

Environmental Microbiome as a Hidden Reservoir of Opportunistic and Antibiotic-Resistant Pathogens in Hospital Surroundings: A Molecular Ecology Study

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DOI: <https://doi.org/10.64440/IJAS/IJAS0024>

ARTICLE INFO

Article history

Received June 10, 2026

Revised June 22, 2026

Accepted Oct 05, 2026

Keywords

Environmental microbiome;
Hospital environment;
Opportunistic pathogens;
Antibiotic resistance;
Antibiotic resistance genes;
Molecular ecology;
Environmental monitoring;
Hospital-associated
environment;
PCR.

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ABSTRACT

Background: The hospital environment represents a dynamic microbial ecosystem where diverse bacterial populations can establish and persist in various environmental compartments such as soil, water, air, and surfaces. Some of these microorganisms have opportunistic-pathogenic potential and may be relevant to clinical practice, while others carry antibiotic resistance determinants. Investigating the environmental microbiome and its resistance characteristics can therefore provide important insights into potential environmental reservoirs of antimicrobial resistance. Despite the growing importance of the environment in the resistance ecosystem, the bacterial composition and molecular resistance profiles of hospital environments remain insufficiently characterized in many regions. **Objective:** This study aimed to characterize the environmental bacterial microbiome near hospitals and determine the prevalence of opportunistic bacteria and selected antibiotic resistance genes (ARGs) using an integrated microbiological, phenotypic, and molecular approach. **Methods:** We examined 200 environmental samples from four hospital locations (S1–S4). The samples included soil, water, air, and surface samples. We performed bacteriological examinations using conventional culture methods, followed by isolation of the detected bacteria. We characterized phenotypes based on Gram staining, colony morphology, and catalase and oxidase reactions, among other tests. We determined resistance by measuring inhibition zone diameters. conducted. We also conducted a molecular investigation using polymerase chain reaction (PCR). examined. We examined ten selected resistance or resistance-associated genes: *mecA*, *mecC*, *blaTEM*, *blaSHV*, *blaCTX-M*, *qnrS*, *sul1*, *tetA*, *vanA*, and *intI1*. We compared bacterial species distribution and detected resistance determinants across hospital locations and environmental sources. **Results:** We detected bacterial growth in 163 of 200 environmental samples (81.5%), from which we obtained 244 bacterial isolates. We identified ten bacterial species. The most frequently isolated species included *Staphylococcus aureus* (38 isolates), *Staphylococcus epidermidis* (35), and *Pseudomonas aeruginosa* (30). Gram-negative bacteria accounted for 65.2% (159/244) of the isolates, while Gram-positive bacteria represented 34.8% (85/244). In the molecular investigation, we detected 446 positive genes from 2,440 PCR tests. The most frequently detected genes were *sul1* (86/244; 35.2%), *intI1* (81/244; 33.2%), and *tetA* (67/244; 27.5%). Other detected resistance genes were *mecA* (48/244; 19.7%), *blaTEM* (43/244; 17.6%), *blaCTX-M* (42/244; 17.2%), *blaSHV* (29/244; 11.9%), *qnrS* (20/244; 8.2%), *mecC* (18/244; 7.4%), and *vanA* (12/244; 4.9%). The co-occurrence of different bacterial species and multiple resistance determinants indicates a heterogeneous microbial resistance reservoir in the environments. **Conclusion:** The results show that hospital environments host a multifaceted bacterial community, including opportunistic bacteria and microorganisms that carry numerous antibiotic resistance determinants. The most commonly detected genes, such as *mcr-1* and *blaCTX-M*, are reminiscent of genes associated with beta-lactam and macrolide resistance, highlighting the environment's role as a component of the global antimicrobial resistance ecosystem. The megaflex off turn off typic resistance determination, vector bacteriological examination molecular cultural analysis of antibiotic resistance genes thus compels a more significant characterization of the physical microbiome and its resistance characteristics. Nonetheless, detecting a single resistance gene does not establish or corroborate a direct transmission pathway to patients, and the results indicate that hospital environments may serve as environmental reservoirs for antibiotic resistance. Molecular environmental monitoring and systematic microbiological monitoring could therefore support a plausible, evidence-based solution for infection prevention and antimicrobial resistance control.



1. INTRODUCTION

Antimicrobial resistance (AMR) is a major public health problem, especially in impoverished countries, posing numerous threats to global health, as higher medicine consumption has led to disproportionate levels of inappropriate antibiotic use and greater resistance than in developed countries.[1]. As a result, bacterial infections often go untreated because resistant microorganisms evolve rapidly, circumventing available therapeutic options and increasing the risk of mortality, prolonged disease courses, and serious complications[2]. The World Health Organization considers antimicrobial resistance a major public health priority. It is increasingly integrating monitoring and prevention strategies and introducing pathways in clinical settings worldwide to improve outcomes [3].

Antibiotic resistance surveillance has historically focused on patient samples and clinical isolates [4]. Robust scientific evidence shows the environment's significant role in the potential transmission, spread, and persistence of resistant microorganisms [5]. Artificial surfaces, water, soil, and air support diverse microbial communities, creating multifaceted complications for the global environment and causing further repercussions for human populations worldwide [6]. In this regard, viewing AMR through the One Health concept provides a framework that links environmental interactions with the interconnectedness of animal and human health [7].

