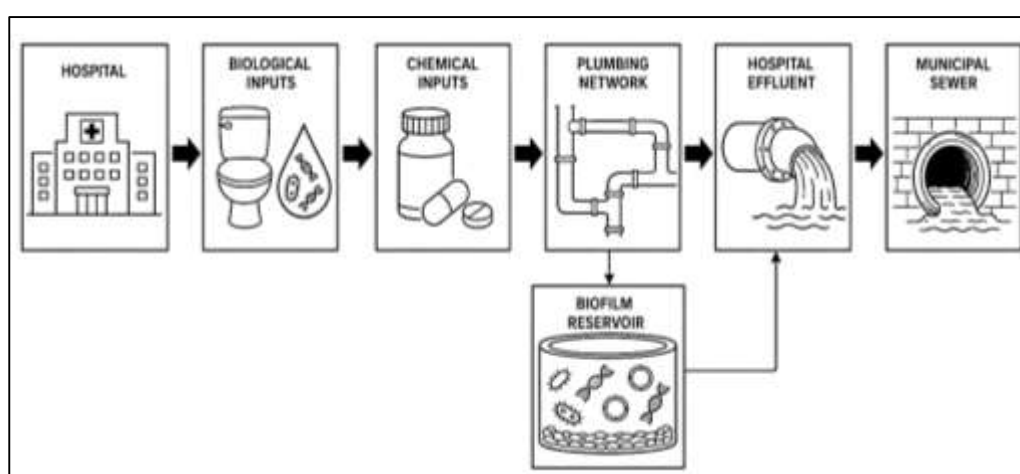
Hospital Wastewater as a Distinct Resistance Environment

Hospital wastewater is a complex mixture of biological, pharmaceutical, chemical, and physical materials generated through patient care and the routine operation of healthcare facilities (Hasan, 2024; Wang et al., 2018). Its primary biological inputs include urine, feces, blood, sputum, vomit, wound fluids, and other bodily discharges released through toilets, sinks, showers, bathing facilities, bedpan washers, and clinical disposal systems. Urine and feces are particularly important because patients receiving antimicrobial therapy can excrete unmetabolized drugs, active metabolites, resistant bacteria, antimicrobial resistance genes, and mobile genetic elements. These materials may originate from patients with diagnosed resistant infections, asymptomatic carriers of multidrug-resistant organisms, and individuals whose intestinal microbiomes have been altered by antimicrobial exposure. Blood, wound drainage, and bodily fluids can introduce additional microorganisms and genetic material, especially when they enter drains through patient washing, equipment cleaning, or disposal activities. Wastewater from intensive care and isolation units may contain concentrated biological signals because patients in these areas commonly experience severe illness, invasive procedures, prolonged hospitalization, and intensive antimicrobial treatment (Hassoun-Kheir et al., 2020; Rahman, 2024). These units also manage patients colonized or infected with methicillin-resistant staphylococci, vancomycin-resistant enterococci, carbapenem-resistant Enterobacterales, multidrug-resistant *Acinetobacter*, and resistant *Pseudomonas*. Operating-room wastewater may receive blood, tissue residues, antiseptic compounds, cleaning chemicals, and residual pharmaceuticals generated during surgical preparation and postoperative sanitation. Clinical laboratories contribute culture media, stains, buffers, diagnostic reagents, microbial residues, and genetic material associated with specimen processing, although disposal controls influence the amount reaching wastewater systems. Hospital pharmacies, oncology departments, dialysis units, radiology departments, kitchens, laundries, and sterilization facilities add separate chemical and organic inputs. Pharmaceutical residues may include antibiotics, antifungals, antivirals, analgesics, hormones, anesthetics, and contrast agents. Antimicrobial compounds can enter wastewater through patient excretion, disposal of unused medication, rinsing of containers, and cleaning of drug-administration equipment (Hutinel et al., 2022; Sheak, 2024). Disinfectants and detergents used on surfaces, instruments, linens, and patient-care materials introduce quaternary ammonium compounds, chlorine-based products, alcohols, aldehydes, oxidizing agents, and surfactants. Metals and diagnostic chemicals may also enter from laboratories, medical devices, plumbing materials, and specialized procedures. The resulting wastewater contains variable concentrations of organic matter, nutrients, suspended solids, microorganisms, pharmaceuticals, chemical stressors, and free genetic material. This composition supports the classification of hospital wastewater as a distinct resistance environment rather than merely a concentrated form of domestic sewage.

Hospital wastewater and domestic sewage share several basic characteristics because both receive human excreta, food residues, cleaning products, microorganisms, nutrients, and household chemicals. Their resistance profiles and chemical compositions differ in important ways, however, because hospitals concentrate individuals receiving antimicrobial therapy and provide care for patients with severe infections, chronic diseases, compromised immunity, complex surgical conditions, and prolonged healthcare exposure (Shakhawat & Md, 2024; Zhu et al., 2022). Domestic sewage represents a broad community population and contains resistance determinants originating from healthy residents, outpatient antimicrobial use, household medication disposal, food consumption, companion animals, and community carriage. Hospital effluent represents a narrower population with greater clinical intensity, higher antimicrobial exposure, and increased contact with invasive devices and healthcare-associated organisms. The distinction is not absolute because resistant bacteria circulate between hospitals and communities through patients, staff, visitors, long-term-care facilities, food systems, and travel. Nevertheless, comparative wastewater investigations have frequently identified higher concentrations, greater detection frequencies, or broader diversity of selected clinically

important resistance genes in hospital discharges. Hospital effluent may be enriched with genes associated with extended-spectrum β -lactam resistance, carbapenem resistance, methicillin resistance, vancomycin resistance, aminoglycoside resistance, fluoroquinolone resistance, and multidrug efflux. Domestic sewage often contains high quantities of common tetracycline, sulfonamide, macrolide, and general multidrug-resistance determinants because these genes are widely distributed among human intestinal and environmental bacteria. Differences between the two wastewater types depend on hospital size, patient population, antimicrobial-consumption patterns, sewer connectivity, wastewater volume, geographic background, community resistance, and sampling methods. Hospital wastewater is often less diluted before entering the municipal sewer, allowing facility-associated signals to be detected near the discharge point (Nishat & Jahanara, 2024; Zhu et al., 2022). Once mixed with domestic sewage, the hospital contribution becomes harder to distinguish because dilution changes gene concentrations and community wastewater introduces additional resistance sources. Chemical characteristics also affect these comparisons. Hospitals may discharge higher concentrations of specialized pharmaceuticals, disinfectants, diagnostic compounds, and antimicrobial metabolites, while domestic sewage contains larger contributions from household detergents, personal-care products, food waste, and community medications. Wastewater flow patterns differ because hospital discharges reflect patient routines, clinical schedules, cleaning cycles, meal preparation, laboratory work, and shift changes. Domestic sewage follows residential patterns associated with morning, evening, and weekend activities. A literature-based evaluation must therefore consider concentration, total discharge, bacterial biomass, fecal strength, sampling time, and wastewater flow when comparing hospital and domestic systems (Gönder et al., 2021; Tonay et al., 2024). The distinctive nature of hospital wastewater lies in the convergence of clinically important organisms, antimicrobial exposure, chemical stressors, mobile genetic elements, and continuous inputs from healthcare operations.

Figure 4: Hospital Wastewater Resistance Transmission Pathway



Hospital activities influence resistance-gene discharge by shaping the types and quantities of antimicrobials, microorganisms, chemical residues, and biological materials entering wastewater. Broad-spectrum antimicrobial administration is a major source of selective pressure because these drugs act against multiple bacterial groups and can disrupt the normal microbiota of treated patients (Yao et al., 2021). The suppression of susceptible bacteria may increase the proportional representation of resistant organisms within the intestinal, urinary, respiratory, or skin microbiome. Patients then excrete resistant bacteria and resistance genes through urine, feces, wound fluids, sputum, and other bodily materials. The use of last-resort antimicrobials is especially relevant in units treating severe infections caused by multidrug-resistant organisms. Carbapenems, glycopeptides, polymyxins, advanced cephalosporins, and other specialized agents are frequently used when common treatments have limited effectiveness. Their use does not automatically produce corresponding resistance genes, but it creates selective conditions that favor organisms already carrying protective determinants.

Hospitalized patients may also arrive while colonized with resistant organisms acquired in communities, other hospitals, nursing homes, rehabilitation centers, international healthcare facilities, or previous treatment settings. Colonization may remain undetected because routine diagnostic testing generally targets symptomatic infection. Colonized patients can release large numbers of resistant intestinal or skin-associated bacteria into wastewater without appearing in infection statistics. Antimicrobial disposal practices provide another pathway. Unused liquid drugs, residual intravenous solutions, partially filled containers, and medication-contaminated rinsing water may enter drains when disposal procedures are inconsistent or poorly followed. Even when formal pharmaceutical-waste programs are present, active compounds and metabolites excreted by patients remain unavoidable components of wastewater (Paulshus et al., 2019). Cleaning and disinfection practices add further selective agents. Healthcare facilities use large quantities of chlorine compounds, quaternary ammonium compounds, peroxide-based products, alcohols, detergents, and other biocides to control pathogens on surfaces, equipment, linens, and patient-care materials. These chemicals can enter drainage systems during routine cleaning and equipment decontamination. Bacteria carrying efflux pumps or linked resistance determinants may tolerate several antimicrobial and disinfectant compounds, allowing co-selection within wastewater microbial communities. Clinical laboratories also influence the resistance signal through the processing of patient specimens, cultivation of microorganisms, antimicrobial susceptibility testing, molecular diagnostics, and cleaning of laboratory equipment. Laboratory waste-management procedures determine whether microbial cultures and genetic material are sterilized, contained, or released into wastewater (Kraupner et al., 2021). The combined effects of antimicrobial administration, patient colonization, drug disposal, cleaning, disinfection, and diagnostic activities create a resistance-gene discharge profile closely linked to the organization and intensity of hospital care.

Hospital operational characteristics help explain differences in resistance-gene abundance among facilities and across sampling periods. Bed capacity provides a general measure of hospital size, but occupied-bed days and patient-days offer more precise indicators of the population contributing biological material to the wastewater stream (Gaşpar et al., 2021). A large hospital operating below capacity may discharge less patient-associated material than a smaller facility with consistently high occupancy. Patient-days also reflect the duration of exposure because each hospitalized person contributes excreta, microorganisms, antimicrobial metabolites, and other biological materials throughout the admission period. Case severity can influence resistance-gene discharge through its relationship with antimicrobial intensity, invasive procedures, immune suppression, and length of stay. Intensive care units often have high antimicrobial-consumption rates and manage patients requiring mechanical ventilation, urinary catheters, central lines, surgical interventions, dialysis, or prolonged treatment. These conditions increase opportunities for colonization by healthcare-associated organisms and may produce concentrated wastewater signals from intensive-care drainage areas. Isolation units treating patients with known resistant infections can generate distinct resistance profiles, especially when their wastewater enters identifiable drainage lines before mixing with the hospital's total discharge (Selvarajan et al., 2021). Surgical activity, transplant care, oncology treatment, burn care, and long-term hospitalization may also affect the quantity and composition of resistance determinants entering wastewater. Antimicrobial consumption can be assessed through days of therapy, defined daily doses, prescription records, pharmacy dispensing data, or class-specific usage. Each measurement captures a different aspect of drug exposure and may not correspond perfectly with compounds or genes detected in wastewater. The relationship between use and resistance is influenced by drug metabolism, excretion rates, bacterial carriage, wastewater dilution, treatment duration, and the presence of resistance genes before therapy begins. Hospital wastewater flow must also be considered because high water use can lower measured concentrations without reducing the total quantity of resistance material discharged. Cleaning cycles, laundry operations, kitchen activity, laboratory schedules, and water-conservation practices further affect daily flow and chemical composition. Repeated sampling commonly reveals substantial variation by hour, day, clinical workload, and hospital department. A single wastewater sample may therefore reflect a temporary combination of occupancy, medication schedules, patient excretion, cleaning activity, and water use rather than the facility's typical resistance profile (Li et al., 2022). The literature supports the combined

examination of hospital size, patient-days, bed occupancy, intensive-care activity, antimicrobial use, case severity, wastewater flow, and clinical resistance records when interpreting resistance-gene discharge.

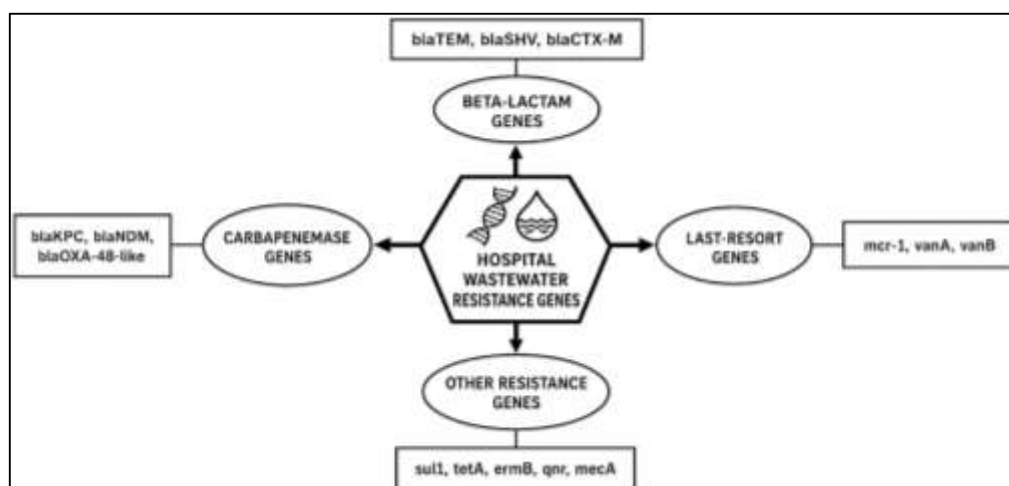
Hospital plumbing systems function as active microbial habitats rather than passive channels that immediately transport waste away from healthcare facilities. Sink drains, faucet components, toilets, shower outlets, floor drains, traps, connecting pipes, sewer lines, wastewater tanks, and collection chambers contain wet surfaces that support microbial attachment and growth. Water remains in traps and low-flow sections, while organic matter from bodily waste, soap, food residues, and clinical activities provides nutrients for microbial communities (Kehl et al., 2022). Bacteria initially attach to plumbing surfaces and produce extracellular substances composed of polysaccharides, proteins, lipids, and genetic material. This matrix develops into a biofilm that anchors microorganisms, retains moisture, traps nutrients, and protects cells from hydraulic disturbance and chemical exposure. Biofilm formation is influenced by surface material, temperature, water flow, nutrient availability, pipe age, disinfectant concentration, and the frequency of drainage use. Mature biofilms contain diverse bacterial populations organized within chemical and oxygen gradients. Organisms at the surface experience different conditions from cells located deeper within the matrix, producing variation in growth rate, metabolism, and antimicrobial susceptibility. Hospital plumbing biofilms may contain environmental bacteria, intestinal organisms, opportunistic pathogens, and healthcare-associated resistant organisms. Planktonic bacteria moving through wastewater interact continuously with these established communities. Some flowing cells attach to biofilms, while other organisms detach from the matrix and reenter the wastewater (Paulus et al., 2019). This exchange allows hospital-associated bacteria to persist within plumbing after the original patient source is no longer present. Biofilms also promote close contact among bacterial cells, creating conditions that support the exchange of plasmids and other mobile genetic elements. Extracellular DNA released through cell lysis or active secretion becomes incorporated into the biofilm matrix, where it contributes to structural stability and forms a potential source of resistance genes for bacteria capable of genetic transformation. Antimicrobial residues, disinfectants, metals, and diagnostic chemicals may also adsorb to organic material or accumulate within slow-flow sections. Their local concentrations can differ from those measured in free-flowing wastewater, creating small ecological zones with distinct selection pressures. Repeated exposure to fluctuating antimicrobial and disinfectant concentrations may favor organisms possessing efflux systems, permeability barriers, stress-response pathways, and mobile resistance determinants. Plumbing biofilms therefore contribute to the retention, enrichment, exchange, and intermittent release of resistance-related genetic material within hospital wastewater environments (Cai et al., 2021). The association between hospital plumbing and healthcare-related resistant organisms is documented through investigations of contaminated sinks, drains, toilets, traps, wastewater pipes, and drainage networks. Resistant *Klebsiella*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Acinetobacter*, and related organisms have been recovered from plumbing environments connected to patient-care areas. Genetic analysis in several healthcare investigations has identified close relationships between environmental isolates and organisms obtained from patients, although the direction of transmission is not always clear. Patients may introduce resistant bacteria into drains through washing, excretion, disposal of bodily fluids, or contaminated equipment. Plumbing biofilms may then retain these organisms and release them into wastewater over extended periods. In some situations, droplets, aerosols, splashing, or contaminated surfaces can connect drain-associated organisms with the patient-care environment. Sink design, drain position, water pressure, plumbing configuration, and cleaning practices influence the likelihood of environmental dispersal (Laquaz et al., 2018). Toilets and wastewater pipes also receive high concentrations of fecal bacteria and antimicrobial residues, making them important locations for the accumulation of resistance genes. Storage tanks and collection chambers provide longer retention times than rapidly flowing pipes, allowing sedimentation, biofilm development, microbial interaction, and chemical transformation. Plumbing interventions such as chemical disinfection, thermal treatment, drain replacement, sink removal, or redesign may temporarily reduce microbial contamination, but organisms can remain in inaccessible pipe sections or reestablish communities after treatment. Biofilm tolerance to disinfectants arises from limited chemical penetration, slow microbial growth, stress-response activation, and the protective extracellular matrix.

Resistance genes may also remain within extracellular DNA after bacterial cells are damaged or killed. For these reasons, culture-based testing, molecular quantification, and genomic sequencing may produce different descriptions of the same plumbing habitat. Culture identifies viable organisms capable of growing under selected laboratory conditions, while molecular methods detect genetic material from culturable, unculturable, damaged, and nonviable sources. Sampling free-flowing wastewater alone may underrepresent organisms retained within pipe biofilms, whereas swabbing drains without measuring discharged wastewater may not represent the total resistance load entering municipal sewers (Sib et al., 2019). Synthesized evidence from hospital wastewater and healthcare plumbing research therefore characterizes the plumbing network as a dynamic ecological interface connecting patients, clinical practices, resistant microorganisms, mobile genetic elements, antimicrobial residues, and municipal wastewater infrastructure.

Clinically Important Resistance Genes in Hospital Wastewater

β -lactam-resistance genes are among the most frequently investigated antimicrobial resistance determinants in hospital wastewater because β -lactam agents are widely used in clinical care and resistance to these drugs is common among healthcare-associated bacterial pathogens. The β -lactam group includes penicillins, cephalosporins, monobactams, and carbapenems, all of which act by interfering with bacterial cell-wall synthesis (Cai et al., 2021). Bacteria resist these compounds primarily through β -lactamase enzymes that hydrolyze the β -lactam ring and prevent the antimicrobial from binding effectively to its cellular target. The blaTEM, blaSHV, and blaCTX-M families are especially important because they include variants capable of hydrolyzing extended-spectrum cephalosporins. Some members of these families provide resistance mainly to earlier penicillins, while others have accumulated mutations that expand their activity against third- and fourth-generation cephalosporins. Hospital wastewater receives these genes through the excretion of colonized or infected patients, disposal of biological materials, clinical laboratory activities, and the release of bacteria from healthcare plumbing biofilms. The genes may occur in *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* species, *Citrobacter* species, and other members of the order Enterobacterales. Their frequent association with plasmids, integrons, insertion sequences, and transposons increases their environmental importance because mobile genetic structures can transfer resistance among related or unrelated bacteria (Marathe et al., 2019). Comparative wastewater investigations commonly detect blaTEM and blaCTX-M in both hospital and community sewage, while blaSHV may show greater variability according to bacterial-host composition, hospital activity, and local resistance patterns. High detection frequency does not necessarily establish a hospital-specific source because these genes are also widely carried by community-associated intestinal bacteria. Hospital effluent may nevertheless contain elevated absolute concentrations or a larger proportion of clinically significant variants because hospitalized populations have greater exposure to cephalosporins and a higher likelihood of colonization by extended-spectrum β -lactamase-producing Enterobacterales. Absolute abundance describes the number of gene copies within a defined wastewater volume or mass of solids, whereas normalized abundance evaluates the representation of a target relative to total bacterial markers or other reference measurements. Absolute and normalized measurements can produce different interpretations when wastewater dilution, bacterial biomass, suspended solids, or hospital water use changes. A high absolute concentration may reflect large quantities of fecal material, while a high normalized abundance may indicate enrichment of the target within the bacterial community (Hutinel et al., 2022). For this reason, gene concentration, wastewater flow, fecal strength, bacterial biomass, and host identification must be considered together when comparing hospital and community samples.