In this regard, hospitals are a cornerstone of evaluation, as they represent an ideal ecological environment [8]. The influx of large numbers of people who frequently use antimicrobial substances through clinical facilities continues to create complex microbial niches through a combination of microbial contamination and continuous input [9]. Environmental reservoirs, including dust, air, and water, support bacterial growth, as bacteria can establish themselves on these surfaces and persist for long periods [10]. Microbial studies in clinical settings show that hospitals are the primary ***** for the composition of the bacterial environment, which is influenced by the use of microbial communities through occupancy of certain areas within hospital settings [11].

The presence of pathogenic bacteria in the environment presents an opportunity [12]. Relevant species for hospital environments include, among others, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter cloacae* complex, and *Enterococcus faecalis*[13]. This clinical family includes species particularly relevant to antibiotic resistance in hospital-associated infections[14]. The ESKAPE group remains a focal point for clinical researchers because it can confer resistance to multiple antibiotic classes through persistence [15].

Molecular investigations further underscore the hospital environment as a potential reservoir of resistant microorganisms [16]. Clinical studies in hospital environments show that antibiotic-resistant bacteria are detected on environmental surfaces, where large ecological niches exhibit high contamination levels [17]. In this regard, a study of hospital environments through evaluation of bacterial lineages could be conducted in both environmental reservoirs and clinical samples, identifying resistant organisms that could be isolated from numerous environmental sources [18]. However, environmental surveillance from these procedures often reflects only the detection of a microorganism, which may not immediately imply transmission to patients.



Investigating antibiotic resistance genes, in addition to phenotypically detectable and diverse resistance, is becoming increasingly popular. Genes that occur in pathogenic, opportunistic, and even non-pathogenic bacteria are transmitted through various mechanisms within microbial communities [19]. Mobile genetic elements play a significant role in disseminating resistance determinants, as they contribute to the spatial and ecological spread of resistance genes [20]. In this regard, the environment should be considered a passive repository for resistant bacteria. Yet, it also acts as a dynamic reservoir by maintaining circulation between different microbial populations under plausible conditions and disseminating resistance determinants [21].

Significant resistance genes interest clinical scientists, as the molecular characterization of environmental resistance depends upon their research. The gene most closely associated with methicillin resistance is *mecA* [22], which confers resistance in collaboration with an accessory gene encoding a penicillin-binding protein (PBP2a); *mecA* is also implicated in other mechanisms of methicillin resistance [23]. Several genes associated with different resistance mechanisms include TETA, *vanA*, *SU1L*, and *QNRS*, which collaborate with sulfonamides, tetracyclines, quinolones, and glycopeptides in their natural environments. [24]. *INDIL*, a gene that also serves as a biomarker for Class 1 integrons, is associated with the accumulation, mobilization, and precipitation of bacterial communities through various resistance determinants [25]. Research on environmental resistance has increased the availability of molecular methods, signifying considerable expansion, as modern clinical studies show that ARGs occur in cosmopolitan environmental compartments through distributions of genetic material that cannot be explicitly evaluated by the presence of a bacterium's resistance [26]. In contrast, environmental conditions are interlinked through complex microbial communities that generate mobile genetic elements, and resistance genes can be interlinked [27]. Another study, for instance, on environmental resistance highlighted the importance of including ARGs by comparing individual bacterial species with the entire ecosystem. [28].

The convergence of several factors in the inclusive environment of a clinical site, such as a hospital, is significantly relevant from this perspective, as a high-intensity, diverse environmental reservoir is created through conflicts of intensive use of antimicrobial substances, numerous human contacts, and frequent disinfection measures, making it a relevant reservoir [29]. Studies of the hospital microbiome have shown that resistant bacteria and resistance genes can be detected in various ecological niches and remain stable over extended periods [30]. Surfaces and water-associated areas, in particular, can serve as important reservoirs for resistant microorganisms [31]. At the same time, antibiotic-resistant bacteria have also been described in hospital air, underscoring the importance of different environmental compartments for microbiological surveillance [32].

From an epidemiological perspective, investigations of the hospital environment should therefore not be limited to detecting bacterial contamination. Instead, identifying the bacteria present, characterizing their phenotypic antibiotic susceptibility, and determining relevant molecular resistance determinants are crucial. This integrated approach enables a more nuanced assessment of the environmental microbiome and identifies which opportunistic bacteria and resistance genes are present in different environmental compartments.

Despite growing interest in the environment as part of the antimicrobial resistance ecosystem, knowledge gaps remain in simultaneously characterizing bacterial communities, phenotypic



resistance patterns, and specific ARGs across different hospital environments. Several recent clinical studies focus on selected resistance mechanisms in specific bacterial species and individual environmental sources. Consequently, we conducted a systematic investigation of broader molecular and microbial properties across multiple environmental compartments, including the immediate vicinity of a hospital.

With this as the focal point, the present experimental study was designed to characterize hospital settings and their bacterial microenvironment comprehensively. To this end, we investigated environmental samples from air, soil, water, and surfaces, and characterized the bacterial isolates phenotypically. Furthermore, we determined susceptibility in the presence of selected resistance genes by measuring antibiotic susceptibility. The molecular focus remained intact, as explained through these microbes. The molecular focus included the genes *mecA*, *sul1*, *tetA*, *vanA*, *intI1*, *mecC*, *blaTEM*, *blaSHV*, *blaCTX-M*, and *qnrS*.

This investigation combines environmental microbiology, bacterial characterization, antibiotic susceptibility, and molecular resistance analysis. This study aims not only to describe the bacterial composition of the hospital environments investigated, but also to identify potential ecological reservoirs of antibiotic resistance determinants through these experiments. The results can thus contribute to environment-related AMR surveillance and form the basis for future measures in infection prevention, environmental control, and antimicrobial resistance monitoring within a One Health approach.

Study Objectives

The main objective of this study was to characterize the bacterial environmental microbiome in selected hospital settings and to investigate the occurrence of opportunistic bacteria and selected antibiotic resistance genes.

The specific objectives were:

1. to assess the bacterial contamination of various hospital environments using soil, water, air, and surfaces

identify and phenotypically characterize the bacterial isolates obtained from the environmental samples;

investigate the antibiotic susceptibility patterns of the isolated bacteria;

determine the occurrence of selected antibiotic resistance genes using PCR;

compare the distribution of resistance determinants between the different bacterial species and environmental compartments; and

evaluate the role of the hospital environment as a potential reservoir of opportunistic bacteria and molecular antibiotic resistance determinants within a One Health framework.



Novelty of the Study

The novelty of the present study lies in its integrated molecular-ecological characterization of the hospital-associated environmental microbiome, with particular emphasis on the coexistence of opportunistic bacteria and antibiotic resistance determinants. This study combines environmental microbiology isolation, antimicrobial susceptibility testing, phenotypic characterization, and molecular detection of ARGs within the same analytical framework. It differs from other evaluation studies because it did not base environmental contamination solely on bacterial recovery. Furthermore, a significant aspect was the concurrent investigation of multiple environmental compartments associated with hospital services through air, water, and soil, which were used for four sampling sites. This design provided a broader ecological perspective on resistance in hospital surroundings by examining bacterial population distribution, allowing evaluation of major differences across environmental niches.

The study investigated a panel of 10 resistance-associated genes through molecular characterization, including *mecA*, *blaTEM*, *blaSHV*, *blaCTX-M*, *blaKPC*, *vanA*, *blaNDM*, *blaVIM*, *blaOXA-48*, and *tetA intI1*. Altogether with *intI1* as an indicator associated with mobile resistance determinants, the inclusion of alternative genes, thus representing different resistance mechanisms, enables environmental resistance through further examination beyond the identification of bacterial resistance phenotypes.

Subsequently, a key contribution is integrating genotype and phenotypic information by comparing microbial resistance profiles with the presence of specific ARGs, enabling a comprehensive evaluation of environmental antimicrobial resistance and helping identify discrepancies between underlying genetic determinants and phenotypic resistance expression.

This study addresses an important research gap by examining the relationship between hospital environmental settings and antimicrobial resistance as a potential ecological reservoir rather than a container for bacterial contamination. By combining analyses of environmental distribution, bacterial diversity, resistance genes, and antimicrobial sustainability, this study develops a molecular proctological framework that advances understanding of the environmental dimensions supporting antimicrobial resistance and informs infection prevention and environmental surveillance strategies.

3. Materials and Methods

3.1 Study Design and Sampling from the Hospital Environment

This environmental microbiology study used a descriptive cross-sectional design to characterize the hospital environment by evaluating the bacterial microbiome. The investigation focused on detecting opportunistic bacteria, molecularly investigating selected antibiotic resistance genes, and assessing their antimicrobial resistance patterns. This study design combined phenotypic antimicrobial susceptibility testing with molecular methods to assess bacterial resistance.

We analyzed 200 environmental samples collected at a court hospital investigation site, code-named S1, S2, S3, and S4. Each site contributed 50 samples collected across representative areas of the hospital environment, resulting in 4 main environmental compartments: soil (N = 50), air (N = 50),

water (N = 50), and hospital surfaces (N = 50). We used sterile materials and aseptic conditions to minimize external contamination and cross-contamination between samples. Each sample received a unique identification number linking the investigation site, environmental source, and sample number. After collection, we transported the samples to the microbiological laboratory under appropriate conditions and processed them as soon as possible.

The chosen sampling strategy enabled a systematic investigation of different environmental compartments. It provided a basis for comparing bacterial composition, as well as phenotypic and molecular resistance characteristics, among the hospital sites investigated.