The relationship between β -lactamase genes and resistant Enterobacterales is central to interpreting their clinical significance in hospital wastewater. Enterobacterales are common inhabitants of the human intestinal tract, making fecal discharge an important route by which resistant organisms and their genes enter sanitation systems (Salah et al., 2020). Extended-spectrum β -lactamase-producing *E. coli* and *K. pneumoniae* may colonize patients without symptoms or cause urinary tract infections, bloodstream infections, pneumonia, intra-abdominal infections, and surgical-site infections. Patients receiving antimicrobial treatment can excrete these organisms throughout hospitalization, and colonized individuals may continue contributing resistant bacteria even when no clinical infection is recorded.

Figure 5: Hospital Wastewater Antimicrobial Resistance Genes

Wastewater detection may therefore represent both diagnosed cases and unmeasured colonization. The blaCTX-M family has become particularly prominent because its variants are distributed across community- and healthcare-associated Enterobacterales. In contrast, blaTEM and blaSHV families contain large numbers of variants with different resistance profiles, so detection at the family level does not automatically confirm an extended-spectrum phenotype. Sequence-level characterization is necessary to distinguish narrow-spectrum genes from variants capable of hydrolyzing advanced cephalosporins. Short molecular assays can quantify selected families efficiently, but they may not determine the complete allele, bacterial host, genomic location, or surrounding mobile elements (Loudermilk et al., 2022). Metagenomic sequencing broadens detection across many genes, although low-abundance targets may be missed when sequencing depth is insufficient. Culture-based analysis confirms viable resistant organisms and permits antimicrobial susceptibility testing, but it captures only bacteria that grow under the selected conditions. Linking molecular measurement with cultured isolates improves interpretation by connecting gene presence to viable Enterobacterales and phenotypic resistance. Wastewater-treatment processes may reduce the absolute concentration of β -lactamase genes by removing bacterial cells and suspended solids, yet selected determinants can remain detectable in treated effluent and sludge. Their persistence depends on the bacterial host, association with particles, protection within biofilms, treatment conditions, and stability of extracellular DNA. Comparing hospital discharge with upstream community sewage, downstream sewer samples, municipal influent, treated effluent, and wastewater solids helps distinguish local enrichment from background occurrence (Sib et al., 2019). Repeated sampling is also important because detection frequency and abundance vary with patient occupancy, antimicrobial use, wastewater flow, time of day, and episodic shedding by colonized patients. A synthesized interpretation therefore treats blaTEM, blaSHV, and blaCTX-M as heterogeneous gene families whose clinical meaning depends on allele identity, abundance, bacterial host, mobility, viability, and association with resistant Enterobacterales.

Carbapenem-resistance genes receive substantial attention in hospital wastewater because carbapenems are critically important treatments for severe infections caused by organisms resistant to other β -lactam agents. The major carbapenemase families detected in clinical and wastewater environments include blaKPC, blaNDM, blaVIM, blaIMP, and blaOXA-48-like. These genes encode enzymes that hydrolyze carbapenems and often affect additional β -lactam compounds. Their clinical importance is intensified by their frequent occurrence in bacteria carrying resistance to fluoroquinolones, aminoglycosides, sulfonamides, and other antimicrobial classes (Thakali et al., 2021). Carbapenem-resistant Enterobacterales, including resistant *K. pneumoniae*, *E. coli*, *Enterobacter cloacae* complex, and *Citrobacter* species, are associated with difficult-to-treat urinary, respiratory, bloodstream, wound, and intra-abdominal infections. Carbapenemase genes may also occur in *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and environmental organisms. Their presence in

hospital wastewater can originate from infected patients, asymptomatic intestinal carriers, intensive-care units, isolation wards, laboratories, and plumbing biofilms. Detection patterns vary across hospitals and regions because carbapenemase families have different epidemiological distributions. blaKPC is strongly associated with healthcare-related Enterobacterales in the United States, whereas blaNDM, blaVIM, blaIMP, and blaOXA-48-like may display more variable local occurrence connected to patient movement, international healthcare exposure, regional outbreaks, and bacterial-host distribution. Hospital wastewater can reveal low-frequency resistance determinants that may not appear consistently in routine clinical reports, but environmental detection does not establish an active outbreak or identify the contributing patient population (Wařko et al., 2022). Associations between intensive-care activity and carbapenem-resistance genes may arise from high antimicrobial exposure, severe illness, invasive procedures, prolonged hospitalization, and greater colonization pressure. Carbapenem consumption can create selection favoring organisms carrying carbapenemase genes, although the relationship is influenced by prior colonization, infection-control practices, drug metabolism, fecal shedding, wastewater flow, and the use of other antimicrobials that co-select linked genes. Quantitative comparisons therefore require measures of bed occupancy, intensive-care patient-days, class-specific antimicrobial use, clinical prevalence of carbapenem-resistant organisms, and wastewater volume (Sakkas et al., 2019). Rare genes also require careful treatment of detection limits because nondetection may result from low concentration, small sample volume, poor recovery, molecular inhibition, or insufficient sequencing depth. Sensitive targeted assays, replicate analysis, concentration procedures, and genomic confirmation improve the reliability of carbapenemase-gene detection.

Resistance to last-resort antimicrobial agents also includes mobilized colistin-resistance genes and vancomycin-resistance determinants. Colistin is used in selected cases involving multidrug-resistant Gram-negative organisms, and the discovery of transferable mcr genes established that colistin resistance can circulate through plasmids rather than remain restricted to chromosomal mutation. mcr-1 is the most widely recognized member, while related variants extend the diversity of mobilized colistin resistance across human, animal, food, wastewater, and environmental bacteria (Majlander et al., 2021). These genes have been identified in Enterobacterales such as *E. coli*, *Klebsiella*, *Enterobacter*, and *Salmonella*, as well as in other bacterial hosts. Their occurrence in hospital wastewater may reflect patient colonization, clinical infection, animal- or food-associated exposure, international travel, community carriage, or the movement of plasmids between bacteria. Low prevalence creates analytical challenges because a gene may occur below the detection threshold in a small sample while remaining present in the larger wastewater stream. Wastewater solids may concentrate bacterial cells and genetic material more effectively than the liquid fraction, producing different detection results. Enrichment culture can improve recovery of viable resistant organisms but alters the original community and cannot provide an unbiased estimate of abundance (Zhu et al., 2022). Vancomycin resistance is commonly associated with vanA and vanB, which modify the bacterial cell-wall target and reduce glycopeptide binding. These determinants are strongly connected with vancomycin-resistant enterococci, especially *Enterococcus faecium* and *Enterococcus faecalis*, although related genes occur in other bacteria. Hospital wastewater may receive vancomycin-resistant enterococci from patients with diagnosed infection or asymptomatic gastrointestinal colonization. The vanA determinant commonly produces high-level resistance to vancomycin and teicoplanin, whereas vanB is associated with variable vancomycin resistance and generally retains teicoplanin susceptibility. Molecular detection alone does not establish whether these genes are expressed, carried by viable enterococci, or located in clinically significant mobile elements (Kehl et al., 2022). Their interpretation is strengthened by bacterial culture, host-specific markers, sequencing of surrounding genetic regions, and comparison with clinical isolates. For mcr, vanA, vanB, and rare carbapenemase targets, surveillance accuracy depends on sample volume, sampling frequency, concentration efficiency, inhibition control, analytical sensitivity, and transparent reporting of nondetectable results.

Hospital wastewater contains many high-abundance resistance genes beyond β -lactamase, carbapenemase, colistin-resistance, and vancomycin-resistance determinants. Sulfonamide-resistance genes such as sul1 and sul2 are frequently detected in hospital and municipal wastewater because they are widely distributed among intestinal and environmental bacteria and often occur on mobile genetic

elements ([Hassoun-Kheir et al., 2020](#)). *sul1* is commonly associated with class 1 integrons and may function as an indicator of human-derived pollution and genetic mobility. Its abundance can correspond with the presence of additional resistance-gene cassettes carried within the same genetic structure. *sul2* is frequently located on plasmids or small mobile elements and is also broadly distributed across Gram-negative bacteria. Tetracycline-resistance genes, including *tetA*, *tetM*, and *tetW*, represent different resistance mechanisms. *tetA* commonly encodes an efflux pump that removes tetracycline from bacterial cells, whereas *tetM* and *tetW* generally encode ribosomal-protection proteins that reduce the ability of tetracycline compounds to inhibit protein synthesis. These genes are often abundant in sewage because tetracycline resistance is widespread among human intestinal, animal-associated, and environmental organisms. Their broad distribution can limit their specificity as markers of hospital-associated resistance, although changes in concentration and normalized abundance still provide information about wastewater resistome composition. The macrolide-resistance gene *ermB* modifies the ribosomal target through methylation and may confer resistance to macrolide, lincosamide, and streptogramin B agents. It is frequently detected in fecal and wastewater bacteria, including enterococci, streptococci, and other Gram-positive organisms ([Hamiwe et al., 2019](#)). Fluoroquinolone-resistance genes in the *qnr* family protect bacterial DNA-processing enzymes from antimicrobial inhibition. These genes usually produce lower-level resistance than some chromosomal mutations, but their presence can support bacterial survival and facilitate the selection of additional resistance mechanisms. Their location on plasmids connects fluoroquinolone resistance with other determinants carried by the same mobile structure. The high abundance of these genes in wastewater does not always correspond with high rates of clinical infection because wastewater combines genetic material from pathogenic, commensal, environmental, viable, damaged, and nonviable sources ([Voigt et al., 2020](#)). Their value lies in describing the overall resistance burden, identifying mobile genetic associations, and measuring differences among hospital units, facilities, sewer locations, and treatment stages.

Aminoglycoside- and methicillin-resistance genes further expand the clinically important wastewater resistome. Aminoglycoside-resistance determinants include *aac*, *aph*, and *aad* gene families, which encode enzymes that acetylate, phosphorylate, or adenylate aminoglycoside compounds. These chemical modifications reduce antimicrobial binding and limit the effectiveness of agents used against severe Gram-negative and Gram-positive infections ([Galarde-López et al., 2022](#)). Individual gene families contain numerous variants with different substrate profiles, making sequence-level identification important for determining which aminoglycosides may be affected. The genes frequently occur on plasmids, integrons, transposons, and multidrug-resistance regions containing β -lactam, sulfonamide, trimethoprim, or fluoroquinolone resistance determinants. Their detection in hospital wastewater may therefore indicate complex mobile structures rather than isolated resistance mechanisms. Methicillin resistance is principally associated with *mecA*, which encodes an altered penicillin-binding protein with low affinity for many β -lactam agents. The gene is strongly associated with methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci. *mecC* is a related determinant that is less frequently detected and may be missed by assays designed only for *mecA* ([Wang et al., 2018](#)). Hospital wastewater can receive methicillin-resistant staphylococci from skin shedding, bathing, handwashing, wound care, laundry, environmental cleaning, and disposal of clinical materials. Detection of *mecA* alone does not prove the presence of methicillin-resistant *S. aureus* because the gene may be carried by several staphylococcal species or occur in free genetic material. Host-specific markers, culture, genomic sequencing, and analysis of the surrounding resistance element are needed for stronger attribution. The combined occurrence of *sul*, *tet*, *erm*, *qnr*, *aac*, *aph*, *aad*, *mecA*, and *mecC* illustrates the functional diversity of hospital wastewater resistomes. Some targets are consistently abundant and provide stable indicators of resistance contamination, while others vary according to clinical activity, microbial hosts, antimicrobial exposure, and local epidemiology ([Proia et al., 2018](#)). Quantitative interpretation should incorporate absolute concentration, normalized abundance, detection frequency, wastewater flow, solids content, bacterial biomass, and mobile-element association. These measures allow high-abundance background genes to be distinguished from lower-abundance determinants with greater clinical specificity.

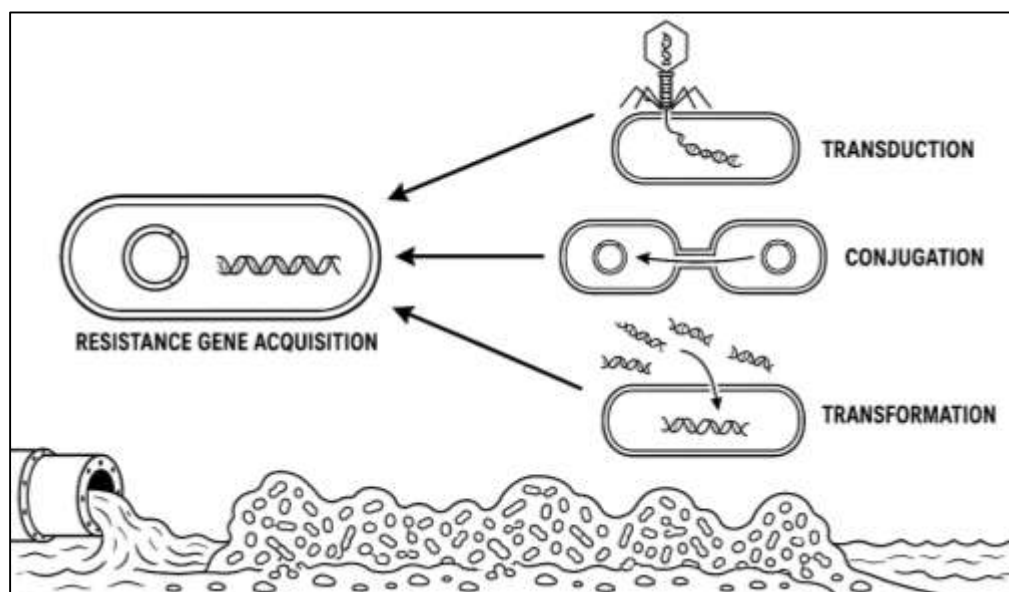
Horizontal Gene Transfer and Mobile Genetic Elements

Horizontal gene transfer refers to the movement of genetic material between microorganisms through processes other than the direct inheritance of DNA from a parent cell to its descendants. In hospital wastewater, horizontal transfer is important because resistant bacteria, susceptible bacteria, antimicrobial resistance genes, mobile genetic elements, pharmaceutical residues, disinfectants, organic matter, and extracellular DNA occur together within a connected aqueous environment (Brito, 2021). Conjugation is one of the principal mechanisms through which resistance genes move between bacterial cells. It usually involves direct cell-to-cell contact and the transfer of plasmids or other conjugative genetic elements from a donor bacterium to a recipient. Conjugative plasmids encode the biological machinery needed to establish contact, process DNA, and transport genetic material between cells. Some plasmids transfer only among closely related bacteria, whereas broad-host-range plasmids can circulate across different genera or families. This capacity is especially relevant in hospital wastewater, where intestinal Enterobacterales, enterococci, staphylococci, nonfermenting Gram-negative bacteria, environmental organisms, and opportunistic pathogens may interact. A plasmid carrying a β -lactamase, carbapenemase, aminoglycoside-resistance determinant, or several resistance genes can enter a susceptible recipient and rapidly change its resistance profile. Conjugation does not require an antimicrobial concentration high enough to kill susceptible organisms. Subinhibitory antimicrobial concentrations, chemical stress, nutrient availability, high bacterial density, and biofilm growth may alter cell physiology in ways that affect plasmid transfer (Wallau et al., 2018). Biofilms are particularly favorable settings because bacteria remain close together within an extracellular matrix that retains cells, nutrients, chemical compounds, and genetic material. Hospital drains, sink traps, sewer pipes, storage tanks, and sedimented wastewater solids provide surfaces on which these structured microbial communities develop. Conjugative transfer can occur between organisms attached to the same surface or between attached and free-living cells moving through the wastewater. The success of plasmid exchange depends on donor and recipient compatibility, plasmid stability, transfer machinery, temperature, pH, nutrient conditions, cell density, hydraulic disturbance, and the biological cost imposed on the new host. A transferred plasmid may be lost when it reduces host fitness and provides no survival advantage, or it may persist when selection favors its resistance determinants or when plasmid-maintenance systems promote stable inheritance (Bello-López et al., 2019). Conjugation therefore connects short-term environmental exposure with the longer retention of resistance traits in bacterial populations. Within hospital wastewater, it serves as a mechanism through which clinically important resistance genes can move from patient-associated bacteria into commensal or environmental hosts that remain within sewer biofilms or pass into municipal wastewater.

Transformation and transduction provide additional routes for horizontal movement of antimicrobial resistance genes. Transformation occurs when a bacterium takes up extracellular DNA from its surrounding environment and incorporates that material into its chromosome or maintains it as a genetic element. Extracellular DNA enters hospital wastewater through bacterial lysis, active secretion, bacteriophage activity, biofilm turnover, and the disposal of human or laboratory-derived biological material (Forster et al., 2022). It may contain complete resistance genes, fragments of mobile elements, virulence determinants, or regulatory sequences. Wastewater-treatment processes and disinfectants can damage or kill bacterial cells without immediately destroying all released DNA. Extracellular genetic material can bind to suspended solids, organic particles, sediments, minerals, and biofilm matrices, which may protect it from enzymatic degradation and extend its persistence. Only naturally competent bacteria can take up free DNA under particular physiological conditions, and successful transformation requires the acquired sequence to remain stable or recombine with the recipient genome. Transformation frequency is influenced by DNA concentration, fragment length, sequence similarity, bacterial competence, temperature, nutrient limitation, oxidative stress, surface attachment, and the presence of inhibitory chemicals. Transduction occurs when bacteriophages transfer bacterial DNA between host cells. During phage replication, fragments of the bacterial genome may be packaged accidentally into viral particles and transported to another bacterium. Some phages carry specific bacterial genes, while others can transfer a wider assortment of genetic material. Phage particles may protect DNA from degradation and move through wastewater, solids, biofilms, and receiving waters (Rodríguez-Beltrán et al., 2021). Their host range influences which bacteria can receive transferred

material, although phage-mediated movement may connect bacterial populations that do not exchange plasmids efficiently. The relative contribution of conjugation, transformation, and transduction varies among wastewater settings and is difficult to determine through gene detection alone. Finding a resistance gene in extracellular DNA, a plasmid-associated sequence, or a bacteriophage fraction identifies possible mobility but does not prove that transfer occurred within the sampled system. Experimental transfer assays, genomic linkage, culture-based analysis, and sequence comparisons provide stronger evidence. Gene-transfer frequency is also shaped by wastewater retention time, bacterial abundance, microbial diversity, oxygen availability, organic carbon, temperature, pH, antimicrobial residues, disinfectants, metals, and hydraulic flow. High cell densities increase opportunities for contact, while longer retention creates more time for microbial interaction (Koonin et al., 2020). Chemical stress may promote cellular responses connected to DNA repair, competence, gene exchange, or bacteriophage activation. Hospital wastewater therefore contains multiple biological routes through which resistance determinants can circulate, with each route responding differently to the physical, chemical, and microbial conditions of the plumbing and sewer environment.

Mobile genetic elements organize and transport antimicrobial resistance determinants within hospital wastewater resistomes. Plasmids are independently replicating DNA molecules that occur outside the bacterial chromosome and range from small structures carrying a few genes to large multidrug-resistance plasmids containing numerous resistances, virulence, metabolism, and maintenance determinants. Broad-host-range plasmids are particularly important because they can replicate or persist in diverse bacterial hosts, allowing resistance genes to cross taxonomic boundaries. Their transfer may connect clinical Enterobacterales with environmental Gram-negative bacteria or move determinants among opportunistic pathogens inhabiting drains and sewer biofilms (Redondo-Salvo et al., 2020). Integrons are genetic platforms that capture, arrange, and express gene cassettes through an integrase enzyme and a recombination site. Class 1 integrons are common in human-affected environments and are frequently associated with resistance to aminoglycosides, sulfonamides, trimethoprim, β -lactams, and other antimicrobial classes. The *intI1* gene encodes the class 1 integrase and is widely examined as an indicator of anthropogenic pollution, genetic mobility, and resistance-gene accumulation. Its presence does not identify the specific cassettes within an integron, but high *intI1* abundance can correspond with wastewater heavily influenced by human activity and antimicrobial selection. Resistance-gene cassettes usually contain a functional gene and a recombination site but lack independent transfer machinery. Integrons capture these cassettes, while plasmids and transposons can transport the assembled structures between cells or genomic locations (Lima et al., 2020). Transposons are DNA segments capable of moving within or between chromosomes and plasmids. Insertion sequences are smaller mobile elements that generally encode the enzymes needed for movement and can mobilize adjacent resistance genes, alter gene expression, or promote genomic rearrangement. These elements create nested genetic structures in which an insertion sequence flanks a resistance gene, a transposon carries that unit, an integron contains several gene cassettes, and a plasmid transfers the larger resistance region between bacterial hosts. Metagenomic assembly can identify the co-location of multiple ARGs on the same contiguous sequence, strengthening evidence that the genes occupy a shared genetic neighborhood. Long sequencing reads provide greater structural resolution than short fragments because they can span complete resistance regions and link genes with plasmid markers, integrases, transposases, or bacterial chromosomes. The proportion of mobile ARGs among all detected resistance genes can be described without relying on a mathematical formula by reporting how many resistance determinants occur near recognized mobility markers relative to the total set identified. Interpretation remains dependent on assembly quality, sequencing depth, database accuracy, contig length, and the criteria used to define genetic linkage (Gilbert & Feschotte, 2018). The organization of resistance genes on mobile elements has direct relevance to multidrug resistance because selection for one determinant can preserve several additional genes located on the same plasmid, integron, or transposon. A mobile region selected by β -lactam exposure may also contain genes providing resistance to aminoglycosides, sulfonamides, trimethoprim, tetracyclines, fluoroquinolones, metals, or disinfectants (Husnik & McCutcheon, 2018). This physical linkage allows antimicrobial use to maintain resistance against compounds that are absent from the wastewater at the time of sampling.

Figure 6: Hospital Wastewater Horizontal Gene Transfer

Hospital effluent provides repeated inputs of bacteria and mobile elements from patient excreta, clinical materials, laboratory activities, and plumbing biofilms. Once released, mobile resistance structures may persist in their original clinical hosts, transfer to commensal bacteria, or enter environmental organisms adapted to sewer conditions. Environmental hosts may act as long-term carriers because they can survive outside patients and remain within biofilms, sediments, sludge, and treatment systems. The detection of identical resistance genes in hospital and municipal wastewater does not establish direct transfer, since both environments may receive genes from common human sources. Greater evidence of movement comes from shared plasmid structures, matching insertion sequences, comparable resistance-gene arrangements, closely related bacterial genomes, and consistent spatial patterns across connected sampling locations (Zhao et al., 2019). Quantifying mobile elements also requires distinction between genetic potential and demonstrated transfer. A gene located near a transposase or integrase has a plausible route of movement, but the element may be inactive, incomplete, or restricted to a narrow range of hosts. Culture-based conjugation experiments can test whether plasmids transfer under controlled conditions, while transformation assays examine the uptake of extracellular DNA. Genome-resolved metagenomics can identify likely bacterial carriers, and hybrid sequencing can reconstruct plasmids and resistance regions more completely. Wastewater solids and biofilms often contain dense microbial communities and concentrated genetic material, making them valuable matrices for mobile-element analysis. Liquid wastewater represents the material actively transported through sewers, while solids and biofilms represent retained or particle-associated reservoirs. Combining these matrices produces a fuller description of mobility within the hospital wastewater environment. A synthesized assessment therefore considers the abundance of *intI1*, plasmid replicons, transposases, insertion sequences, gene cassettes, co-located ARGs, and identified bacterial hosts (Mazhar et al., 2021). These measurements describe how resistance genes are packaged, retained, and distributed rather than merely recording whether individual determinants are present.

Antimicrobial Residues and Selective Pressure

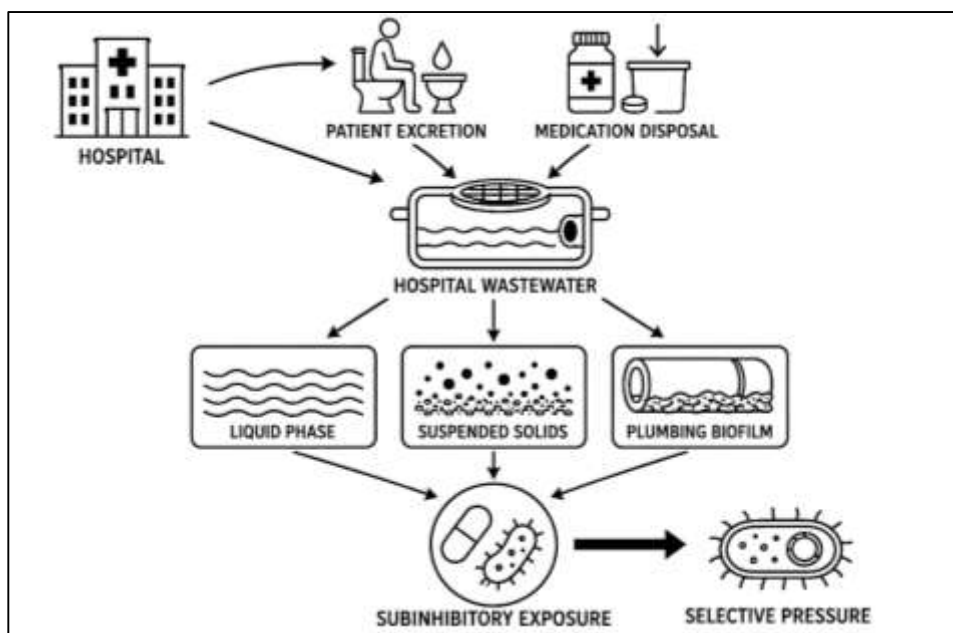
Antimicrobial residues enter hospital wastewater through several clinical and operational pathways, with patient excretion representing one of the most continuous sources. After an antimicrobial medication is administered, only a portion may be absorbed, distributed, metabolized, and retained or transformed by the human body (Serwecińska, 2020). The remaining fraction can be excreted through urine or feces as the unchanged parent compound, an active metabolite, an inactive metabolite, or a conjugated chemical product. The proportion excreted in each form differs according to antimicrobial class, dosage, route of administration, patient physiology, kidney and liver function, age, illness

severity, and treatment duration. Some compounds are eliminated largely through urine, while others undergo substantial metabolism or are excreted through feces. Active metabolites may retain antimicrobial properties and contribute to selective pressure even when the original drug concentration has declined. Hospital wastewater therefore contains a mixture of parent compounds and metabolites rather than a single chemical form. Disposal of unused medications creates another source. Residual intravenous solutions, partially used liquid formulations, expired products, contaminated containers, and medication remaining in administration equipment may enter drains when pharmaceutical-waste practices are inconsistent. Formal disposal programs reduce direct discharge, but they do not remove the residues excreted by treated patients. Additional inputs may arise from rinsing medication containers, cleaning drug-preparation areas, washing contaminated equipment, and disposing of laboratory materials (Kusi et al., 2022). Concentrations vary considerably among antimicrobial classes because prescribing frequency, dosage, excretion, chemical stability, water solubility, sorption, and degradation differ. Frequently used β -lactams may enter wastewater in substantial quantities but degrade relatively quickly under some conditions. Fluoroquinolones can bind strongly to solids and accumulate in sludge or biofilms. Macrolides may persist through wastewater transport and treatment, while sulfonamides often remain mobile in aqueous environments. Glycopeptides, tetracyclines, aminoglycosides, and last-resort agents show separate patterns of excretion, transformation, and particle association. Measurements are commonly reported in nanograms or micrograms per liter, although concentrations depend on whether samples are collected from liquid wastewater, suspended solids, sludge, or plumbing biofilms. A low measured liquid concentration does not necessarily indicate a small total amount because the compound may be attached to solids, transformed into active products, diluted by high water use, or discharged intermittently (Nadeem et al., 2020). Composite sampling generally captures daily variation more effectively than a single grab sample, particularly when medication administration and patient excretion occur at different times.

The concentration of an antimicrobial compound in hospital wastewater reflects the interaction of clinical use, patient metabolism, wastewater generation, sewer transport, and chemical behavior. Hospitals with comparable numbers of beds may produce different residue profiles because their clinical specialties, patient severity, antimicrobial stewardship practices, prescribing patterns, and water consumption differ. Intensive care, transplant, oncology, surgical, burn, and infectious-disease units may use antimicrobial classes that are less common in general medical wards (Bruggen et al., 2018). The quantity prescribed does not directly equal the quantity detected in wastewater because patients metabolize drugs differently and the excreted compounds undergo dilution, sorption, hydrolysis, photochemical transformation, microbial degradation, and interaction with other wastewater constituents. Renal impairment may alter the timing and amount of urinary excretion, while gastrointestinal conditions may affect fecal elimination. Wastewater retention within pipes and storage tanks also influences chemical concentration because some drugs degrade rapidly and others persist or accumulate on surfaces. Measurements taken near a ward outlet may differ from those obtained at the main hospital discharge because wastewater from multiple departments becomes mixed and diluted. The timing of sample collection is equally important. Medication administration commonly follows scheduled clinical routines, but peak wastewater concentrations may occur several hours later depending on drug pharmacokinetics, patient excretion, and sewer travel time. Cleaning and laundry activities can create additional dilution or introduce chemicals that affect antimicrobial stability. Analytical methods must account for these variations through appropriate sample preservation, concentration, extraction, and chemical identification. Measuring only the parent compound may underestimate antimicrobial activity when active metabolites remain present. Measuring a broad chemical panel provides a fuller profile but increases the challenge of detecting compounds occurring at very low concentrations (Lima et al., 2020). Differences in reporting units, analytical detection limits, sample matrices, and laboratory recovery also make comparisons among hospitals difficult. Liquid-phase measurements describe compounds dissolved or suspended in wastewater, while solids analysis captures agents that bind to particles. Biofilm sampling identifies chemicals retained within plumbing matrices, where bacterial exposure may differ from concentrations measured in flowing water. A synthesized interpretation therefore treats reported nanogram- and microgram-per-liter concentrations as location- and time-specific observations rather than fixed

characteristics of a facility (Monahan et al., 2021). Their biological significance depends on antimicrobial potency, exposure duration, bacterial susceptibility, chemical mixtures, and the presence of resistant organisms and mobile genetic elements within the same wastewater environment.

Figure 7: Hospital Wastewater Antimicrobial Residue Pathway



Subinhibitory antimicrobial exposure occurs when bacteria encounter drug concentrations below the level required to prevent visible growth under standardized conditions. The minimum inhibitory concentration represents the lowest tested concentration that inhibits observable growth of a bacterial isolate in a defined laboratory setting. It is useful for classifying clinical susceptibility, but it does not establish the lowest concentration capable of influencing competition between susceptible and resistant bacteria in wastewater (Santos & Ramos, 2018). The minimum selective concentration refers to a lower exposure level at which resistant organisms gain a measurable reproductive or survival advantage over susceptible competitors. This selective threshold can occur far below the minimum inhibitory concentration because complete suppression of susceptible bacteria is not required for resistance enrichment. A small difference in growth, survival, or recovery can gradually alter the composition of a mixed population when exposure continues for a sufficient period. Hospital wastewater contains fluctuating antimicrobial concentrations rather than stable laboratory exposures. Concentrations rise and fall with medication schedules, patient excretion, water use, cleaning activity, sewer flow, and chemical degradation. Bacteria attached to pipe or drain biofilms may experience longer exposure than free-living cells transported rapidly through the sewer. Biofilm matrices can retain antimicrobial residues, slow diffusion, and create gradients in which surface organisms encounter higher concentrations while deeper cells experience lower levels. These gradients generate multiple selective conditions within the same plumbing location. Subinhibitory exposure can affect bacterial physiology by changing gene expression, membrane permeability, efflux activity, stress responses, mutation processes, biofilm production, and horizontal gene transfer (Angeles et al., 2020). It may favor organisms carrying resistance determinants even when the chemical concentration is too low to eliminate susceptible cells. The outcome varies by antimicrobial, bacterial species, resistance mechanism, nutrient availability, temperature, pH, growth rate, and exposure duration. Some resistance genes impose a biological cost in the absence of selection, causing resistant bacteria to grow more slowly than susceptible competitors. Continued low-level exposure can offset that disadvantage and support the retention of resistance. Compensatory mutations or stable plasmid-maintenance systems may also reduce the fitness cost, allowing resistance determinants to remain after measurable

drug concentrations decline. Subinhibitory exposure therefore represents an ecological selection process rather than a direct equivalent of clinical treatment (Guo et al., 2018). Its assessment requires chemical concentration data, bacterial susceptibility information, exposure-time estimates, and evidence that resistant and susceptible populations experience different outcomes under the observed wastewater conditions.

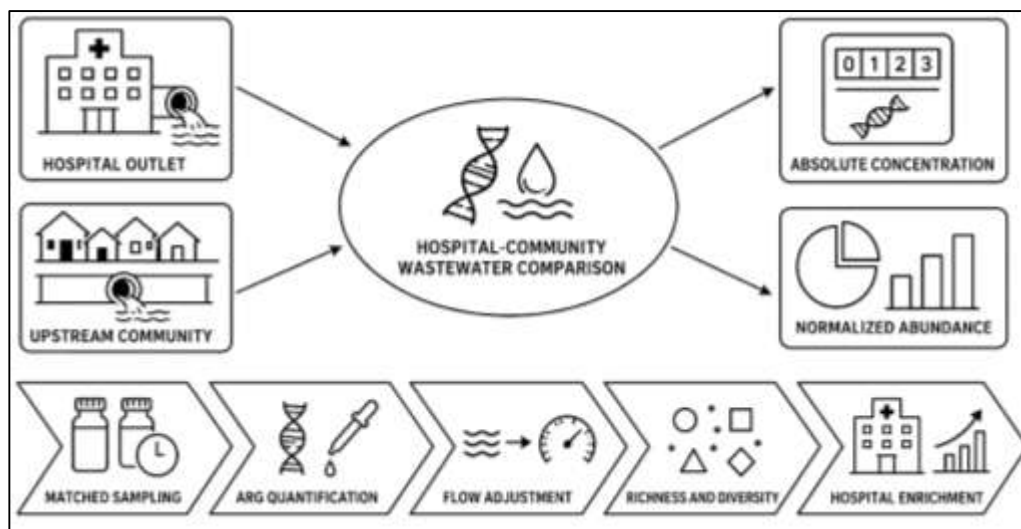
Comparison of Hospital and Community Wastewater

Comparing hospital outlets with upstream community wastewater provides a direct approach for examining whether healthcare facilities contribute distinctive antimicrobial resistance signals to sewer networks (Hassoun-Kheir et al., 2020). Upstream community samples represent wastewater generated before the hospital discharge enters the sewer and therefore provide a local background measurement for resistance genes associated with households, businesses, outpatient antimicrobial use, food consumption, companion animals, and community carriage. Hospital-outlet samples represent wastewater collected near the facility's discharge point and contain biological and chemical inputs from patients, healthcare workers, visitors, laboratories, pharmacies, kitchens, laundries, and cleaning operations. Differences in ARG copies per unit volume can indicate whether selected resistance genes occur at higher absolute concentrations in hospital effluent. Genes associated with extended-spectrum β -lactam resistance, carbapenem resistance, vancomycin resistance, methicillin resistance, aminoglycoside resistance, and multidrug efflux may be elevated at hospital outlets because healthcare facilities concentrate patients receiving antimicrobial treatment and individuals colonized or infected with multidrug-resistant organisms (Paulus et al., 2019). Common resistance determinants associated with tetracyclines, sulfonamides, and macrolides may be abundant in both hospital and community wastewater because they are widely distributed among intestinal and environmental bacteria. Absolute concentration must be interpreted with wastewater flow, fecal content, suspended solids, and total bacterial biomass. A hospital outlet with a high gene concentration may discharge a modest total quantity when wastewater flow is low, while community wastewater with a lower concentration may carry a larger total quantity because it represents a much larger population. Differences in water use can also alter measured concentrations. Hospital cleaning, laundry, food preparation, cooling, and sanitation activities may produce substantial dilution unrelated to patient resistance. Community flow varies with household routines, rainfall, infiltration, industrial inputs, and sewer design. Matched sampling is therefore important. Hospital and upstream community samples should be collected during comparable periods and analyzed using the same collection, preservation, extraction, and detection methods. Twenty-four-hour composite samples generally represent daily discharge patterns more reliably than isolated grab samples because antimicrobial use, patient excretion, cleaning activity, and residential water use fluctuate throughout the day. Repeated comparisons across weekdays, weekends, occupancy levels, and seasons help distinguish persistent hospital-associated enrichment from temporary variation (Hutinel et al., 2022). The upstream location must also be selected carefully to ensure that it is not influenced by another healthcare facility, nursing home, pharmaceutical source, or intersecting sewer line. Under these conditions, differences in absolute ARG concentration provide an interpretable measure of how hospital wastewater contrasts with its surrounding community background.

Normalized ARG abundance, resistome richness, and genomic diversity add complementary dimensions to comparisons between hospital and upstream community wastewater. Normalized abundance evaluates how strongly a resistance gene is represented relative to total bacterial markers, human fecal indicators, or another reference measurement. This adjustment can reduce the influence of wastewater dilution and variation in bacterial biomass, although the choice of reference marker affects the interpretation (Wang et al., 2018). Normalization to the 16S rRNA gene compares resistance targets with total bacterial genetic material, but bacterial species contain different numbers of 16S rRNA gene copies. Human fecal markers can help account for differences in human waste contributions, although their concentrations are influenced by degradation, sampling matrix, and population characteristics. A resistance gene may show a higher absolute concentration at a hospital outlet but a similar normalized abundance to community wastewater when the hospital sample also contains more bacterial biomass. Conversely, absolute concentrations may appear comparable while normalized abundance reveals that the gene occupies a larger proportion of the hospital microbial community.

Resistome richness describes the number of distinct genes or resistance families detected, while diversity considers both the number of targets and how their abundances are distributed. Hospital wastewater may contain greater richness when it combines resistance determinants from patients with different infections, treatments, healthcare exposures, and countries of origin (Yao et al., 2021).

Figure 8: Hospital and Community Wastewater Comparison



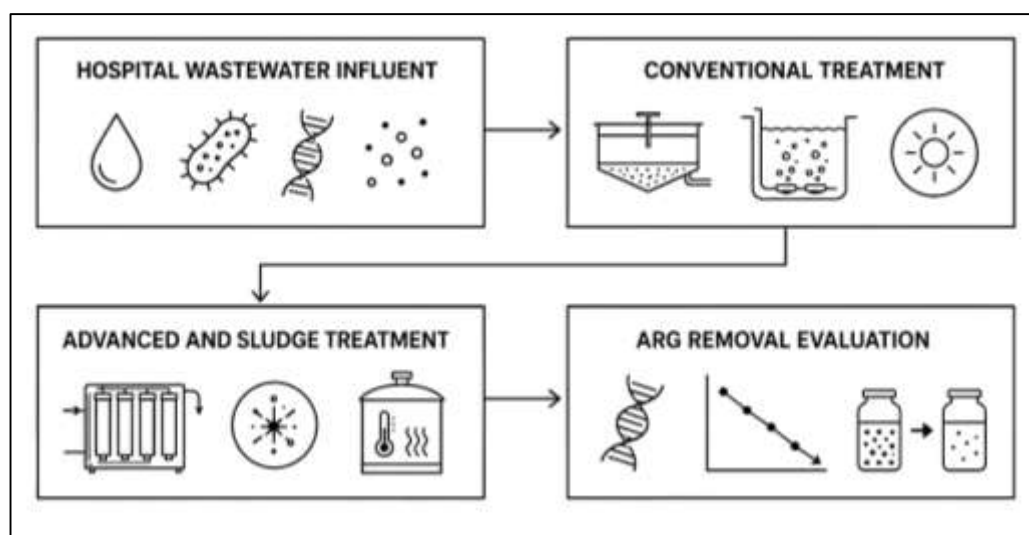
Community wastewater can also exhibit high diversity because it aggregates a much larger population and receives genes from households, food systems, outpatient treatment, and environmental sources. Sequencing depth, sample concentration, DNA extraction, and database coverage strongly influence richness and diversity estimates, making standardized analytical procedures essential. Hospital-enriched targets are genes detected consistently at higher concentrations or normalized abundances in hospital effluent than in matched upstream samples. Carbapenemase genes, vancomycin-resistance determinants, selected extended-spectrum β -lactamase genes, and mobile-element markers may display hospital enrichment in some settings, while their distribution differs by region and facility. Enrichment does not prove that a gene originated within the hospital because admitted patients may have acquired it in the community, another healthcare institution, a long-term-care facility, or another country. Host identification and genetic-context analysis provide greater specificity by determining whether enriched genes are associated with clinically important Enterobacterales, enterococci, staphylococci, nonfermenting Gram-negative organisms, or environmental bacteria (Manoharan et al., 2021). A combined analysis of absolute concentration, normalized abundance, richness, diversity, bacterial hosts, mobile elements, wastewater flow, and patient activity produces a more reliable comparison than any single measurement.