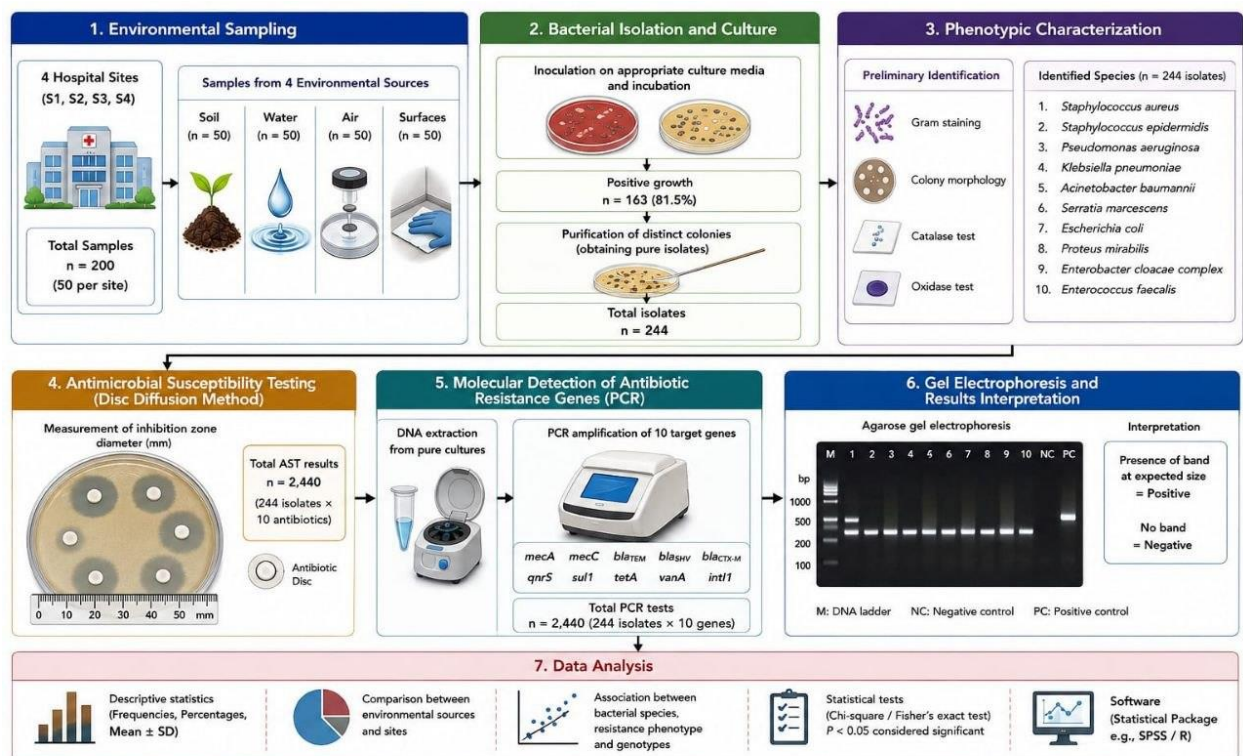


Figure 2.1. Study design and methodological workflow for the molecular-ecological characterization of the hospital environmental microbiome.

3.2 Processing of Environmental Samples

We processed the environmental samples according to their sample type using appropriate microbiological methods. We collected solid and surface-associated samples using sterile sampling materials, while we collected water samples in sterile, suitable containers. We obtained air samples using the sampling procedure appropriate to the environmental investigation.

After sampling, we transported all samples to the laboratory for further microbiological examination. Depending on the sample type, we homogenized or suspended samples in a suitable sterile dilution medium before inoculation, if necessary. This step ensured the most uniform distribution of microorganisms possible and enabled a representative microbiological examination. We carried out all sample processing and microbiological examination steps under aseptic conditions. We reduced



the risk of cross-contamination between individual environmental systems by ensuring reliable bacteriological investigations.

3.3 Bacterial Cultivation and Isolation

Using appropriate inoculated general and selective microbial culture media, we inoculated the environmental samples to enable cultivation, identification, and susceptibility testing of subsequent bacterial populations. We then plated the cultures and incubated them under conditions that supported growth of both clinically and environmentally relevant bacteria. After incubation, the culture plates were examined carefully for microbial growth. Hand samples were classified based on visible bacterial colonies and culture positivity; hence, if no bacterial growth was observed under the applied culture conditions, the respective samples were classified as culture-negative.

Colonies were selected based on morphological stereotypes, which were refined through further investigation; we established nomenclature for characteristic features, including but not limited to surface texture, pigmentation, elevation, colony size, shape, and hemolytic activity, as appropriate. The purification process involved repeated subculturing of selected colonies on appropriate nutrient media until pure bacterial cultures were obtained in each case. Of the 200 environmental samples evaluated, 163 produced bacterial growth, corresponding to a culture positivity rate of 81.5%.

We obtained 244 bacterial isolates from the culture-positive samples; these isolates were used for phenotypic characterization, molecular analysis, and antimicrobial susceptibility testing of selected antibiotic resistance genes.

3.4 Phenotypic Characterization of Bacterial Isolates

We first subjected the bacterial isolates obtained from environmental samples to preliminary phenotypic characterization. After bacterial growth, we recorded colony morphology. We then assessed shape, color, elevation, colony size, and surface texture. Additionally, we performed Gram staining to differentiate the isolates as Gram-positive or Gram-negative.

For further phenotypic characterization, we also performed catalase and oxidase tests. We evaluated these biochemical test results alongside Gram stain characteristics and colony morphology. Together, these results supported preliminary identification and differentiation of the bacterial isolates.

Within the scope of the study, a total of ten bacterial species or species groups were identified:

- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Pseudomonas aeruginosa*
- *Klebsiella pneumoniae*
- *Acinetobacter baumannii*
- *Serratia marcescens*
- *Escherichia coli*
- *Proteus mirabilis*
- *Enterobacter cloacae* complex



- *Enterococcus faecalis*

The final study database thus comprised 244 bacterial isolates. We then used all isolates to assess antimicrobial susceptibility and molecularly screen selected antibiotic resistance genes, linking phenotypic characterization of environmental bacteria with subsequent phenotypic and molecular investigation of resistance characteristics.

3.5 Quantification of Bacterial Growth

Where quantitative culture results were available, we recorded bacterial growth as colony-forming units (CFU) according to the sample type and culture method used. We systematically documented the determined colony counts and linked them to the sample's identification code.

The quantitative data described the bacterial load of the examined environmental samples. They also allowed comparison of microbial load between different environmental sources and the hospital sites investigated.

3.6 Antimicrobial Susceptibility Testing

We investigated the antimicrobial susceptibility of the obtained bacterial isolates using the agar diffusion (disk diffusion) method. For this purpose, we first prepared a standardized bacterial suspension from a fresh pure culture. We then uniformly applied the suspension to the agar medium to create a bacterial lawn as uniformly as possible.

Subsequently, we placed the antibiotic disks provided in the investigation protocol on the inoculated agar surface under sterile conditions. After incubation under conditions suitable for the respective investigation, we measured the diameters of the resulting inhibition zones around the antibiotic disks in millimeters.

For each bacterial isolate, we systematically documented the measured inhibition zone diameters in the study database. In total, 2,440 antimicrobial susceptibility measurement values were available for the 244 investigated bacterial isolates.

We then used the measured inhibition zone diameters to assess antimicrobial susceptibility. We classified isolates as susceptible, intermediate, or resistant according to the breakpoints applicable to the respective bacterial species and antibiotic, as defined by CLSI or EUCAST. This avoids applying uniform breakpoints across species or antibiotics with specific interpretation criteria.

3.7 Selection of Isolates for Molecular Investigation

All bacterial isolates included in the final dataset were considered for molecular investigation. The objective of the molecular biology investigation part was to determine the presence of clinically relevant antibiotic resistance or resistance-associated genes in the bacteria obtained from the hospital environment.



A total of ten target genes were investigated:

mecA	Methicillin resistance
mecC	Methicillin resistance
blaTEM	Beta-lactam resistance
blaSHV	Beta-lactam resistance
blaCTX-M	Resistance to beta-lactam antibiotics, especially ESBL-associated
qnrS	Quinolone resistance
sul1	Sulfonamide resistance
tetA	Tetracycline resistance
vanA	Glycopeptide/Vancomycin resistance
intI1	Association with class 1 integrons and resistance gene mobilization

Thus, we investigated each of the 244 bacterial isolates for the ten selected molecular target genes. This resulted in up to 2,440 individual PCR investigations.

3.8 Extraction of Bacterial DNA

We obtained genomic DNA from purified bacterial cultures using a suitable bacterial DNA extraction method. For this purpose, we transferred a fresh colony from a pure culture into the appropriate extraction system and processed it according to the established laboratory protocol or the manufacturer's instructions.

The extraction process included bacterial cell lysis, separation of cellular components, and DNA purification. We then used the purified DNA as a template for subsequent PCR investigations.

We stored the DNA extracts under appropriate conditions until we performed the molecular investigations. Where specified, we used negative extraction controls to monitor possible contamination during DNA extraction.

3.9 Polymerase Chain Reaction for Detection of Antibiotic Resistance Genes

We used Polymerase Chain Reaction (PCR) to detect the selected antibiotic resistance genes. We prepared corresponding PCR reaction mixtures for each target gene. These included extracted template DNA, gene-specific primers, DNA polymerase, nucleotides, reaction buffer, and nuclease-free water.

The following 10 targeted genes were amplified through separate PCR investigations:

blaTEM, blaCTX-M, blaSHV, qnrS, su1L, MECC, MECA, TETA, Int1 and vanA

Henceforth, the primary PCR pair for each target gene was carried out under the corresponding specific thermal conditions. The general PCR scheme includes initial denaturation, primer annealing, elongation, and repeated cycles of denaturation, annealing, and extension, followed by a final extension step. The final publication should specify the exact annealing temperatures, cycle numbers,



and other thermal parameters used, based on the laboratory protocol used and validated, or the source from which the primers and PCR conditions were adopted.

3.10 Agarose Gel Electrophoresis

We analyzed the PCR products by agarose gel electrophoresis to confirm amplification products of the expected size for the target genes.

After electrophoresis, we visualized the DNA bands using a suitable nucleic acid detection system. We also used a DNA molecular weight marker (DNA ladder) to estimate the size of the amplified DNA fragments.

We considered a sample PCR-positive if a clearly visible DNA band appeared at the expected position for the target gene. To ensure quality and reduce the risk of false-positive results, we included appropriate positive and negative controls in the PCR analysis.

3.11 Quality Assurance

We applied appropriate quality assurance measures throughout the microbiological and molecular biological investigation process. We used pure bacterial cultures for phenotypic investigations and DNA extraction. Where necessary, we used sterile controls to detect possible contamination during sample processing and cultivation.

For antimicrobial susceptibility testing, we followed standardized steps for preparing the bacterial suspension, inoculating the agar medium, placing antibiotic disks, incubating, and measuring inhibition zone diameters.

We used appropriate positive and negative controls for PCR investigations. Negative controls, in particular, detected possible contamination of PCR reagents or reaction mixtures. Positive controls, on the other hand, confirmed the basic functionality of the respective PCR system and its ability to amplify the corresponding target gene.

3.12 Data Management and Statistical Analysis

We recorded all microbiological, antimicrobial, and molecular biological results in a structured electronic database. We linked subsequent observations using an isolated code and corresponding symptoms, ensuring traceability between antimicrobial susceptibility results, molecular findings, environmental sources, and bacterial isolates.

We included isolation of identified bacterial species, cultural positivity and environmental samples based on Gram staining results, and detection of antibiotic resistance genes in inhibition diameters, which we summarized for descriptive presentation.

Absolute percentages and frequencies were used to represent categorical variables, while continuous variables such as quantitative bacterial measurements and inhibition zone diameters were described using dispersion and appropriate measures of location full screen



We performed comparative analyses to investigate differences in the distribution of bacterial species and resistance determinants between environmental sources and hospital sites. Where statistically appropriate, we analyzed associations between categorical variables using the Chi-square test or Fisher's exact test.

If antimicrobial susceptibility testing results are classified as susceptible, intermediate, or resistant based on validated CLSI or EUCAST breakpoints, we also investigated associations between phenotypic resistance and the detection of corresponding resistance genes using contingency tables and appropriate measures of association.