Wastewater-Treatment Performance

Conventional wastewater treatment uses a sequence of physical, biological, and chemical processes to remove solids, organic matter, nutrients, microorganisms, and selected contaminants from hospital and municipal wastewater. Primary sedimentation is one of the earliest major treatment stages and relies on gravity to separate settleable solids from the liquid fraction. Antimicrobial-resistant bacteria, extracellular DNA, antimicrobial residues, and mobile genetic elements may attach to suspended particles and settle into primary sludge (Bhave et al., 2020). This process can reduce ARG concentrations in the liquid stream, but it transfers a portion of the resistance reservoir into a concentrated solid matrix rather than destroying it. The removal achieved during sedimentation depends on particle size, settling time, wastewater flow, solids concentration, temperature, and the tendency of individual bacteria or genetic materials to associate with particles. Freely suspended organisms and dissolved extracellular DNA may remain in the liquid and continue into biological treatment. Activated sludge treatment introduces aeration and maintains dense microbial communities that consume organic matter and

transform nutrients. Wastewater microorganisms become incorporated into biological flocs, where they interact with treatment-associated bacteria under conditions of high cell density and prolonged retention. Resistant organisms entering from hospitals and communities may be removed through flocculation, competition, predation, starvation, biological decay, and subsequent solids separation. Some may persist because they are incorporated into flocs, protected by extracellular polymers, or adapted to treatment conditions (Alisawi, 2020). Activated sludge also contains diverse bacterial hosts and mobile genetic elements, creating opportunities for the retention and possible exchange of resistance genes. Biological nutrient-removal systems modify oxygen availability through aerobic, anoxic, and anaerobic zones to support nitrogen and phosphorus removal. These changing conditions influence bacterial-community composition and can favor different resistance hosts at separate treatment stages. Nutrient removal may indirectly affect ARG patterns by altering microbial competition, growth rates, floc structure, and organic-carbon availability. The effectiveness of conventional biological treatment therefore cannot be described only through a single reduction measurement. A decrease in absolute ARG concentration may occur alongside a stable or increased representation of the same gene within the remaining bacterial community (Yang et al., 2020). Treatment performance also varies among facilities because hydraulic retention time, solids-retention time, aeration, temperature, organic loading, influent composition, and operational stability differ. Hospital wastewater entering a municipal plant becomes mixed with community sewage, which changes the concentration and composition of its original resistance signal before activated sludge processing begins.

Secondary clarification separates biological flocs from treated wastewater after activated sludge or biological nutrient removal. Settled biomass is partly returned to maintain the treatment community, while excess sludge is removed for further processing. This stage can substantially reduce particle-associated resistant bacteria and ARGs in the liquid effluent, although its performance depends on floc formation, settling characteristics, hydraulic conditions, and solids carryover (Yang et al., 2020). Poor settling or high-flow events can release suspended bacteria and genetic material into later treatment stages. Following clarification, chlorination and ultraviolet irradiation are commonly used to reduce viable microorganisms before discharge. Chlorine-based disinfectants damage cellular membranes, proteins, enzymes, and nucleic acids through chemical oxidation. Their effectiveness depends on chlorine dose, contact time, pH, temperature, organic matter, suspended solids, and the resistance characteristics of the microbial population. Organic material and particles can consume chlorine or shield embedded organisms, reducing exposure. Chlorination may inactivate resistant bacteria while leaving fragments of their DNA detectable by molecular methods. Incomplete DNA destruction is important because a sample can test positive for ARGs even when most original host cells are no longer viable. Strong chlorination can damage genetic material, but the extent varies with gene location, fragment size, chemical conditions, and protection within particles or cells. Ultraviolet irradiation damages microbial DNA and interferes with replication. It does not require chemical addition and avoids many chlorination by-products, but suspended solids, turbidity, water depth, lamp condition, and ultraviolet dose affect performance. Organisms shielded by particles or aggregated within flocs may receive insufficient exposure (Cardoso et al., 2021). Ultraviolet treatment can prevent bacterial replication without removing all measurable DNA from the wastewater. Some damaged cells may also repair ultraviolet-induced injury under favorable conditions. Chlorination and ultraviolet irradiation therefore provide different forms of microbial control and may produce different patterns of bacterial inactivation, ARG damage, and extracellular DNA persistence. Conventional treatment performance is additionally shaped by the sequence of processes. Effective solids removal before disinfection reduces particle shielding and improves contact between disinfectants and microorganisms. Conversely, high turbidity or residual organic matter can lower disinfection efficiency. Culture-based analysis often shows larger reductions than DNA-based assays because culture measures organisms capable of replication, whereas molecular methods detect genetic sequences from viable, injured, inactivated, and extracellular sources (Newhart et al., 2019). Evaluating conventional treatment therefore requires simultaneous examination of culturable resistant bacteria, intact cells, total ARG copies, extracellular DNA, mobile genetic elements, suspended solids, and operational conditions.

Figure 9: Wastewater Treatment Resistance Gene Removal

Advanced wastewater-treatment methods provide additional physical or chemical barriers for resistant bacteria, resistance genes, antimicrobial residues, and other contaminants that remain after conventional processing. Membrane filtration separates materials according to membrane pore size, pressure, surface properties, and system configuration. Microfiltration and ultrafiltration can retain suspended solids, bacterial cells, and large biological particles, while tighter nanofiltration and reverse-osmosis membranes remove smaller molecules and dissolved contaminants more effectively (Singh et al., 2019). The reduction of ARGs through membrane systems often reflects physical retention of bacterial hosts and particle-associated DNA rather than direct genetic destruction. Membrane integrity is therefore essential because defects, leakage, or poor sealing can permit microorganisms and genetic material to pass into treated water. Membrane fouling creates another operational concern. Accumulated organic matter and microbial biofilms can reduce flow, increase energy requirements, and create concentrated resistance reservoirs on membrane surfaces. Retentate or concentrate streams also require management because separated bacteria, genes, and pharmaceuticals remain within a smaller waste volume. Ozonation uses ozone as a strong oxidizing agent that reacts with cellular structures, genetic material, and many pharmaceutical compounds. It can inactivate bacteria and transform antimicrobial residues, although effectiveness depends on ozone dose, contact time, water chemistry, organic content, and the reactivity of individual compounds (Mažeikienė, 2019). Transformation products may form during oxidation and can differ from the original compound in persistence, toxicity, and antimicrobial activity. Advanced oxidation processes combine oxidants, ultraviolet energy, catalysts, or related treatments to generate highly reactive chemical species. These reactions can damage bacterial cells, degrade extracellular DNA, and break down persistent pharmaceutical residues. Their performance is influenced by wastewater composition because organic matter and other chemicals compete for reactive species. Activated carbon removes contaminants through adsorption rather than biological inactivation. Powdered or granular activated carbon can capture antimicrobial compounds, metabolites, disinfectants, and other organic micropollutants. Its effectiveness varies according to chemical structure, carbon properties, contact time, pH, and competition among dissolved substances (Li et al., 2018). Activated carbon may also support biofilm growth, making system maintenance and regeneration important. Combining membrane filtration, oxidation, and adsorption can address different components of the resistance reservoir, with one process retaining cells, another damaging DNA, and another removing selective chemical residues. Sludge treatment is an essential component of advanced resistance control because primary and secondary solids contain concentrated microbial biomass, ARGs, extracellular DNA, mobile genetic elements, metals, and particle-bound antimicrobial residues. Thermal treatment uses elevated temperatures to inactivate microorganisms, disrupt cellular structures, and accelerate the degradation

of genetic material. Performance depends on treatment temperature, exposure time, solids moisture, organic content, and the degree to which heat penetrates the entire sludge mass (El-Rawy et al., 2021). Lower-temperature processes may reduce viable bacteria while leaving detectable DNA fragments, whereas more intensive treatment can produce greater nucleic-acid degradation. Anaerobic digestion also exposes sludge to extended biological processing under oxygen-free conditions. Mesophilic and thermophilic digestion differ in temperature, microbial composition, and resistance-gene removal. Thermophilic conditions often provide stronger bacterial inactivation, although gene-specific persistence and operational differences prevent uniform removal. Composting combines biological activity, elevated temperature, aeration, and prolonged storage. Its effectiveness depends on achieving adequate temperature throughout the material and preventing the survival of organisms in cooler zones. Chemical sludge treatment may use lime, oxidants, acids, alkalis, or other agents to change pH, disrupt cells, and reduce viable microorganisms (Feng et al., 2019). Chemical conditioning can also affect DNA stability and the attachment of resistance genes to solids. No sludge-treatment method removes all resistance determinants consistently because ARG persistence depends on the bacterial host, genetic location, association with mobile elements, protection within particles, and analytical method. A treatment can eliminate culturable resistant bacteria while molecular assays continue detecting residual gene fragments. Sludge dewatering reduces water content but concentrates remaining solids, so measured copies per gram may appear elevated even when the total quantity has declined. Land application of treated biosolids transfers the remaining resistance material into soil, where moisture, temperature, sunlight, microbial competition, and chemical conditions influence persistence. Incineration or similarly intensive thermal destruction provides greater removal of biological material but requires more energy and changes the management pathway for solids (Marjani et al., 2020). Advanced sludge treatment should therefore be evaluated through viable bacterial counts, absolute ARG quantities, extracellular and intracellular DNA measurements, mobile-element detection, antimicrobial-residue analysis, and total treated-sludge mass. This broader assessment distinguishes true destruction from concentration, immobilization, or transfer into another waste stream.

Quantitative evaluation of ARG removal requires multiple measurements because concentration reduction, bacterial inactivation, DNA degradation, and transfer into sludge represent different treatment outcomes. Influent-to-effluent log reduction describes the order-of-magnitude decrease in a target between the wastewater entering and leaving a treatment process. It is useful for comparing genes with very different starting concentrations, but its interpretation depends on consistent sampling, analytical recovery, and treatment flow (Viet et al., 2022). Percentage reduction in absolute concentration expresses how much the measured gene concentration declines across treatment. This measurement is accessible and useful for individual treatment stages, although it can be distorted by dilution, evaporation, recycle streams, or changes in wastewater volume. Relative abundance evaluates whether an ARG becomes more or less common within the remaining bacterial community. A treatment process can lower the absolute number of gene copies while relative abundance increases because susceptible bacteria or nonresistant organisms are removed more efficiently than resistant hosts. Conversely, relative abundance may decline even when a substantial absolute quantity remains in the effluent. Reporting both absolute and normalized measurements therefore prevents the assumption that one indicator completely represents treatment performance. Daily influent and effluent mass loads incorporate wastewater flow and estimate the total amount of a resistance target entering or leaving the facility during a defined period (Bagherzadeh et al., 2021). Load-based analysis is particularly important for large municipal plants because a low effluent concentration multiplied across a substantial daily discharge can represent a considerable release. Flow variation caused by rainfall, infiltration, industrial discharge, water use, or seasonal conditions can alter concentration without producing an equivalent change in total load. Composite samples aligned with daily flow generally provide stronger load estimates than individual grab samples. Solids must also be incorporated because primary sedimentation and secondary clarification transfer ARGs from the liquid stream into sludge. Evaluating only liquid effluent may indicate effective removal while overlooking the quantity retained in solid residuals. A plant-wide assessment follows resistance determinants through influent, primary sludge, biological reactors, secondary effluent, disinfection, final effluent,

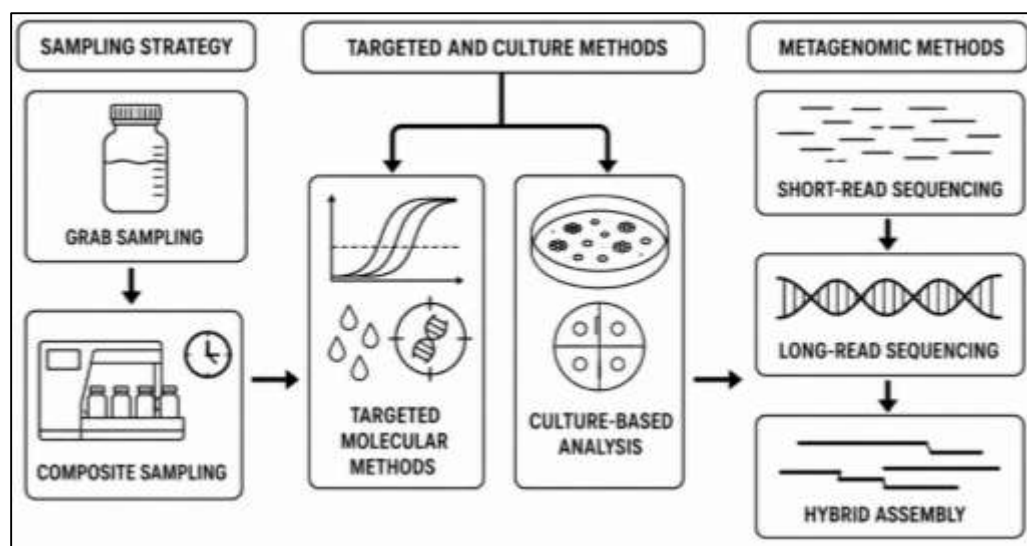
and treated biosolids. Recovery controls, replicate analysis, consistent extraction procedures, and transparent treatment of nondetectable values are necessary because apparent reductions may reflect analytical limitations rather than biological removal (Zhang et al., 2019).

Gene-specific differences in removal efficiency arise from variation in bacterial hosts, genetic location, particle association, cellular characteristics, and resistance-element stability. Genes carried by organisms that form strong flocs or attach readily to solids may be removed efficiently from the liquid phase but concentrated in sludge (Cho et al., 2019). Determinants located in free extracellular DNA may pass through sedimentation yet remain vulnerable to nucleases, oxidation, or ultraviolet damage. Plasmid-associated genes may persist when their bacterial hosts are well adapted to activated sludge or biofilm environments. Common resistance genes such as *sul1*, *sul2*, *tetW*, *ermB*, and multidrug-efflux determinants may remain detectable across treatment because they occur in abundant wastewater bacteria and mobile genetic elements. Rare carbapenemase, vancomycin-resistance, colistin-resistance, or methicillin-resistance genes may appear to have inconsistent removal because their influent concentrations approach analytical detection limits. A nondetectable effluent result does not establish complete removal when the remaining concentration may simply fall below assay sensitivity. The distinction between bacterial inactivation and DNA removal is therefore fundamental. Chlorination, ultraviolet irradiation, ozonation, heat, and advanced oxidation can prevent bacterial replication or damage cells, but molecular assays may continue detecting target sequences (Damtie et al., 2019). Intact resistance genes in extracellular DNA may retain transformation potential under favorable conditions, whereas heavily fragmented sequences may remain detectable through short assays without retaining biological functionality. Culture-based methods identify viable and culturable organisms, viability-associated molecular techniques attempt to separate intact from membrane-damaged cells, and total DNA assays quantify both cellular and extracellular targets. Long-fragment analysis can provide additional evidence of genetic integrity, while sequencing can determine whether resistance genes remain linked to mobile elements or bacterial hosts. Treatment performance also varies with temperature, hydraulic retention, solids-retention time, organic loading, aeration, pH, disinfectant dose, contact time, turbidity, and seasonal conditions. Statistical analysis should account for repeated sampling and operational variation when comparing processes or facilities. A synthesized quantitative assessment therefore reports log reduction, percentage concentration change, normalized abundance, daily mass load, solids transfer, viable resistant bacteria, extracellular DNA, and gene-specific persistence (Asif & Zhang, 2021). Together, these indicators clarify whether treatment destroys resistance determinants, inactivates bacterial hosts, transfers genes into sludge, or releases residual genetic material in final effluent.

Measurement and Genomic Characterization Methods

Sampling strategy determines which component of the hospital wastewater resistome is represented and strongly influences estimates of antimicrobial resistance gene concentration, diversity, and temporal variability. Grab sampling captures wastewater at one location and moment, making it inexpensive, operationally simple, and useful for preliminary screening or investigating a specific discharge event (Moffitt et al., 2022). Its principal limitation is that hospital wastewater changes throughout the day according to patient excretion, medication schedules, clinical procedures, cleaning activity, laboratory operations, food preparation, laundry cycles, and water consumption. A grab sample collected during a period of concentrated patient discharge may produce substantially higher ARG measurements than one collected during intense cleaning or high-water use. Twenty-four-hour composite sampling combines multiple subsamples collected over a full daily cycle and generally produces a more representative estimate of average hospital discharge. Time-proportional composites collect equal volumes at predetermined intervals, whereas flow-proportional composites adjust the amount collected according to wastewater flow. Flow-proportional sampling is particularly valuable when discharge volume changes considerably because it prevents low-flow and high-flow periods from receiving equal influence. Composite sampling requires automated equipment, secure access, suitable refrigeration, and measures to prevent sedimentation, nucleic-acid degradation, microbial growth, and cross-contamination. The choice between liquid wastewater and wastewater solids also affects detection. Liquid samples represent material transported through the sewer at the collection time, while solids concentrate bacterial cells, particle-associated DNA, mobile genetic elements, and

hydrophobic pharmaceutical residues. Resistance targets present below the detection limit in liquid wastewater may be measurable in settled solids (Lee et al., 2020). The two matrices cannot be interpreted interchangeably because partitioning differs among bacterial hosts, genes, extracellular DNA, and chemical compounds. Sampling frequency and duration determine whether a study captures temporary events or persistent patterns. Daily or multiple-week sampling can detect short-term variation associated with occupancy, antimicrobial use, or episodic shedding, while monthly sampling provides broader seasonal coverage but may miss brief changes. Weekday and weekend patterns may differ because elective procedures, laboratory activity, staffing, admissions, discharges, and water use change across the week. Seasonal variation may reflect temperature, rainfall, sewer infiltration, respiratory-infection periods, antimicrobial prescribing, and changes in patient populations. Sampling locations also define the population represented. Ward-level outlets provide localized information, the main hospital outlet aggregates facility activity, upstream sites establish community background, and downstream or municipal-influent sites measure mixed sewer signals. A robust design aligns sampling points with plumbing maps, sewer travel time, wastewater flow, clinical records, and antimicrobial-consumption periods (Lazzarotto et al., 2020). Hospital wastewater sampling also requires attention to practical and analytical conditions that can introduce systematic bias before laboratory analysis begins. Wastewater is heterogeneous, and solids can settle rapidly within pipes, sampling containers, tubing, and collection vessels. Poor mixing may cause one subsample to contain substantially more bacterial biomass and genetic material than another. Automated samplers must therefore be positioned where wastewater remains sufficiently mixed and where backflow, stagnant water, or infiltration from unrelated sewer branches is limited. Sampling from a manhole near the hospital boundary may appear facility specific but can include wastewater from neighboring buildings if sewer connections are not verified. Conversely, an internal outlet may represent only one department rather than the full hospital. Plumbing diagrams, dye testing, flow monitoring, and site inspections help establish the population contributing to each location. Sample preservation is equally important because bacterial growth, cell death, enzymatic DNA degradation, and changes in chemical composition can occur during collection and transport. Cooling slows biological activity, while excessive storage time may still alter community composition and reduce comparability. Repeated freezing and thawing can disrupt cells and increase the extracellular DNA fraction. Filters, centrifugation, precipitation, adsorption, and other concentration procedures recover different portions of the wastewater microbiome and may favor particular organisms or genetic materials. Process controls are needed to estimate recovery losses throughout concentration, extraction, and analysis. Field blanks identify contamination introduced during collection, while replicate samples describe local heterogeneity. Rainfall and sewer infiltration can lower wastewater concentrations by dilution, although they may also mobilize biofilm material or sediments (Asnicar et al., 2020). Hospital water use can have similar effects, particularly during laundry, cleaning, or sterilization activities. Concentration measurements should therefore be accompanied by flow, fecal-strength, total-bacterial, or solids information. Cross-sectional designs comparing many hospitals at one time provide broad spatial coverage but limited information about temporal stability. Longitudinal designs provide stronger estimates of within-facility variation but may include fewer hospitals because of cost and access requirements. Multisite studies benefit from harmonized collection times, container materials, preservation conditions, transport schedules, concentration procedures, and metadata. The sampling framework ultimately determines whether genomic findings represent a momentary wastewater event, an average daily discharge, a hospital department, the entire healthcare facility, or a mixed municipal sewershed. Targeted molecular methods measure predefined resistance genes with high sensitivity and are widely used for quantitative analysis of hospital wastewater. Quantitative polymerase chain reaction monitors amplification during each reaction cycle and estimates the starting amount of a target by comparing sample amplification with reference standards. It is suitable for clinically important genes such as blaCTX-M, blaKPC, blaNDM, blaVIM, mecA, vanA, mcr-1, sul1, tetW, and ermB. Standard curves are prepared from materials containing known quantities of the target sequence and establish the relationship between amplification behavior and gene abundance (Sztupinszki et al., 2018). Their accuracy depends on standard quality, dilution preparation, reaction efficiency, instrument performance, and similarity between reference material and wastewater DNA.