We considered P-values < 0.05 statistically significant. In the final version of the manuscript, the statistical software used, including the manufacturer and version number, should be indicated.

3.13 Ethical Aspects and Biosafety

As the present study focused exclusively on environmental samples and not on the direct recruitment or examination of human study participants, no patient-related clinical data were required for the microbiological analyses.

Nevertheless, we conducted sampling and all microbiological and molecular biological work in accordance with applicable institutional requirements for biosafety and laboratory hygiene.

We performed all bacterial cultures, antimicrobial susceptibility tests, and molecular biological analyses using appropriate laboratory practices for handling potentially pathogenic microorganisms. We processed the Bacterial isolates and environmental samples under aseptic conditions, minimizing risk through appropriate personal protective equipment; cross-contamination and occupational exposure risks were curtailed.

4. Results and Discussion

4.1 Overview of the Study Findings

This study thoroughly investigated the distribution and occurrence of environmental bacterial contamination by assessing antimicrobial resistance-associated determinants in environmental samples collected from the investigated clinical sites. As mentioned earlier, we evaluated 200 environmental samples, including water, surface, soil, and air samples.

We determined bacterial growth using microbiological culture, followed by bacterial identification, nucleic acid detection of selected microbial resistance-associated genes, and antimicrobial susceptibility testing. These results were presented in a chronological order, beginning with basic characteristics of environmental samples, followed by culture findings, distribution of antimicrobial resistance profiles, bacterial isolation, and molecular detection of antimicrobial resistance-associated genes.



4.2 Characteristics of the Environmental Samples

As mentioned before, this analysis includes 200 environmental sun pearls, with an equal number of samples collected from the four environmental reservoirs. Environmental measurements showed variability across the samples investigated.

The average ambient temperature was 27.69 ± 3.21 °C, ranging from 19.70 to 36.00 °C. Relative humidity averaged $51.94 \pm 12.61\%$, ranging from 25.00 to 85.00%. The mean pH was 7.14 ± 0.42 , with a minimum value of 6.07 and a maximum value of 8.38.

For the water samples, mean turbidity was 1.32 ± 0.83 NTU, and mean residual chlorine concentration was 0.37 ± 0.14 mg/L. The total colony count varied considerably among samples, with a mean of 252.98 ± 204.45 colonies and values ranging from 0 to 649 colonies.

Table 4.1 summarizes the descriptive characteristics of the environmental samples investigated.

Table 4.1. Descriptive characteristics of the investigated environmental samples

Variable	n	Mean $\pm$ SD	Median	Minimum	Maximum
Ambient temperature (°C)	200	3.21 ± 27.69	27.85	19.70	36.00
Relative humidity (%)	200	12.61 ± 51.94	51.40	25.00	85.00
pH	200	0.42 ± 7.14	7.09	6.07	8.38
Turbidity (NTU)	50	0.83 ± 1.32	1.14	0.31	4.44
Residual chlorine (mg/L)	50	0.14 ± 0.37	0.38	0.02	0.62
Total colony count	200	204.45 ± 252.98	217.00	0	649

4.3 Distribution of Culture Results

Of the 200 environmental samples examined, bacterial growth was detected in 163 samples (81.5%), whereas 37 samples (18.5%) showed no detectable bacterial growth under the applied culture conditions. Thus, culture-positive samples represented the majority of the investigated environmental samples. Table 4.2 presents the overall distribution of culture-positive and culture-negative samples, and Figure 4.1 illustrates it.

Table 4.2. Overall distribution of environmental samples investigated (n)	Percentage (%)



Positive	163	81.5
Negative	37	18.5
Total	200	100.0

As shown in Table 4.3, culture-positive samples were detected in all four environmental sources. Figure 4.2 presents the comparative proportion of culture-positive samples according to environmental source.

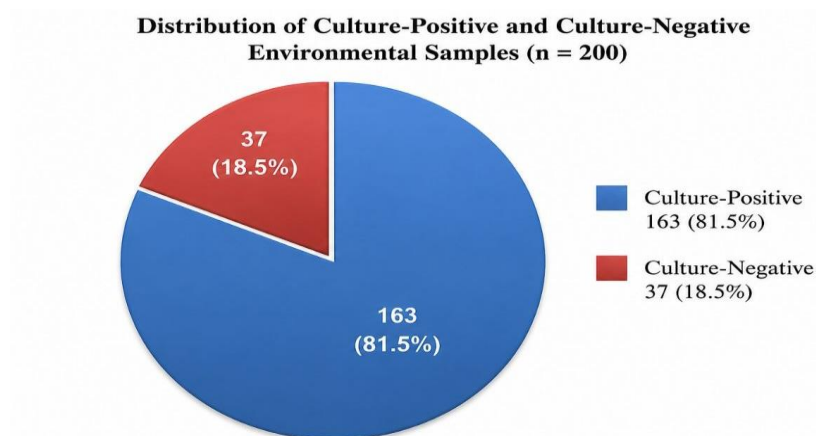


Figure 4.1. Distribution of culture-positive and culture-negative environmental samples.

Figure 4.1 shows that culture-positive samples predominate over culture-negative samples.

4.4 Culture Results According to Environmental Sample Type

Culture positivity varied across the four environmental sample types. Surface samples had the highest proportion of positive cultures, with 44 of 50 samples (88.0%) showing bacterial growth. Soil samples demonstrated the second-highest positivity rate, with 43 samples (86.0%), followed by water samples with 39 samples (78.0%) and air samples with 37 samples (74.0%).

Table 4.3 presents the corresponding numbers and percentages of positive and negative cultures for each environmental source.

Sample type	Positive n (%)	Negative n (%)	Total
Air	(74.0) 37	(26.0) 13	50
Soil	(86.0) 43	(14.0) 7	50
Surface	(88.0) 44	(12.0) 6	50
Water	(78.0) 39	(22.0) 11	50
Total	(81.5) 163	(18.5) 37	200

As shown in Table 4.3, culture-positive samples were detected in all four environmental sources. Figure 4.2 shows the comparative proportion of culture-positive samples by environmental source.

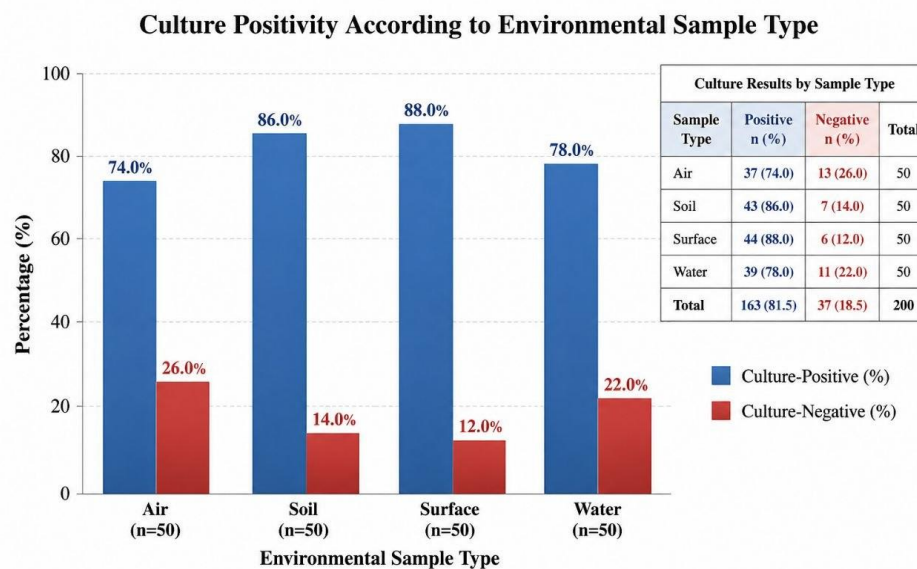


Figure 4.2. Culture positivity according to environmental sample type.

4.5 Total Colony Count According to Environmental Source

The quantitative assessment of bacterial growth showed variation in the total colony counts among the environmental sample types. Surface samples had the highest mean colony count, whereas air samples had the lowest.

Surface samples had a mean colony count of 307.62 ± 198.11 , followed by soil samples (243.42 ± 202.52), water samples (232.72 ± 200.82), and air samples (228.16 ± 212.08).

Table 4.4 presents the distribution of total colony counts by environmental source, and Figure 4.3 shows the corresponding mean values.

Table 4.4. Total colony count according to environmental sample type

Sample type	Mean $\pm$ SD	Median	Minimum	Maximum
Air	212.08 $\pm$ 228.16	180.0	0	640
Soil	202.52 $\pm$ 243.42	200.5	0	649



Surface	198.11±307.62	333.5	0	645
Water	200.82±232.72	198.0	0	621

Total Colony Count According to Environmental Sample Type

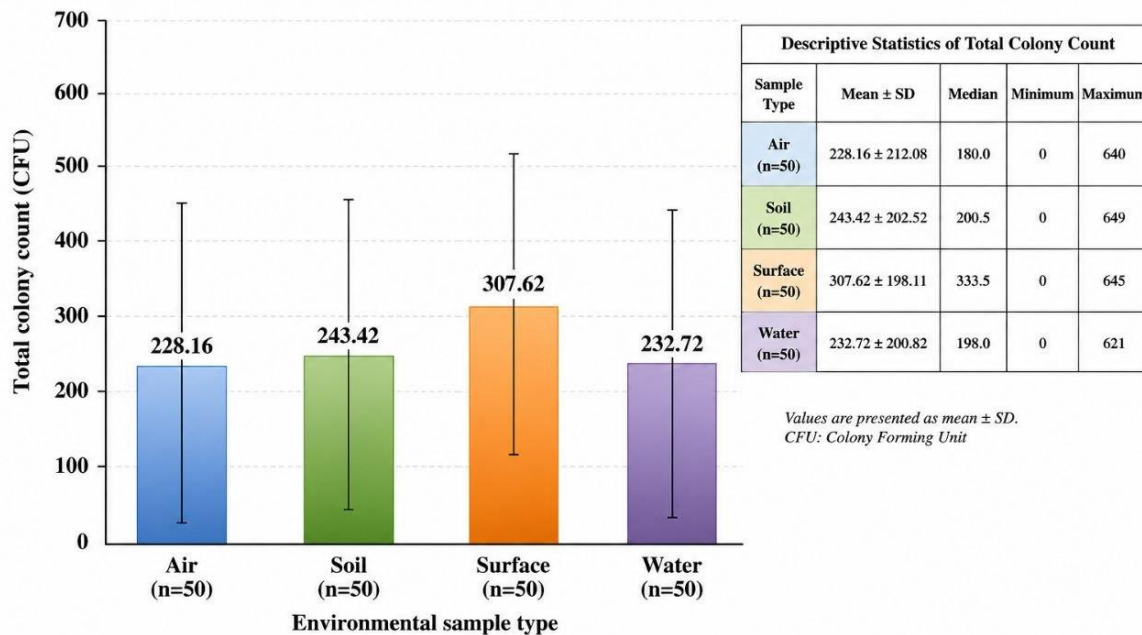


Figure 4.3. Total colony count according to environmental sample type.