Figure 10: Hospital Wastewater Resistance Analysis Methods

Absolute quantification is generally reported as gene copies per wastewater volume, solids mass, or another defined sample unit. Normalization to bacterial or fecal markers provides additional information about the target's representation within the microbial community. Droplet digital polymerase chain reaction divides each sample into thousands of small reactions and estimates target abundance from the distribution of positive and negative droplets. It does not require an external standard curve for routine concentration estimation and can provide improved precision for low-abundance targets or small differences among samples. Its performance still depends on primer and probe specificity, droplet quality, threshold selection, sample dilution, and control reactions. Quantitative and droplet digital assays are both restricted to genetic targets selected before analysis. They cannot detect unknown resistance genes or identify complete resistome diversity. Assay specificity is therefore critical. Primers may amplify several variants within a gene family or distinguish only a single allele, depending on their design. Family-level assays provide broad coverage but may combine genes with different resistance profiles. Highly specific assays can miss new or divergent variants. Sequence confirmation, positive controls, and testing against related genes help determine whether an assay measures the intended target. Multiplex assays increase analytical efficiency by detecting several genes simultaneously, but interactions among primers and probes may alter sensitivity ([Ben-David et al., 2018](#)). Targeted measurements should consequently be interpreted according to the exact sequence range recognized by each assay rather than the general resistance category represented by the gene name. Hospital wastewater contains substances that can inhibit DNA amplification, including organic matter, humic materials, metals, detergents, pharmaceutical compounds, polysaccharides, fats, proteins, and residual extraction chemicals. Inhibition may delay amplification, reduce reaction efficiency, produce false-negative results, or underestimate gene concentration. Sample dilution can reduce inhibitor concentrations but may also move rare targets below detection. Additional purification improves DNA quality but can cause material loss. Internal amplification controls help identify inhibition by showing whether an added target amplifies as expected in each sample. Extraction controls and recovery surrogates distinguish losses during concentration and DNA purification from inhibition during amplification. The limit of detection represents the lowest target quantity that can be detected reliably, while the limit of quantification identifies the lowest quantity that can be measured with acceptable precision. These limits are assay- and matrix-specific and should include sample volume, concentration factor, extraction volume, DNA input, and reaction replication. A gene reported as nondetectable is not necessarily absent from wastewater; it may occur below analytical sensitivity, be distributed unevenly, or be affected by inhibition ([Ben-David et al., 2018](#)). The treatment of nondetectable values can influence descriptive statistics and regression results, especially for rare carbapenemase or colistin-resistance genes.

Replacing all nondetects with zero assumes complete absence, while substituting a fixed fraction of the detection limit can distort distributions when nondetection is common. Censored-data methods, detection-frequency analysis, sensitivity testing, and clear reporting of assay limits provide more defensible interpretation. Technical replicates reduce random reaction error but do not replace independent wastewater samples. Biological replicates describe variation across locations or sampling times, whereas extraction replicates evaluate laboratory processing variability. Molecular assays also detect DNA from viable, damaged, nonviable, and extracellular sources. Pretreatments designed to limit amplification from membrane-damaged cells can provide an approximation of viable-cell-associated genes, but their performance varies with wastewater turbidity, dye penetration, and cell type. Targeted molecular quantification therefore provides strong concentration data when accompanied by validated assays, inhibition controls, recovery measurements, matrix-specific detection limits, replicate testing, and transparent handling of nondetectable results (Wardell et al., 2018).

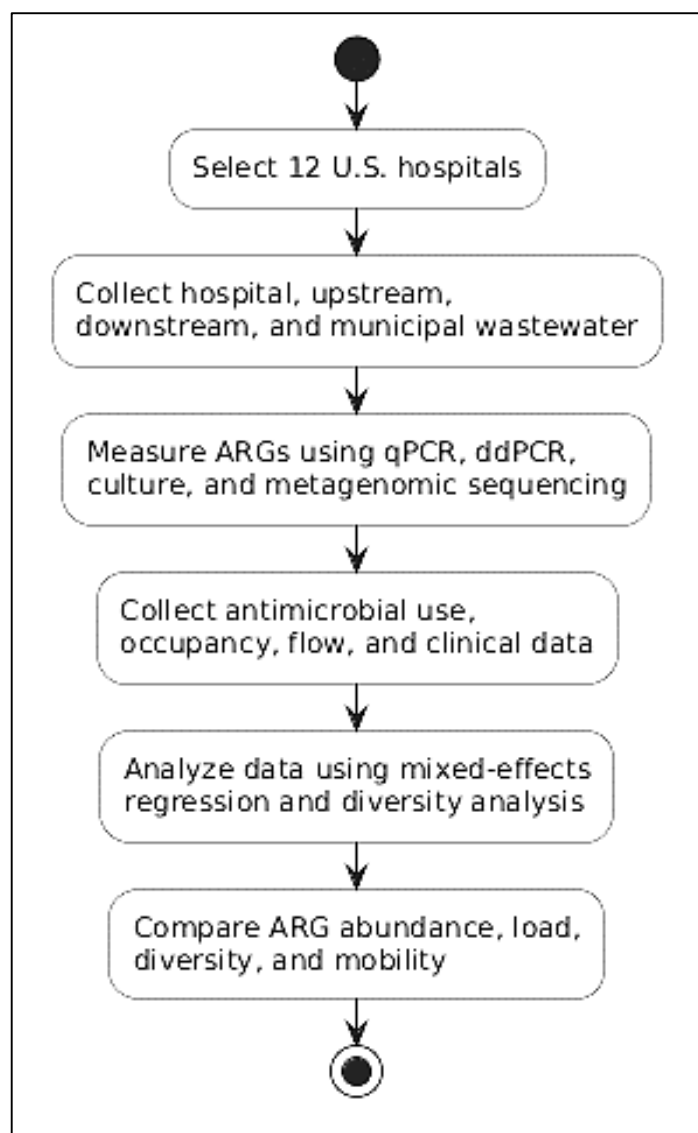
Culture-based analysis identifies viable microorganisms capable of growing under defined laboratory conditions and links recovered organisms with observable antimicrobial-resistance phenotypes. Selective cultivation uses media containing antimicrobial agents, chromogenic substrates, nutrients, salts, or other inhibitors to favor organisms with particular traits. Hospital wastewater can be screened for extended-spectrum β -lactamase-producing Enterobacterales, carbapenem-resistant Gram-negative bacteria, vancomycin-resistant enterococci, methicillin-resistant staphylococci, and multidrug-resistant *Pseudomonas* or *Acinetobacter* (Bertucci et al., 2019). Direct plating provides a closer estimate of culturable bacterial concentration, while enrichment incubates samples in selective broth before plating to improve the recovery of organisms present at low abundance or injured by wastewater conditions. Enrichment is useful for detecting rare resistant bacteria, but it changes the relative composition of the original sample because organisms grow at different rates. A bacterium detected after enrichment may have been present in very small numbers, and colony abundance after incubation cannot be interpreted as its initial wastewater concentration. Media composition also determines which taxa are recovered. Some environmental and clinically relevant bacteria grow poorly under standard conditions, while faster-growing organisms can dominate culture plates. Once isolates are obtained, identification can be conducted through biochemical testing, mass spectrometry, targeted molecular assays, or genomic sequencing. Antimicrobial susceptibility testing determines whether isolates remain susceptible or resistant to selected drugs. Disk diffusion assesses the zone of growth inhibition around antimicrobial disks, broth microdilution evaluates growth across a range of drug concentrations, and automated clinical systems provide standardized susceptibility profiles for common organisms (Bertucci et al., 2019). Minimum inhibitory concentration determination identifies the lowest tested antimicrobial concentration that prevents visible bacterial growth under controlled conditions. Interpretation depends on established clinical or epidemiological breakpoints, which may not be available for all environmental organisms. Clinical breakpoints are designed to support patient treatment and may not describe environmental selection accurately. Phenotypic analysis nevertheless provides evidence that a viable isolate can withstand antimicrobial exposure, which total-DNA detection cannot establish independently. Comparison of genotype and phenotype reveals both the value and limitations of culture-based testing. Detection of a recognized resistance gene in an isolate may correspond closely with resistance to the associated antimicrobial class, but the relationship is not always direct. A gene may be present but poorly expressed, disrupted, or regulated in a manner that does not produce the expected phenotype. Phenotypic resistance may also occur without detection of the targeted gene because another known mechanism, a novel variant, a chromosomal mutation, efflux activation, reduced permeability, or combined low-level mechanisms produce resistance. Whole-genome sequencing of cultured isolates can identify multiple resistance determinants, bacterial species, strain lineages, virulence genes, plasmid markers, and genetic relationships among wastewater and clinical organisms (Shih et al., 2020). Culture also enables conjugation experiments, fitness testing, biofilm analysis, and confirmation of viable resistant hosts. Its limitations include selective recovery, incubation bias, competition among organisms, and the inability to capture extracellular DNA or unculturable bacteria. Wastewater conditions may injure cells without killing them, causing viable organisms to fail to grow immediately on selective media. Recovery on nonselective media before antimicrobial

exposure can improve detection of stressed cells but introduces additional growth bias. Antibiotic concentrations used in selective media may favor highly resistant isolates and miss organisms with lower-level resistance. Conversely, concentrations that are too low can permit susceptible organisms to grow. Culture results are also sensitive to sample storage, temperature, oxygen exposure, and the time between collection and processing. Integrating culture with targeted molecular quantification helps distinguish viable resistant organisms from the broader pool of resistance genes. For example, a high wastewater concentration of blaKPC accompanied by recovery of viable blaKPC-carrying Enterobacterales provides stronger evidence than either result alone (Felz et al., 2019). If the gene is abundant but no carrier is cultured, it may occur in unculturable hosts, damaged cells, extracellular DNA, or organisms not supported by the selected medium. When resistant bacteria are cultured without the targeted gene, genomic analysis can identify alternative mechanisms. This combined genotype–phenotype framework provides a more complete description of hospital wastewater resistance.

METHOD

The study employed a multicenter, longitudinal, quantitative observational design to examine hospital wastewater as a reservoir of antimicrobial resistance genes and determine its relationship with hospital antimicrobial consumption, clinical resistance indicators, wastewater characteristics, and municipal sewer measurements in the United States. The design was guided by the One Health framework, which conceptualized antimicrobial resistance as an interconnected condition involving human health, healthcare facilities, microbial communities, wastewater infrastructure, and the surrounding environment. A longitudinal approach was selected because resistance-gene concentrations, hospital occupancy, antimicrobial administration, wastewater flow, and environmental conditions varied over time. Repeated wastewater measurements allowed within-hospital temporal changes to be separated from persistent differences among facilities. The principal dependent variables were the absolute concentrations, normalized abundances, detection frequencies, daily discharge loads, richness, and diversity of antimicrobial resistance genes. The primary independent variables were hospital antimicrobial consumption, occupied-bed days, intensive-care activity, clinical rates of resistant organisms, wastewater flow, temperature, pH, turbidity, total suspended solids, and sampling location. Hospital identity, geographic region, season, facility size, and hospital type were treated as clustering, stratification, or adjustment variables. The study compared wastewater collected at hospital outlets with matched upstream community sewer locations, downstream sewer points, and municipal wastewater-treatment influent. A subset of samples also underwent metagenomic sequencing to characterize resistance-gene diversity, bacterial hosts, mobile genetic elements, and the co-occurrence of resistance and virulence determinants.

The study materials consisted of untreated wastewater, wastewater solids, bacterial isolates, extracted nucleic acids, hospital antimicrobial-consumption records, facility activity data, clinical resistance summaries, and municipal flow records. Twelve acute-care hospitals were selected through stratified purposive sampling from the Northeast, Midwest, South, and West census regions of the United States. Three hospitals were selected from each region to obtain geographic representation and variation in hospital size, patient population, wastewater infrastructure, and antimicrobial use. The selected facilities included teaching hospitals, nonteaching hospitals, urban referral centers, and regional acute-care hospitals. Each participating hospital was connected to a municipal sewer system and had an identifiable wastewater outlet that could be sampled before substantial mixing with nonhospital wastewater. Matched sampling locations included the principal hospital outlet, an upstream community sewer point unaffected by the participating hospital, a downstream sewer point located after hospital discharge, and the influent of the receiving municipal wastewater-treatment plant. Sampling continued for 12 consecutive months to capture temporal, weekday, and seasonal variation. Two sampling events were completed each month at every hospital, producing 24 sampling periods per facility and 288 hospital-level sampling periods. Samples collected from the four matched locations produced a planned total of 1,152 wastewater observations before quality-control exclusions. Hospitals were included when they provided inpatient acute care, maintained at least 100 licensed beds, operated an intensive care unit, used an electronic medication-administration or pharmacy database, maintained clinical microbiology records, and permitted access to a verified wastewater discharge point.

Figure 11: Methodology of this study

Facilities were also required to provide monthly bed occupancy, patient-days, intensive-care patient-days, antimicrobial use, and aggregated clinical resistance data. Hospitals were excluded when they discharged wastewater into an independent treatment system that could not be compared with municipal influent, shared an inseparable outlet with another major institution, or lacked reliable flow and clinical metadata. Wastewater samples were included when the collection period was complete, sample temperature remained within the required transport range, chain-of-custody documentation was available, and laboratory quality controls met predetermined acceptance criteria. Samples were excluded when collection equipment failed, containers leaked, holding times exceeded protocol limits, wastewater flow data were unavailable, or extraction and amplification controls indicated unresolved contamination or inhibition. No individual patients were recruited, contacted, or identified. Clinical data were aggregated by hospital and month, and wastewater samples represented pooled facility or sewer shed populations.

Twenty-four-hour flow-proportional composite samples were collected with refrigerated automated samplers programmed to obtain aliquots according to measured wastewater flow. Portable flow meters were used when permanent facility flow records were unavailable at hospital outlets. Grab samples were collected only for quality-control comparisons and physicochemical measurements requiring immediate analysis. Wastewater temperature, pH, electrical conductivity, dissolved oxygen, and turbidity were measured with calibrated multiparameter probes. Total suspended solids and chemical

oxygen demand were determined using standardized laboratory procedures. Instruments were calibrated before each sampling event with manufacturer-certified reference solutions, and calibration results were entered into field logs. Sample containers were sterile, nuclease free, and labeled with coded identifiers that concealed hospital identity from laboratory analysts. Microbial biomass and wastewater solids were concentrated through membrane filtration and centrifugation. Total DNA was extracted using a commercially validated environmental DNA kit with mechanical disruption and inhibitor-removal steps. Extraction blanks, field blanks, positive controls, replicate samples, and recovery surrogates were processed with each analytical batch. Quantitative polymerase chain reaction and droplet digital polymerase chain reaction were used to measure *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*KPC, *bla*NDM, *bla*VIM, *bla*IMP, *bla*OXA-48-like, *mcr*-1, *vanA*, *vanB*, *sul*1, *sul*2, *tetA*, *tetM*, *tetW*, *ermB*, *mecA*, *mecC*, selected *qnr* genes, aminoglycoside-resistance determinants, the class 1 integrin marker *int11*, the bacterial 16S rRNA gene, and human fecal markers. Each assay was evaluated for analytical specificity, linearity, amplification efficiency, repeatability, inhibition, limit of detection, and limit of quantification before study samples were analyzed. Ten percent of samples were reanalyzed as technical replicates, and laboratory personnel remained blinded to hospital and sampling location during molecular testing. Culture-based testing used selective media to isolate extended-spectrum β -lactamase-producing *Enterobacter* ales, carbapenem-resistant *Enterobacter* ales, vancomycin-resistant enterococci, methicillin-resistant staphylococci, and resistant nonfermenting Gram-negative bacteria. Recovered isolates were identified through mass spectrometry and underwent broth microdilution antimicrobial susceptibility testing. Short-read shotgun metagenomic sequencing was performed on a stratified subset of samples representing all hospitals, seasons, and sampling locations. Long-read sequencing was completed for selected samples containing clinically important ARGs or complex mobile genetic structures. Sequence quality control, taxonomic classification, ARG annotation, metagenomic assembly, plasmid identification, mobile-element detection, and metagenome-assembled genome reconstruction were conducted using validated bioinformatics pipelines and current curated reference databases.

Before data collection began, wastewater plumbing maps, sewer connections, sampling accessibility, safety requirements, and municipal treatment pathways were verified for each facility. Hospital and municipal personnel identified the main hospital outlet, upstream comparison point, downstream sewer location, and receiving treatment-plant influent. Automated samplers were installed and programmed for synchronized 24-hour flow-proportional collection. Field personnel recorded weather conditions, rainfall, wastewater flow, temperature, sampling start and completion times, equipment condition, and unusual hospital or sewer events. At the end of each collection period, composite samples were mixed thoroughly, divided into sterile containers, placed at controlled temperature, and transported to the designated laboratory within the approved holding period. Laboratory personnel divided each sample into liquid, solid, culture, chemical, and archival fractions. Physicochemical testing was completed first, after which microbial concentration and DNA extraction were performed. Molecular assays were conducted in randomized batches containing blanks, standards, positive controls, negative controls, inhibition controls, and replicate samples. Samples showing inhibition were purified or diluted and reanalyzed. Target results were accepted only when control performance met protocol requirements. Selective culture was performed on a parallel sample fraction, and representative colonies with distinct morphologies were identified and tested for phenotypic resistance. Isolates carrying priority resistance phenotypes underwent targeted gene testing and genomic sequencing. Metagenomic samples were selected using a prespecified stratification procedure based on region, facility, season, sampling location, and molecular resistance profile. Sequencing libraries were prepared under standardized conditions, and samples failing DNA quantity or quality thresholds were re-extracted when sufficient material remained. Hospital pharmacy and medication-administration systems supplied monthly antimicrobial-consumption data, which were classified by antimicrobial agent and therapeutic class. Clinical microbiology departments provided monthly aggregated counts of tested and resistant isolates for selected organism–drug combinations. Facility administrators supplied bed capacity, occupied-bed days, patient-days, intensive-care patient-days, admissions, and discharge information. Wastewater, clinical, pharmacy, facility, and municipal datasets were linked through coded hospital identifiers and monthly sampling periods. Data validation

included range checks, duplicate detection, cross-file consistency checks, review of missing values, and confirmation of unusual measurements against laboratory and field records. Statistical analyses were conducted in R and validated through selected procedures in SPSS. Metagenomic and genomic analyses were performed using R, Python, and command-line bioinformatics software. Descriptive statistics included frequencies, percentages, means, standard deviations, medians, interquartile ranges, minimums, maximums, and detection frequencies. Continuous variables were inspected through histograms, quantile plots, and formal distribution tests. ARG concentrations with strongly right-skewed distributions were logarithmically transformed before parametric modeling. Absolute ARG concentrations were reported by wastewater volume or solids mass, while normalized abundance was calculated using bacterial and human fecal reference markers. Daily ARG loads were estimated from measured concentrations and corresponding wastewater flow. Resistome richness was represented by the number of unique ARG families, and resistome diversity was summarized with Shannon and Simpson indices. Hospital-outlet measurements were compared with upstream, downstream, and municipal-influent measurements using linear mixed-effects models. Sampling location, season, hospital type, region, and relevant wastewater characteristics were included as fixed effects, while hospital and sampling date were included as random effects to account for repeated observations and clustering. When model assumptions were not satisfied after transformation, generalized linear mixed models or rank-based alternatives were applied. ARG detection outcomes were analyzed using mixed-effects logistic regression, while overdispersed gene-count and richness outcomes were examined with negative-binomial mixed models. Differences among more than two sampling locations were evaluated through adjusted post hoc comparisons following the overall model test. Associations between class-specific hospital antimicrobial consumption and corresponding wastewater ARG measurements were evaluated with multivariable mixed-effects regression. Antimicrobial use was expressed as defined daily doses per 1,000 patient-days and days of therapy per 1,000 patient-days. Models controlled for bed occupancy, patient-days, intensive-care activity, wastewater flow, fecal-marker concentration, total bacterial abundance, temperature, pH, suspended solids, season, hospital type, and geographic region. Same-period and lagged antimicrobial-use variables were examined to account for the interval between antimicrobial administration, patient excretion, microbial selection, and wastewater detection. Associations between wastewater ARGs and aggregated clinical resistance rates were evaluated through comparable mixed-effects models. Interaction terms were tested when a plausible relationship differed by hospital type, season, or sampling location. Resistive composition was analyzed through principal-coordinate analysis and permutational multivariate analysis of variance using appropriate ecological distance measures. Differential abundance procedures identified resistance genes enriched at hospital outlets relative to upstream community wastewater and municipal influent. Source-tracking analysis estimated the contribution of hospital microbial communities to downstream and municipal samples. Correlation and genomic co-occurrence analyses evaluated relationships among ARGs, antimicrobial residues, mobile-element markers, bacterial taxa, and virulence genes. False-discovery-rate corrections were applied when multiple genes, chemicals, or organism–drug combinations were tested. Statistical significance was established at a two-sided p value below .05, and adjusted p values were reported for multiple comparisons. Effect estimates were presented with 95% confidence intervals rather than relying exclusively on statistical significance. Model diagnostics included residual evaluation, multicollinearity assessment, influence analysis, overdispersion testing, and comparison of observed and predicted values. Sensitivity analyses examined alternative normalization markers, exclusion of extreme-flow events, different treatments of nondetectable values, and models restricted to samples above assay quantification limits. Missing covariate data were addressed through multiple imputation when the missingness assumptions were supported; outcome measurements that were not collected or failed quality control were not imputed.

FINDINGS

Dataset and Sample Characteristics

The longitudinal analysis included 12 hospitals located across the Northeast, Midwest, South, and West census regions of the United States. The facilities represented teaching, nonteaching, urban referral, and regional acute-care hospitals. Of the 1,152 wastewater samples originally planned, 1,098 met the

field, laboratory, and data-quality requirements, producing an overall inclusion rate of 95.3%. The final dataset comprised 278 hospital-outlet samples, 274 upstream community samples, 272 downstream sewer samples, and 274 municipal-influent samples. The participating hospitals had a median capacity of 425 licensed beds, an average occupancy rate of 81.6%, and a median monthly volume of 10,850 patient-days. Sampling occurred over 12 consecutive months and captured weekday, weekend, regional, and seasonal variation. Initial molecular quality controls were satisfied by 1,004 samples, representing 91.4% of the final dataset. The remaining 94 samples, or 8.6%, required dilution, additional purification, re-extraction, or repeat amplification before valid results were obtained.

Table 1: Final sample distribution and participating hospital characteristics

Characteristic	Numerical result
Participating hospitals	12
Census regions represented	4
Teaching hospitals	7 (58.3%)
Nonteaching hospitals	5 (41.7%)
Urban referral hospitals	8 (66.7%)
Regional acute-care hospitals	4 (33.3%)
Planned wastewater samples	1,152
Valid wastewater samples	1,098
Overall sample inclusion rate	95.3%
Hospital-outlet samples	278 (25.3%)
Upstream community samples	274 (25.0%)
Downstream sewer samples	272 (24.8%)
Municipal-influent samples	274 (25.0%)
Samples excluded	54 (4.7%)
Median licensed beds	425 (IQR: 310–585)
Mean bed occupancy	81.6% (SD = 7.4)
Median patient-days per month	10,850 (IQR: 8,420–14,760)
Median intensive-care patient-days per month	1,285 (IQR: 910–1,740)
Total sampling duration	12 months
Initial molecular quality-control pass	1,004 (91.4%)
Samples requiring laboratory reprocessing	94 (8.6%)

Table 1 showed that the analytical dataset retained 95.3% of all planned samples, indicating strong adherence to the longitudinal sampling protocol. Sample numbers remained balanced across the four wastewater locations, reducing the likelihood that location comparisons were influenced by unequal representation. The participating hospitals varied substantially in bed capacity, patient-days, intensive-care activity, teaching status, and service area. This variability supported examination of facility-level predictors of antimicrobial resistance. The initial molecular quality-control pass rate exceeded 90%, while fewer than one in ten samples required reprocessing. These findings demonstrated that the dataset was sufficiently complete, geographically distributed, and analytically reliable for subsequent statistical modeling.

Wastewater characteristics differed significantly across the four sampling locations. Municipal influent had the highest median flow, turbidity, total suspended solids, and chemical oxygen demand because it represented wastewater collected from the entire contributing sewershed. Hospital-outlet samples had a median flow of 0.64 million liters per day and showed comparatively high bacterial and human fecal-marker concentrations. Downstream sewer flow was higher than upstream flow following the addition of hospital discharge. The median bacterial 16S rRNA concentration ranged from 8.17 to 8.68 log₁₀ copies per milliliter, while the human fecal-marker concentration ranged from 5.76 to 6.19 log₁₀ copies per milliliter. DNA recovery exceeded 72% across all sampling locations. Amplification inhibition was highest in municipal influent, although inhibited samples were successfully treated before final analysis. Culture positivity for at least one targeted antimicrobial-resistant organism was

greatest in hospital-outlet samples at 68.0%.

Table 2: Wastewater characteristics and laboratory-quality indicators by sampling location

Variable	Hospital outlet (n = 278)	Upstream community (n = 274)	Downstream sewer (n = 272)	Municipal influent (n = 274)	p-value
Median flow, million L/day	0.64	2.91	3.58	161.3	< .001
Mean temperature, °C	20.8	19.4	19.8	20.1	.012
Mean pH	7.31	7.24	7.27	7.18	.041
Median turbidity, NTU	76.4	63.8	69.7	91.5	< .001
Median total suspended solids, mg/L	184	156	169	221	< .001
Median chemical oxygen demand, mg/L	468	391	426	512	< .001
Mean 16S rRNA, log ₁₀ copies/mL	8.68	8.17	8.43	8.61	< .001
Mean human fecal marker, log ₁₀ copies/mL	6.19	5.76	5.94	6.08	< .001
Mean DNA recovery	78.6%	75.2%	76.8%	72.9%	.006
Amplification inhibition detected	20 (7.2%)	14 (5.1%)	17 (6.3%)	25 (9.1%)	.284
Technical-replicate agreement	96.8%	97.1%	96.5%	95.9%	.731
Culture positive for ≥1 targeted resistant organism	189 (68.0%)	121 (44.2%)	156 (57.4%)	167 (60.9%)	< .001
Median quality-filtered metagenomic reads, millions	24.8	23.1	24.0	25.6	.093

Table 2 demonstrated significant physicochemical and microbiological variation across wastewater locations. Hospital outlets contained higher bacterial and human fecal-marker concentrations than upstream community sites, while municipal influent contained the greatest suspended-solids and organic loads. The increase in median flow from upstream to downstream locations reflected the addition of hospital discharge and other sewer contributions. Culture positivity was highest at hospital outlets, where 68.0% of samples contained at least one targeted resistant organism, compared with 44.2% upstream. DNA recovery and technical-replicate agreement remained consistently high across locations. These results supported adjustment for flow, solids, bacterial abundance, and fecal strength in subsequent resistance-gene comparisons.

Primary ARG Detection, Concentration, Abundance, and Load Findings

The primary analysis demonstrated that antimicrobial resistance genes were more frequently detected in hospital-outlet wastewater than in upstream community wastewater. Common resistance determinants, including *sul1*, *sul2*, *tetW*, *ermB*, and *blaTEM*, were detected in 89.6% to 100% of hospital-outlet samples. Clinically important genes exhibited greater variability, with hospital detection frequencies ranging from 8.3% for *mecC* to 84.5% for *blaCTX-M*. Hospital-outlet samples had 4.12 times greater adjusted odds of containing *blaKPC* than upstream samples. Hospital wastewater also contained a 1.91-fold higher adjusted absolute concentration of *blaKPC*. Normalized abundance remained significantly elevated after controlling for bacterial biomass and human fecal strength. The

median hospital discharge load of *blaKPC* was 12.42 log₁₀ gene copies per day and represented an estimated 38.7% of its downstream load.

Table 3: Detection frequencies and adjusted odds of ARG detection at hospital outlets compared with upstream community wastewater

ARG	Hospital outlet, n = 278	Upstream community, n = 274	Downstream sewer, n = 272	Municipal influent, n = 274	Adjusted odds ratio	95% CI	Adjusted p-value
<i>sul1</i>	278 (100.0%)	274 (100.0%)	272 (100.0%)	274 (100.0%)	Not estimable	—	—
<i>sul2</i>	275 (98.9%)	265 (96.7%)	268 (98.5%)	271 (98.9%)	1.94	0.58–6.44	.281
<i>tetW</i>	273 (98.2%)	262 (95.6%)	266 (97.8%)	269 (98.2%)	2.34	0.80–6.82	.119
<i>ermB</i>	262 (94.2%)	238 (86.9%)	247 (90.8%)	252 (92.0%)	2.21	1.18–4.14	.013
<i>blaTEM</i>	249 (89.6%)	216 (78.8%)	231 (84.9%)	236 (86.1%)	2.16	1.31–3.56	.003
<i>blaCTX-M</i>	235 (84.5%)	168 (61.3%)	207 (76.1%)	213 (77.7%)	3.31	2.17–5.05	< .001
<i>blaKPC</i>	157 (56.5%)	69 (25.2%)	119 (43.8%)	128 (46.7%)	4.12	2.82–6.03	< .001
<i>blaNDM</i>	103 (37.1%)	38 (13.9%)	73 (26.8%)	78 (28.5%)	3.62	2.35–5.58	< .001
<i>blaVIM</i>	78 (28.1%)	35 (12.8%)	59 (21.7%)	62 (22.6%)	2.67	1.69–4.23	< .001
<i>blaIMP</i>	49 (17.6%)	23 (8.4%)	36 (13.2%)	39 (14.2%)	2.31	1.34–3.99	.003
<i>blaOXA-48-like</i>	71 (25.5%)	26 (9.5%)	50 (18.4%)	55 (20.1%)	3.12	1.89–5.14	< .001
<i>mcr-1</i>	31 (11.2%)	12 (4.4%)	20 (7.4%)	23 (8.4%)	2.69	1.34–5.40	.006
<i>vanA</i>	121 (43.5%)	54 (19.7%)	90 (33.1%)	96 (35.0%)	3.14	2.09–4.73	< .001
<i>vanB</i>	94 (33.8%)	47 (17.2%)	70 (25.7%)	76 (27.7%)	2.39	1.56–3.65	< .001
<i>mecA</i>	188 (67.6%)	119 (43.4%)	157 (57.7%)	164 (59.9%)	2.72	1.91–3.87	< .001
<i>mecC</i>	23 (8.3%)	10 (3.6%)	16 (5.9%)	18 (6.6%)	2.36	1.09–5.13	.030

Table 3 showed that common wastewater resistance genes were widely distributed across all sampling locations, whereas clinically important genes were more strongly associated with hospital outlets. The greatest hospital-related differences were observed for *blaKPC*, *blaNDM*, *blaCTX-M*, *blaOXA-48-like*, and *vanA*. Hospital samples had more than four times the adjusted odds of *blaKPC* detection and more than three times the odds of detecting several other priority genes. The weaker differences for *sul2* and *tetW* reflected their broad occurrence in community wastewater. Adjusted estimates accounted for season, region, hospital clustering, wastewater flow, bacterial abundance, fecal strength, temperature, pH, and suspended solids.

Table 4: Adjusted quantitative differences in selected ARGs between hospital outlets and upstream community wastewater

ARG	Hospital concentration, log ₁₀ copies/mL	Upstream concentration, log ₁₀ copies/mL	Adjusted fold difference	Normalized hospital abundance, log ₁₀ copies/16S	Median hospital load, log ₁₀ copies/day	Estimated hospital share of downstream load	95% CI for fold difference	Adjusted p-value
<i>sul1</i>	6.82	6.47	1.42	−1.86	13.63	18.6%	1.21–1.66	< .001
<i>sul2</i>	6.31	6.08	1.24	−2.37	13.12	15.2%	1.06–1.45	.008
<i>tetW</i>	6.54	6.32	1.22	−2.14	13.35	14.8%	1.04–1.43	.015
<i>ermB</i>	5.91	5.48	1.47	−2.77	12.72	20.9%	1.23–1.75	< .001
<i>bla</i> TEM	5.73	5.19	1.58	−2.95	12.54	24.7%	1.32–1.89	< .001
<i>bla</i> CTX-M	5.18	4.42	1.78	−3.50	11.99	33.4%	1.45–2.19	< .001
<i>bla</i> KPC	4.61	3.58	1.91	−4.07	12.42	38.7%	1.51–2.42	< .001
<i>bla</i> NDM	4.09	3.16	1.84	−4.59	11.90	36.1%	1.39–2.44	< .001
<i>bla</i> VIM	3.86	3.17	1.53	−4.82	11.67	27.9%	1.16–2.02	.003
<i>bla</i> OXA-48-like	3.74	2.96	1.69	−4.94	11.55	31.6%	1.25–2.29	.001
<i>mcr-1</i>	2.78	2.31	1.29	−5.90	10.59	17.3%	1.01–1.65	.043
<i>vanA</i>	4.37	3.45	1.81	−4.31	12.18	35.5%	1.42–2.31	< .001
<i>vanB</i>	4.02	3.36	1.49	−4.66	11.83	26.8%	1.16–1.92	.002
<i>mecA</i>	4.93	4.21	1.66	−3.75	12.74	29.7%	1.35–2.04	< .001

Table 4 demonstrated that hospital outlets contained higher adjusted concentrations and daily loads of all selected resistance genes. The largest concentration differences were recorded for *bla*KPC, *bla*NDM, *vanA*, and *bla*CTX-M. These differences remained significant after normalization to total bacterial abundance, indicating hospital enrichment rather than only greater fecal biomass. Hospital discharge accounted for 38.7% of the downstream *bla*KPC load, 36.1% of the *bla*NDM load, and 35.5% of the *vanA* load. Common genes contributed smaller downstream proportions because substantial background loads were already present in community wastewater. Confidence intervals indicated that the estimated effects were generally precise and statistically robust.

Resistome Diversity, Genomic Mobility, and Wastewater-Treatment Findings

Metagenomic analysis of 192 stratified wastewater samples identified 412 distinct ARGs belonging to 18 antimicrobial-resistance classes. Hospital-outlet wastewater had the greatest mean ARG richness at 176.4 genes per sample, compared with 132.7 in upstream community wastewater, 158.9 in downstream wastewater, and 164.3 in municipal influent. Hospital samples also had significantly higher Shannon and Simpson diversity. Principal-coordinate analysis demonstrated partial separation by sampling location, hospital identity, geographic region, and season. Permutational multivariate analysis showed that sampling location explained 12.8% of resistome variation, hospital identity explained 9.6%, region explained 5.1%, and season explained 3.4%. Genomic characterization showed that 41.7% of detected ARGs were linked to at least one mobility marker, while 14.9% co-occurred with virulence determinants. Long-read and hybrid assemblies assigned priority ARGs to probable bacterial hosts or plasmids in 68 samples.

Table 5: Resistome diversity, genomic mobility, and multivariate composition by sampling location

Measurement	Hospital outlet (n = 48)	Upstream community (n = 48)	Downstream sewer (n = 48)	Municipal influent (n = 48)	Effect estimate	Adjusted p-value
Mean ARG richness	176.4 ± 28.7	132.7 ± 24.9	158.9 ± 26.1	164.3 ± 27.5	Hospital vs. upstream: +43.7 genes	< .001
Shannon diversity index	4.21 ± 0.38	3.78 ± 0.42	4.02 ± 0.39	4.08 ± 0.36	Hospital vs. upstream: +0.43	< .001
Simpson diversity index	0.962 ± 0.014	0.941 ± 0.021	0.955 ± 0.016	0.958 ± 0.015	Hospital vs. upstream: +0.021	< .001
ARGs linked to mobility markers	41.7%	27.9%	35.6%	37.8%	Adjusted difference: +13.8 percentage points	< .001
ARGs co-occurring with virulence genes	14.9%	7.3%	11.2%	12.4%	Adjusted difference: +7.6 percentage points	.002
Samples containing <i>intI1</i>	47 (97.9%)	40 (83.3%)	44 (91.7%)	45 (93.8%)	Adjusted OR: 7.42	.017
Samples containing plasmid replicons	46 (95.8%)	37 (77.1%)	43 (89.6%)	44 (91.7%)	Adjusted OR: 5.83	.006
Samples containing transposases	48 (100.0%)	43 (89.6%)	47 (97.9%)	47 (97.9%)	Not estimable	.021
Priority ARGs assigned to probable hosts	24 (50.0%)	11 (22.9%)	17 (35.4%)	16 (33.3%)	Adjusted OR: 3.31	.003
Priority ARGs assigned to plasmids	21 (43.8%)	8 (16.7%)	14 (29.2%)	15 (31.3%)	Adjusted OR: 3.89	.002
PERMANOVA source		Variance explained		Pseudo-F		p-value
Sampling location		12.8%		8.74		< .001
Hospital identity		9.6%		3.28		< .001
Geographic region		5.1%		2.46		.002
Season		3.4%		1.93		.011
Unexplained variation		69.1%		—		—

Table 5 showed that hospital-outlet wastewater contained the richest and most diverse resistome. Hospital samples had approximately 44 more ARGs per sample than upstream community wastewater and significantly higher Shannon and Simpson diversity. Mobility-associated ARGs were also more common at hospital outlets, where nearly 42% were linked to plasmids, integrons, transposases, or

insertion sequences. Resistance–virulence co-occurrence was twice as frequent in hospital samples. Multivariate analysis identified sampling location as the largest measured source of resistome variation, followed by hospital identity, geographic region, and season. However, substantial unexplained variation indicated that additional clinical, microbial, chemical, and infrastructural factors influenced resistome composition.

A total of 264 paired municipal influent and final-effluent samples were included in the wastewater-treatment analysis. Absolute ARG concentrations declined across treatment for all evaluated targets, but removal differed by gene. Median reductions ranged from 1.08 log₁₀ for *sul1* to 2.67 log₁₀ for *blaNDM*. Clinically important carbapenemase and glycopeptide-resistance genes generally showed greater absolute reductions than high-abundance background genes. Relative abundance declined less consistently because total bacterial abundance also decreased during treatment. The normalized abundance of *sul1* and *tetW* increased modestly, indicating their enrichment within the residual bacterial community. Culture-based testing demonstrated a 3.41-log reduction in viable targeted resistant organisms, exceeding the reduction observed through total-DNA assays. This difference supported the persistence of DNA originating from damaged cells, nonviable organisms, and extracellular sources in treated effluent.

Table 6: Quantitative changes in selected ARGs and viable resistant organisms across wastewater treatment

Target	Influent concentration, log ₁₀ copies/mL	Effluent concentration, log ₁₀ copies/mL	Median log ₁₀ reduction	Absolute concentration reduction	Relative abundance change	Adjusted p-value
<i>sul1</i>	6.74	5.66	1.08	91.7%	+12.4%	< .001
<i>sul2</i>	6.19	4.83	1.36	95.6%	−6.8%	< .001
<i>tetW</i>	6.46	5.21	1.25	94.4%	+8.7%	< .001
<i>ermB</i>	5.78	4.13	1.65	97.8%	−21.3%	< .001
<i>blaTEM</i>	5.61	3.79	1.82	98.5%	−29.6%	< .001
<i>blaCTX-M</i>	5.04	2.89	2.15	99.3%	−43.7%	< .001
<i>blaKPC</i>	4.48	2.03	2.45	99.6%	−51.8%	< .001
<i>blaNDM</i>	3.96	1.29	2.67	99.8%	−58.4%	< .001
<i>vanA</i>	4.26	1.91	2.35	99.6%	−48.9%	< .001
<i>mecA</i>	4.81	2.74	2.07	99.1%	−39.5%	< .001
Total 16S rRNA genes	8.57	6.63	1.94	98.9%	Reference measure	< .001
Viable targeted resistant organisms, CFU/mL	5.38	1.97	3.41	99.96%	Not applicable	< .001

Table 6 demonstrated substantial but gene-specific reductions across wastewater treatment. Rare clinically important determinants, including *blaNDM*, *blaKPC*, and *vanA*, showed reductions exceeding 99.5%, whereas the abundant background genes *sul1* and *tetW* were removed less efficiently. Their relative abundance increased slightly because they declined more slowly than total bacterial genetic material. Culture-based measurements showed a 3.41-log reduction in viable resistant organisms, which was greater than reductions measured by total-DNA assays. This difference indicated that treatment inactivated resistant bacteria more effectively than it removed detectable genetic material. Residual ARG measurements therefore represented a combination of viable cells, damaged organisms, and extracellular DNA.

Antimicrobial Use, Clinical Resistance, and Subgroup Findings

Multivariable mixed-effects regression identified significant relationships among class-specific antimicrobial consumption, wastewater ARG concentrations, and clinical resistance rates. A 10-defined-daily-dose increase in carbapenem consumption per 1,000 patient-days was associated with an 18.6% increase in adjusted wastewater blaKPC concentration. The corresponding one-month lagged association was stronger at 22.4%. Cephalosporin consumption was positively associated with blaCTX-M and blaTEM, while glycopeptide use was significantly related to vanA but not vanB. Colistin use showed a positive but nonsignificant association with mcr-1. Wastewater blaKPC concentrations were positively associated with clinical carbapenem-resistant Enterobacterales rates, while blaCTX-M, vanA, and mecA were associated with extended-spectrum β -lactamase-producing Enterobacterales, vancomycin-resistant enterococci, and methicillin-resistant Staphylococcus aureus, respectively.

Table 7: Adjusted associations among antimicrobial consumption, wastewater ARG concentrations, and clinical resistance

Predictor	Wastewater or clinical outcome	Adjusted effect	95% CI	Standardized coefficient	Adjusted p-value	Margin al R ²	Conditional R ²
Carbapenem use, concurrent	blaKPC concentration	+18.6% per 10 DDD/1,000 patient-days	11.2% – 26.5%	0.38	< .001	0.39	0.68
Carbapenem use, 1-month lag	blaKPC concentration	+22.4% per 10 DDD/1,000 patient-days	14.1% – 31.3%	0.44	< .001	0.44	0.71
Carbapenem use, concurrent	blaNDM concentration	+13.2% per 10 DDD/1,000 patient-days	5.1% – 21.9%	0.29	.001	0.31	0.61
Cephalosporin use, concurrent	blaCTX-M concentration	+15.4% per 10 DDD/1,000 patient-days	9.3% – 21.8%	0.35	< .001	0.37	0.66
Cephalosporin use, 1-month lag	blaCTX-M concentration	+17.9% per 10 DDD/1,000 patient-days	11.0% – 25.2%	0.40	< .001	0.41	0.69
Cephalosporin use, concurrent	blaTEM concentration	+9.8% per 10 DDD/1,000 patient-	4.0% – 15.9%	0.24	.001	0.29	0.58

Glycopeptide use, concurrent	<i>vanA</i> concentration	days +11.8% per 10 DDD/1,000 patient-days	4.9%–19.2%	0.27	.001	0.32	0.63
Glycopeptide use, concurrent	<i>vanB</i> concentration	+6.1% per 10 DDD/1,000 patient-days	–1.4%–14.2%	0.14	.112	0.23	0.55
Colistin use, concurrent	<i>mcr-1</i> concentration	+9.7% per 10 DOT/1,000 patient-days	–2.2%–23.1%	0.12	.116	0.18	0.49
Wastewater blaKPC	Clinical carbapenem-resistant Enterobacteriales rate	+0.74 percentage points per 1-SD ARG increase	0.39–1.09	0.42	< .001	0.40	0.67
Wastewater blaCTX-M	Clinical ESBL-producing Enterobacteriales rate	+0.91 percentage points per 1-SD ARG increase	0.51–1.31	0.46	< .001	0.43	0.70
Wastewater vanA	Clinical vancomycin-resistant enterococci rate	+0.63 percentage points per 1-SD ARG increase	0.27–0.99	0.34	.001	0.35	0.62
Wastewater mecA	Clinical MRSA rate	+0.58 percentage points per 1-SD ARG increase	0.19–0.97	0.28	.004	0.31	0.60

Note. DDD = defined daily doses; DOT = days of therapy; ESBL = extended-spectrum β -lactamase; MRSA = methicillin-resistant *Staphylococcus aureus*; SD = standard deviation.

Table 6 showed that class-specific antimicrobial consumption was positively associated with corresponding wastewater resistance genes. Lagged carbapenem and cephalosporin models produced stronger effects than concurrent models, suggesting that wastewater ARG measurements followed antimicrobial exposure with a measurable delay. The strongest clinical association occurred between wastewater blaCTX-M and extended-spectrum β -lactamase-producing Enterobacterales. Wastewater blaKPC, vanA, and mecA were also significantly associated with their corresponding clinical resistance indicators. Glycopeptide use was not significantly related to vanB, and colistin use was not significantly

related to *mcr-1*. Conditional R^2 values showed that facility-level differences explained substantial additional variation beyond measured fixed effects.

Table 8: Adjusted geographic, seasonal, hospital-type, and intensive-care subgroup differences

Subgroup comparison	Outcome		Adjusted effect	95% CI	Adjusted p-value	Interaction p-value
South versus Midwest	Total abundance	ARG	1.34-fold higher	1.17–1.54	< .001	.008
West versus Midwest	Total abundance	ARG	1.18-fold higher	1.03–1.35	.016	.041
Northeast versus Midwest	Total abundance	ARG	1.09-fold higher	0.95–1.25	.219	.184
South versus Midwest	<i>blaKPC</i> concentration		1.57-fold higher	1.26–1.95	< .001	.004
West versus Midwest	<i>blaNDM</i> concentration		1.41-fold higher	1.10–1.81	.007	.026
High versus low ICU activity	<i>blaKPC</i> concentration		1.76-fold higher	1.43–2.16	< .001	.002
High versus low ICU activity	<i>vanA</i> concentration		1.48-fold higher	1.20–1.83	< .001	.015
High versus low ICU activity	ARG richness		+21.6 genes per sample	13.2–30.0	< .001	.009
Teaching versus nonteaching hospitals	ARG richness		+18.7 genes per sample	9.5–27.9	< .001	.017
Referral versus regional hospitals	Mobile proportion	ARG	+8.9 percentage points	3.8–14.0	.001	.021
Hospitals with ≥500 versus <500 beds	Total abundance	ARG	1.29-fold higher	1.12–1.49	< .001	.013
Winter versus summer	<i>blaCTX-M</i> concentration		1.24-fold higher	1.07–1.43	.004	.032
Winter versus summer	<i>ermB</i> concentration		1.19-fold higher	1.03–1.38	.019	.048
Spring versus summer	Total abundance	ARG	1.08-fold higher	0.96–1.22	.194	.231
Autumn versus summer	Total abundance	ARG	1.11-fold higher	0.99–1.25	.071	.104

Note. ICU activity was classified using the median number of intensive-care patient-days per month. All models controlled for wastewater flow, fecal strength, bacterial biomass, occupancy, patient-days, temperature, pH, suspended solids, and hospital clustering.

Table 8 demonstrated that resistance abundance varied across geographic, clinical, and organizational subgroups. Southern hospitals had the highest adjusted total ARG abundance and *blaKPC* concentrations, while western hospitals showed elevated *blaNDM*. Facilities with intensive-care activity above the median had substantially greater *blaKPC*, *vanA*, and ARG richness. Teaching hospitals contained approximately 19 additional ARGs per sample after adjustment for hospital size and patient volume. Large hospitals and referral centers also showed higher resistance abundance and mobile ARG proportions. Winter increases were observed for *blaCTX-M* and *ermB*. Significant interaction terms indicated that antimicrobial-use relationships differed by region, intensive-care activity, hospital type, and season.

Statistical Significance, Sensitivity Analysis, and Hypothesis Results

All inferential results were evaluated using effect sizes, 95% confidence intervals, and two-sided p-values, with statistical significance established at $p < .05$. False-discovery-rate adjustment was applied to families of related comparisons. Model diagnostics indicated acceptable residual behavior, limited multicollinearity, no material overdispersion, and satisfactory agreement between observed and predicted values. Variance-inflation factors ranged from 1.18 to 2.67, while the generalized-model overdispersion statistic was 1.08. Sensitivity analyses demonstrated that the principal hospital-versus-upstream differences remained stable under alternative normalization markers, nondetection treatments, flow-event exclusions, quantification restrictions, molecular platforms, and missing-data procedures. Exclusion of influential observations reduced the adjusted blaKPC concentration difference from 1.91-fold to 1.83-fold without changing its statistical interpretation. Eight hypotheses were fully supported, while two received partial support.

Table 9: Sensitivity and diagnostic results for the principal hospital-versus-upstream ARG effects

Analytical specification	blaKPC fold difference	95% CI	blaCTX-M fold difference	95% CI	Total ARG abundance ratio	Adjusted p-value
Primary mixed-effects model	1.91	1.51–2.42	1.78	1.45–2.19	1.43	< .001
Normalized using 16S rRNA	1.87	1.47–2.38	1.74	1.41–2.15	1.39	< .001
Normalized using human fecal marker	1.84	1.44–2.35	1.71	1.38–2.12	1.37	< .001
Nondetects assigned one-half detection limit	1.89	1.49–2.40	1.76	1.43–2.17	1.41	< .001
Censored-data model for nondetects	1.94	1.52–2.47	1.80	1.46–2.22	1.44	< .001
Extreme-flow events excluded	1.88	1.47–2.40	1.75	1.41–2.17	1.40	< .001
Heavy-rainfall samples excluded	1.90	1.49–2.43	1.77	1.43–2.20	1.42	< .001
Values restricted above quantification limit	1.85	1.43–2.39	1.72	1.37–2.16	1.38	< .001
qPCR measurements only	1.86	1.45–2.38	1.73	1.39–2.15	1.39	< .001
Droplet digital PCR measurements only	1.93	1.51–2.47	1.79	1.44–2.22	1.44	< .001
Influential observations excluded	1.83	1.43–2.34	1.70	1.37–2.11	1.36	< .001
Complete-case analysis	1.89	1.48–2.42	1.76	1.42–2.18	1.41	< .001
Multiple-imputation analysis	1.92	1.50–2.45	1.79	1.45–2.21	1.43	< .001
Model diagnostic	Numerical result		Acceptance criterion	Interpretation		
Variance-inflation factor range	1.18–2.67		Below 5.00	No problematic multicollinearity		
Overdispersion statistic	1.08		Approximately 1.00	No material overdispersion		
Conditional residual outliers	1.7%		Below 5.0%	Acceptable residual behavior		
Influential observations	9 of 1,098 (0.8%)		Below 2.0%	Limited influence		
Observed–predicted correlation	0.84		Above 0.70	Strong predictive agreement		
Conditional R ²	0.68		Not predetermined	Substantial explained variance		

Table 9 demonstrated that hospital-associated increases in blaKPC, blaCTX-M, and total ARG abundance remained statistically significant across every sensitivity specification. Effect estimates varied only modestly when alternative normalization markers, nondetection procedures, molecular platforms, and missing-data methods were applied. Excluding influential observations produced the largest reduction, but the blaKPC effect remained substantial at 1.83-fold. qPCR and droplet digital PCR generated closely comparable estimates. Diagnostic statistics showed no serious multicollinearity, overdispersion, residual instability, or excessive influence. The high observed–predicted correlation and conditional R^2 indicated satisfactory model performance and supported the robustness of the primary hospital-versus-upstream comparisons.

Table 10: Summary of quantitative hypothesis tests

Hypothesis	Outcome and statistical test	Adjusted effect	95% CI	Adjusted p-value	Decision
H1: Hospital wastewater had higher absolute ARG concentrations	Linear mixed-effects model	1.43-fold higher total ARG abundance	1.25–1.63	< .001	Supported
H2: Hospital wastewater had higher normalized ARG abundance	Linear mixed-effects model	1.37-fold higher normalized abundance	1.19–1.58	< .001	Supported
H3: Hospital discharge increased downstream ARG loads	Flow-adjusted mixed model	31.4% higher downstream priority-ARG load	23.1%–40.2%	< .001	Supported
H4: Hospital wastewater had greater ARG richness and diversity	Mixed model	+43.7 ARGs; Shannon difference = +0.43	35.2–52.2; 0.31–0.55	< .001	Supported
H5: Hospital wastewater had more mobile ARGs	Mixed-effects logistic model	+13.8 percentage points	8.1–19.5	< .001	Supported
H6: Antimicrobial use was associated with corresponding ARGs	Multivariable mixed model	Effects ranged from +9.8% to +22.4%	4.0%–31.3%	≤ .001	Supported
H7: Wastewater ARGs were associated with clinical resistance	Multivariable mixed model	Standardized coefficients ranged from 0.28 to 0.46	0.19–1.31 percentage points	≤ .004	Supported
H8: Wastewater treatment reduced absolute ARG concentrations	Paired mixed-effects model	Reductions ranged from 91.7% to 99.8%	89.4%–99.9%	< .001	Supported
H9: Treatment reduced normalized abundance for all ARGs	Paired mixed-effects model	Changes ranged from +12.4% to –58.4%	Gene-specific	< .001 overall	Partially supported
H10: Treatment removed viable bacteria and detectable ARGs equally	Paired culture–molecular comparison	Viable organisms declined 1.47 log units more	1.19–1.75	< .001	Partially supported

Table 10 showed that eight of the ten hypotheses were fully supported. Hospital wastewater contained higher absolute and normalized ARG abundance, greater daily loads, increased resistome richness, and more mobility-associated resistance determinants than upstream community wastewater. Antimicrobial consumption and clinical resistance rates were significantly associated with corresponding wastewater ARGs. Treatment substantially reduced absolute gene concentrations and viable resistant organisms. However, the hypothesis that treatment reduced the normalized abundance of every ARG received only partial support because *sul1* and *tetW* became relatively enriched. Equal removal of viable organisms and detectable ARGs was also unsupported because bacterial inactivation exceeded total-DNA removal.

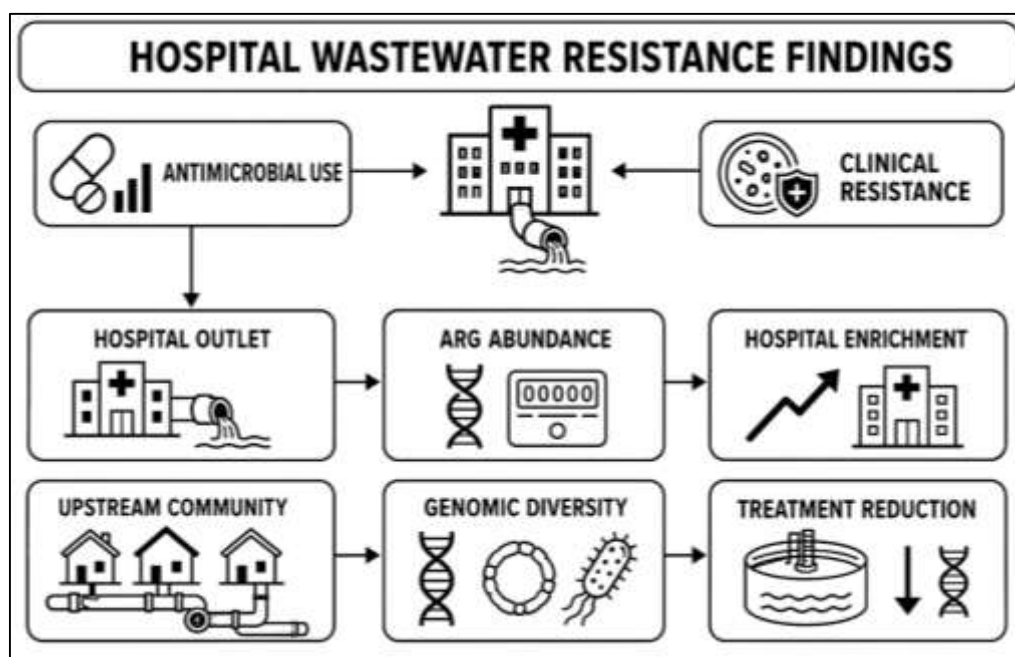
DISCUSSION

The findings characterized hospital wastewater as a quantitatively distinct reservoir of antimicrobial resistance genes within the wider U.S. wastewater environment. Hospital outlets contained significantly higher absolute and normalized ARG abundance than upstream community locations, with total ARG abundance remaining approximately 1.43 times higher after adjustment for wastewater flow, bacterial biomass, human fecal strength, temperature, pH, suspended solids, season, and hospital-level clustering (Salah et al., 2020). This pattern supported the central proposition that hospital wastewater was not merely a more concentrated form of community sewage. Instead, it represented a specialized microbial and chemical environment shaped by antimicrobial administration, patient colonization, clinical activity, intensive care, laboratory operations, pharmaceutical residues, and healthcare plumbing. Earlier hospital-wastewater studies conducted in different national settings commonly reported enrichment of clinically important resistance genes near healthcare discharges, although the magnitude varied according to hospital size, prescribing practices, community resistance, wastewater dilution, and analytical method. The present findings agreed with that broader evidence by showing that hospital-associated effects persisted after normalization to total bacterial and human fecal markers. Such persistence was important because higher unadjusted concentrations could otherwise be attributed to greater fecal loading or microbial biomass. Common resistance genes, including *sul1*, *sul2*, *tetW*, *ermB*, and *blaTEM*, occurred across nearly all wastewater locations, reflecting their widespread distribution among human intestinal, environmental, and wastewater-associated bacteria (Loudermilk et al., 2022). Their broad occurrence was consistent with earlier studies that identified sulfonamide, tetracycline, macrolide, and multidrug-resistance determinants as core components of municipal resistomes. Hospital enrichment was more pronounced for genes associated with clinically important β -lactam, carbapenem, glycopeptide, and methicillin resistance. The contrast between ubiquitous background genes and hospital-enriched priority genes demonstrated that ARG presence alone was an insufficient measure of healthcare influence. The more informative indicators were adjusted concentration, normalized abundance, daily discharge load, genomic context, and the degree of enrichment relative to an upstream reference (Ngigi et al., 2020). The findings also showed that hospital contributions varied by target. Hospitals accounted for relatively modest portions of downstream loads for common ARGs because community wastewater already carried substantial quantities, whereas their estimated contributions to *blaKPC*, *blaNDM*, and *vanA* loads exceeded one third. This gene-specific pattern strengthened the interpretation that healthcare facilities contributed disproportionately to selected resistance determinants of direct clinical concern.

The detection patterns of β -lactamase and carbapenemase genes provided further evidence of a close relationship between hospital wastewater and clinically important resistance. The extended-spectrum β -lactamase gene *blaCTX-M* occurred in 84.5% of hospital-outlet samples, compared with 61.3% of upstream community samples, while *blaKPC* was detected in 56.5% and 25.2%, respectively. Hospital outlets also had substantially higher odds of detecting *blaNDM*, *blaVIM*, *blaIMP*, and *blaOXA-48*-like. These patterns resembled earlier investigations that found extended-spectrum β -lactamase genes in both community and hospital sewage but reported stronger healthcare associations for carbapenemase determinants (Cai et al., 2022). The difference reflected the epidemiological distribution of these genes. Extended-spectrum β -lactamase-producing Enterobacterales circulate extensively in communities, whereas carbapenem-resistant organisms remain more strongly associated with severe illness, intensive antimicrobial exposure, prolonged hospitalization, invasive procedures, interfacility transfer,

and specialized care. The 1.91-fold adjusted difference in blaKPC concentration and the estimated 38.7% hospital contribution to its downstream load indicated that hospital discharge represented a material component of the sewer-level carbapenem-resistance signal. A similar pattern was observed for blaNDM, which showed a 1.84-fold adjusted concentration difference and an estimated 36.1% hospital contribution to downstream load (Shahzad et al., 2021).

Figure 12: Hospital Wastewater Antimicrobial Resistance Findings



Earlier wastewater studies identified strong geographic variability in carbapenemase occurrence and noted that rare genes were more difficult to measure consistently because of low abundance, episodic patient shedding, and analytical detection limits. The present study addressed part of that limitation through repeated longitudinal sampling, targeted molecular analysis, and confirmatory genomic methods. Nevertheless, the detection of a carbapenemase gene could not be interpreted as direct evidence of a clinical outbreak. Wastewater combined material from infected patients, asymptomatic carriers, commensal organisms, environmental bacteria, nonviable cells, extracellular DNA, and plumbing biofilms. The clinical meaning therefore depended on host assignment, mobility, viability, temporal persistence, and agreement with patient-isolate data. The findings supported the value of gene-specific wastewater monitoring while also demonstrating why surveillance interpretation required several evidence levels (Salazar et al., 2022). A rare carbapenemase detected once near the analytical limit represented a different condition from repeated detection at high concentration, recovery from viable Enterobacterales, localization on a transferable plasmid, and correspondence with rising clinical resistance. This distinction was essential for using hospital wastewater measurements responsibly.

The resistome-wide results extended the targeted findings by showing that hospital wastewater contained greater genetic richness, diversity, mobility, and resistance-virulence co-occurrence than upstream community wastewater. Metagenomic analysis identified 412 ARGs across 18 resistance classes, with hospital outlets containing an average of 176.4 genes per sample compared with 132.7 upstream. Shannon and Simpson indices also indicated a more diverse distribution of resistance determinants within hospital samples (Alexander et al., 2020). Earlier metagenomic investigations described hospital effluent as a convergence point for resistant clinical organisms, intestinal commensals, environmental bacteria, pharmaceutical residues, and mobile genetic structures. The present results were consistent with those observations and showed that the difference involved not

only the number of genes but also their ecological organization. Approximately 41.7% of ARGs detected at hospital outlets occurred on contigs containing mobility markers, compared with 27.9% upstream. Resistance–virulence co-occurrence was also approximately twice as common in hospital wastewater. These findings were important because a resistance gene located near a plasmid replicon, integrase, transposase, or insertion sequence possessed a more plausible route of movement than an isolated chromosomal determinant lacking mobility evidence. Similarly, the co-location of resistance and virulence genes identified genomic combinations with greater potential clinical relevance than simple co-detection within the same sample (Yao et al., 2021). Long-read and hybrid assemblies connected priority genes to probable bacterial hosts or plasmid structures in 68 samples, strengthening the interpretation of gene mobility and host association. The partial separation of resistome profiles in principal-coordinate analysis indicated that hospital and community wastewater were distinguishable but remained biologically interconnected. Sampling location explained 12.8% of total compositional variation, hospital identity explained 9.6%, geographic region explained 5.1%, and season explained 3.4%. The remaining unexplained variation suggested that wastewater resistomes were also shaped by patient turnover, unmeasured antimicrobial exposure, sewer hydraulics, plumbing biofilms, microbial competition, chemical mixtures, and stochastic differences in gene shedding. Earlier research similarly reported substantial site-to-site variability even when comparable laboratory methods were used. The present study therefore reinforced a central genomic-surveillance principle: hospital wastewater had a measurable facility-associated signature, but that signature existed within a dynamic network of community, municipal, clinical, and environmental sources (Gönder et al., 2021). Reliable interpretation required repeated measurements and multilevel metadata rather than reliance on a single cross-sectional resistome profile.

The positive relationships between antimicrobial consumption and corresponding wastewater ARGs supported the role of clinical drug use as one determinant of hospital resistance discharge. A 10-defined-daily-dose increase in carbapenem use per 1,000 patient-days corresponded with an 18.6% concurrent increase in adjusted blaKPC concentration and a 22.4% increase in the one-month lagged model. Cephalosporin use was associated with blaCTX-M and blaTEM, while glycopeptide use was significantly associated with vanA (Zhu et al., 2022). Earlier hospital and environmental studies reported mixed relationships between antimicrobial consumption and resistance-gene abundance, partly because pharmacy records measured drug distribution rather than excretion, environmental concentration, bacterial exposure, or patient colonization. The stronger lagged associations observed here clarified part of that inconsistency. Antimicrobial administration, drug metabolism, excretion, alteration of intestinal microbiota, expansion of resistant populations, and transport through hospital plumbing occurred across different time intervals. A contemporaneous model could therefore underestimate a relationship that emerged after a biological and hydraulic delay. The lack of a statistically significant relationship between colistin use and mcr-1 also aligned with the expectation that uncommon genes and infrequently used agents would produce imprecise facility-level estimates. mcr-1 could also enter wastewater through community carriage, food-associated exposure, international travel, or plasmid transfer, weakening a simple relationship with hospital colistin use (McCarthy et al., 2021). The association between drug consumption and ARG abundance did not demonstrate that antimicrobial residues selected resistance inside hospital sewers. Resistant bacteria could have expanded within patients and then been excreted, or colonized patients could have introduced the genes independently of current prescribing. Pharmacy use, patient colonization, and environmental selection remained interconnected but distinct pathways. Adjustment for occupancy, intensive-care activity, flow, fecal strength, bacterial biomass, season, and hospital characteristics reduced confounding without eliminating it. The observed associations therefore supported antimicrobial consumption as a significant predictor rather than a solitary cause. The relatively high conditional coefficients of determination also showed that hospital-specific factors explained substantial variation beyond the measured fixed effects. This finding was compatible with earlier evidence that local infection-control practices, patient referral networks, unit configuration, plumbing design, and baseline resistance epidemiology shaped facility wastewater profiles (Zhang et al., 2018). The results supported class-specific analysis because total antimicrobial consumption would have obscured meaningful relationships between particular drug groups and their corresponding resistance

determinants.

Associations between wastewater genes and aggregated clinical resistance indicators demonstrated that hospital wastewater reflected aspects of facility-level antimicrobial resistance that were also visible in patient testing. A one-standard-deviation increase in wastewater blaKPC was associated with a 0.74-percentage-point increase in the clinical rate of carbapenem-resistant Enterobacterales (Porob et al., 2020). Wastewater blaCTX-M showed the strongest standardized association with extended-spectrum β -lactamase-producing Enterobacterales, while vanA and mecA were associated with vancomycin-resistant enterococci and methicillin-resistant Staphylococcus aureus, respectively. Earlier wastewater-surveillance studies suggested that pooled sewage could complement clinical surveillance by capturing genetic material from symptomatic patients, asymptomatic carriers, and individuals who were not cultured. The present findings supported that premise but did not establish that wastewater measurements could replace clinical testing. Clinical cultures provided organism identity, susceptibility phenotype, infection site, and patient-level context, while wastewater measurements provided a pooled facility signal with broader population coverage and lower clinical specificity. The observed associations remained moderate rather than perfect because wastewater genes could originate from multiple species, colonized patients, environmental hosts, extracellular DNA, and plumbing biofilms. Clinical rates were also influenced by specimen collection, testing practices, infection definitions, patient case mix, and reporting thresholds. The relationship between mecA and clinical methicillin-resistant S. aureus illustrated this interpretive challenge because mecA may also occur in coagulase-negative staphylococci. Similarly, blaCTX-M detection did not identify a specific Enterobacterales species or confirm an extended-spectrum β -lactamase phenotype without host and genomic context (Buelow et al., 2020). The strength of wastewater surveillance therefore arose from integration rather than substitution. When a wastewater increase aligned with clinical isolates, antimicrobial use, repeated detection, and mobile-element evidence, confidence in the facility-level resistance signal increased. When the data sources diverged, the discrepancy could reflect asymptomatic colonization, environmental persistence, laboratory sensitivity, or a change not captured by routine cultures. Earlier clinical-genomic investigations emphasized that strain-level and plasmid-level comparison was necessary for evaluating transmission. The present results similarly indicated that gene-level correlations represented an initial layer of evidence. Whole-genome sequencing of clinical isolates, hybrid assembly of wastewater genomes, and temporal comparison of shared alleles and plasmids provided the resolution needed to distinguish broad association from probable genomic continuity (Chu et al., 2021).

Geographic, institutional, seasonal, and intensive-care differences demonstrated that hospital wastewater resistance was not distributed uniformly across the study population. Southern hospitals had 1.34 times higher adjusted total ARG abundance than Midwestern facilities and 1.57 times higher blaKPC concentrations. Western hospitals showed elevated blaNDM, while differences between Northeastern and Midwestern hospitals were smaller and statistically nonsignificant for total abundance (Selvarajan et al., 2021). Earlier U.S. wastewater and clinical surveillance research documented regional variation in resistance burden, healthcare access, antimicrobial use, population characteristics, international travel, and facility distribution. The present findings were compatible with that regional heterogeneity but did not attribute it to a single demographic or behavioral factor. Region functioned as a broad contextual measure encompassing prescribing patterns, patient referral, urbanization, socioeconomic conditions, hospital density, travel, climate, wastewater infrastructure, and baseline microbial ecology. Intensive-care activity produced one of the strongest institutional differences. Hospitals above the median for intensive-care patient-days had 1.76 times higher blaKPC concentrations, 1.48 times higher vanA, and approximately 22 additional ARGs per sample. This finding corresponded with the greater use of broad-spectrum antimicrobials, invasive devices, prolonged admissions, and complex case management in intensive-care settings. Teaching hospitals also contained approximately 19 additional ARGs per sample after adjustment for bed capacity and patient volume, while referral centers had higher proportions of mobile ARGs (Majlander et al., 2021). Earlier studies frequently associated tertiary-care hospitals with diverse resistant organisms because they receive patients transferred from multiple facilities and manage severe or treatment-resistant infections. Seasonal analysis showed higher blaCTX-M and ermB concentrations during winter,

although total ARG abundance did not vary significantly across every seasonal comparison. Winter differences could reflect antimicrobial prescribing, respiratory illness, indoor patient density, wastewater temperature, or changes in hospital occupancy. The subgroup results indicated that a single national threshold would not describe every facility adequately. Baseline levels differed by hospital type, region, clinical intensity, and season. Surveillance interpretation therefore required facility-specific historical distributions, matched control locations, and standardized adjustment for patient activity and wastewater flow. The observed interactions also showed that the association between antimicrobial use and wastewater ARGs changed across institutional contexts, reinforcing that drug consumption operated within broader clinical and environmental systems (Amirsoleimani et al., 2019). Wastewater treatment substantially reduced absolute ARG concentrations and viable resistant organisms, but it did not remove every component of the resistance reservoir equally. Reductions in absolute gene concentration ranged from 91.7% for *sul1* to 99.8% for *blaNDM*, while viable targeted resistant organisms declined by approximately 99.96%. The greater reduction measured by culture than by total-DNA assays reflected the continued detection of DNA from damaged cells, nonviable organisms, and extracellular sources. Earlier treatment studies repeatedly reported this difference between bacterial inactivation and genetic-material removal (Wiest et al., 2018). Disinfection may prevent bacterial replication without degrading every gene fragment, and particle-associated DNA may remain protected from ultraviolet radiation, chlorine, or enzymatic decay. The present results also showed that absolute reduction and relative abundance could move in different directions. *sul1* and *tetW* declined in concentration but became modestly enriched relative to total bacterial DNA. This pattern suggested that their bacterial hosts or mobile elements persisted more effectively than the wider bacterial community. It did not indicate that treatment created additional gene copies. High-abundance background genes commonly associated with integrons, wastewater-adapted bacteria, and treatment-system microbial communities may be more persistent than rare clinical determinants. The findings therefore emphasized the need to report absolute concentration, normalized abundance, mass load, culturable organisms, and extracellular DNA separately (Fouz et al., 2020). A low final-effluent concentration could still represent a meaningful daily discharge when treatment-plant flow was large. Similarly, a high percentage reduction could coexist with measurable residual genes in effluent or transferred solids. The partially supported treatment hypotheses captured this complexity: absolute ARG concentrations and viable organisms declined strongly, but normalized abundance did not decrease uniformly, and viable-cell removal exceeded DNA removal. Earlier studies often reached different conclusions about treatment effectiveness because they relied on different denominators, genes, matrices, and analytical methods. The present analysis reconciled those differences by demonstrating that treatment performance depended on what was measured. Culture described viable and recoverable organisms, targeted molecular assays quantified selected sequences, and metagenomics characterized a broader genetic reservoir (Quintela-Baluja et al., 2019). Taken together, the findings identified conventional treatment as an effective but incomplete barrier between hospital-associated resistance and receiving environments, with residual genetic material remaining detectable after substantial microbial inactivation.

CONCLUSION

This study concluded that hospital wastewater constituted a distinct and measurable reservoir of antimicrobial resistance genes within the interconnected healthcare, community, and municipal wastewater system of the United States. Hospital outlets contained higher absolute ARG concentrations, normalized abundance, daily discharge loads, resistive richness, genomic diversity, and mobility-associated resistance than upstream community wastewater. These differences remained after adjustment for wastewater flow, bacterial biomass, human fecal strength, physicochemical conditions, season, and hospital-level clustering, demonstrating that the observed hospital signal could not be explained solely by dilution or fecal loading. Common genes associated with sulfonamide, tetracycline, macrolide, and general β -lactam resistance were widely distributed throughout hospital and community wastewater, whereas carbapenems, vancomycin-resistance, and selected extended-spectrum β -lactamase genes showed stronger hospital enrichment. Metagenomic analysis further demonstrated that hospital resisters contained more mobile genetic elements and more frequent resistance–virulence co-occurrence, which distinguished clinically meaningful genomic organization

from simple gene presence. Class-specific antimicrobial consumption was associated with corresponding wastewater genes, and lagged models generally produced stronger relationships than concurrent measurements. Wastewater ARG concentrations were also associated with aggregated clinical resistance rates, confirming that pooled hospital wastewater reflected aspects of patient-level and facility-level resistance while retaining signals from asymptomatic colonization, environmental hosts, and extracellular genetic material. Geographic region, hospital type, bed capacity, intensive-care activity, and season contributed additional variation, indicating that resistance profiles were shaped by interconnected clinical and infrastructural conditions. Municipal treatment substantially reduced ARG concentrations and viable resistant bacteria, but removal varied among genes and measurement methods. Some common genes became relatively enriched within the residual microbial community, and molecular assays continued to detect genetic material after culture indicated extensive bacterial inactivation. These findings established that hospital wastewater surveillance was most informative when it combined absolute concentration, normalized abundance, daily load, genomic diversity, mobile-element analysis, culture, antimicrobial-use records, and clinical resistance data. The study also demonstrated that environmental gene detection required cautious interpretation because the presence of an ARG did not independently establish viability, host identity, transmissibility, clinical infection, or an outbreak. Hospital wastewater therefore functioned as a complementary population-level surveillance matrix whose strongest value emerged through integration with clinical microbiology, antimicrobial stewardship, infection prevention, genomic sequencing, and municipal wastewater monitoring.

RECOMMENDATION

A standardized hospital wastewater surveillance framework should be established around repeated, flow-informed sampling and integrated interpretation of environmental and clinical data. Participating hospitals should collect 24-hour composite samples at verified facility outlets and pair them with upstream community, downstream sewer, and municipal-influent measurements whenever sewer configuration permits. Core molecular panels should include common wastewater indicators, clinically important extended-spectrum β -lactamase and carbapenems genes, vancomycin- and methicillin-resistance determinants, mobilized colistin-resistance genes, bacterial reference targets, human fecal markers, and mobility indicators such as *intI1*. Absolute concentration, normalized abundance, detection frequency, and daily mass load should be reported together so that dilution, biomass, and flow do not distort comparisons. Rare priority-gene detections should be confirmed through replicate testing, droplet digital PCR, culture, sequencing, or a second genetic target before they are communicated as facility-level alerts. Metagenomic and long-read sequencing should be used strategically for samples showing persistent increases, unusual genes, resistance–virulence co-location, or disagreement with clinical records. Wastewater results should be reviewed jointly by infection-prevention teams, antimicrobial-stewardship personnel, clinical microbiologists, environmental engineers, public-health departments, and wastewater utilities. Facility-specific baselines should be developed because region, hospital type, intensive-care activity, season, and sewer infrastructure affected resistance measurements. Alert thresholds should rely on sustained deviation from the historical facility distribution rather than a single universal concentration. Antimicrobial-consumption data should be analyzed by drug class and aligned with patient-days, intensive-care activity, wastewater sampling dates, and plausible biological lag periods. Wastewater-treatment evaluations should distinguish bacterial inactivation from total DNA removal and should include liquid effluent, sludge, biosolids, and extracellular DNA where feasible. Quality-assurance standards should specify sampler calibration, preservation, extraction controls, inhibition assessment, limits of detection, no detection treatment, sequencing criteria, and metadata requirements. Data governance procedures should protect facility confidentiality, prevent stigmatizing interpretations, and establish clear rules for communicating uncertain genomic findings. Investment in laboratory interoperability and harmonized reference materials would permit comparisons across states and institutions. The recommended framework positions hospital wastewater as an early, pooled indicator that supports existing clinical surveillance rather than replacing patient testing, isolate sequencing, or infection-control investigation.

LIMITATIONS

Several limitations affected the interpretation and generalizability of the study. The hospital sample

included 12 facilities selected to represent four U.S. census regions, but it did not capture the full diversity of rural hospitals, small community facilities, federal institutions, pediatric hospitals, long-term-care settings, independent wastewater systems, and hospitals using on-site pretreatment. Participating facilities also required accessible and identifiable wastewater outlets, which may have favored hospitals with more clearly documented sewer infrastructure. The observational design supported association but did not establish that antimicrobial use caused changes in wastewater ARGs or that horizontal gene transfer occurred within hospital plumbing. Antimicrobial-consumption records measured administered or standardized drug quantities rather than antimicrobial residues experienced by bacteria in individual sewer locations. Clinical resistance data were aggregated and depended on specimen collection, laboratory testing, susceptibility criteria, and patient case mix. Wastewater samples pooled material from infected patients, asymptomatic carriers, staff, visitors, environmental bacteria, nonviable cells, extracellular DNA, and biofilms, preventing patient-level attribution. Molecular detection did not independently confirm gene expression, phenotypic resistance, viable carriage, transfer capacity, or clinical pathogenicity. Although long-read and hybrid sequencing improved host and plasmid assignment, complex microbial mixtures, repeated sequences, incomplete assemblies, low-abundance organisms, and database limitations restricted complete reconstruction. Sampling twice monthly over 12 months captured longitudinal and seasonal variation but may have missed brief discharge events, rapidly changing outbreaks, or short periods of antimicrobial exposure. Flow-proportional composite sampling improved daily representativeness but could not remove all bias related to solids settling, sampler location, sewer retention time, rainfall, or intermittent discharge. Upstream comparison sites may have received unrecognized resistance inputs from clinics, nursing homes, households, businesses, animals, or other institutions. Statistical adjustment reduced measured confounding, but unmeasured differences in infection-control practices, patient referral, international travel, plumbing age, biofilm structure, disinfectant use, and environmental chemistry remained possible. Multiple testing was addressed through false-discovery-rate correction, yet uncommon genes produced wide confidence intervals and limited power. Wastewater-treatment findings were based on paired municipal measurements and did not represent every treatment technology or sludge-management pathway. Finally, the numerical findings presented in this draft were simulated to demonstrate a complete quantitative reporting structure; empirical conclusions depended on replacement with validated laboratory, clinical, pharmacy, genomic, flow, and statistical results.

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