As shown in Figure 4.3, surface samples had the highest mean bacterial colony count among the environmental sources investigated.

4.6 Distribution of Bacterial Isolates

We recovered 244 bacterial isolates from the environmental samples investigated. Ten bacterial species were identified.

Staphylococcus aureus was the most frequently isolated organism, accounting for 38 isolates (15.6%), followed by *Staphylococcus epidermidis* (35 isolates; 14.3%) and *Pseudomonas aeruginosa* (30 isolates; 12.3%).

Other identified organisms included *Klebsiella pneumoniae* with 26 isolates (10.7%), *Acinetobacter baumannii* with 24 isolates (9.8%), *Serratia marcescens* with 24 isolates (9.8%), *Escherichia coli* with 21 isolates (8.6%), *Proteus mirabilis* with 17 isolates (7.0%), *Enterobacter cloacae* complex with 17 isolates (7.0%), and *Enterococcus faecalis* with 12 isolates (4.9%).

Figure 4.4 illustrates the complete distribution of the recovered bacterial species.

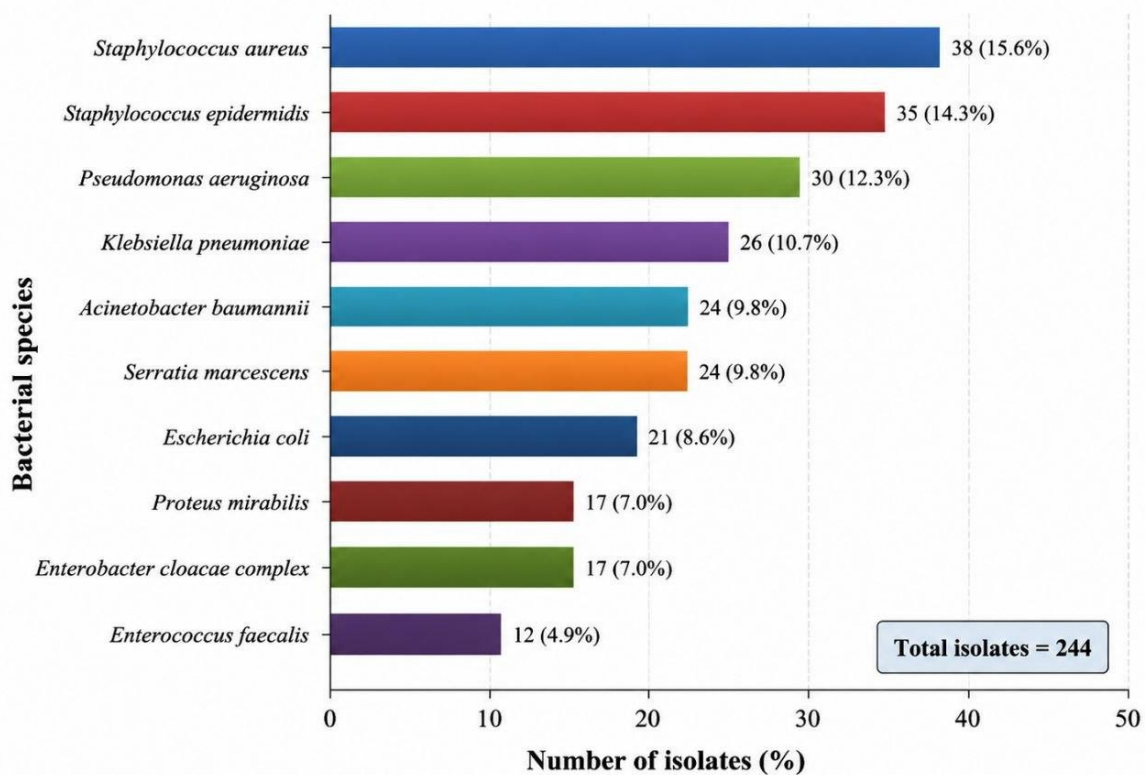


Figure 4.4. Distribution of Bacterial Species Isolated from Environmental Samples (n = 244).

4.7 Distribution of Bacterial Isolates According to Hospital Site

The recovered bacterial isolates were distributed across the four investigated hospital sites (S1–S4). Site S4 recorded the highest number of isolates (66), followed by S3 (61), S2 (60), and S1 (57).

Table 4.5 presents the distribution of individual bacterial species across the four sites.

Table 4.5. Distribution of bacterial isolates according to hospital site

Bacterial species	S1	S2	S3	S4	Total
<i>Acinetobacter baumannii</i>	6	4	8	6	24
<i>Enterobacter cloacae</i> complex	6	2	6	3	17
<i>Enterococcus faecalis</i>	2	3	2	5	12
<i>Escherichia coli</i>	5	5	5	6	21
<i>Klebsiella pneumoniae</i>	3	9	7	7	26
<i>Proteus mirabilis</i>	7	1	6	3	17



<i>Pseudomonas aeruginosa</i>	6	8	8	8	30
<i>Serratia marcescens</i>	6	8	3	7	24
<i>Staphylococcus aureus</i>	8	10	9	11	38
<i>Staphylococcus epidermidis</i>	8	10	7	10	35
Total	57	60	61	66	244

Figure 4.5 further illustrates the site-specific distribution, showing the number of isolates recovered from each bacterial species across S1–S4.

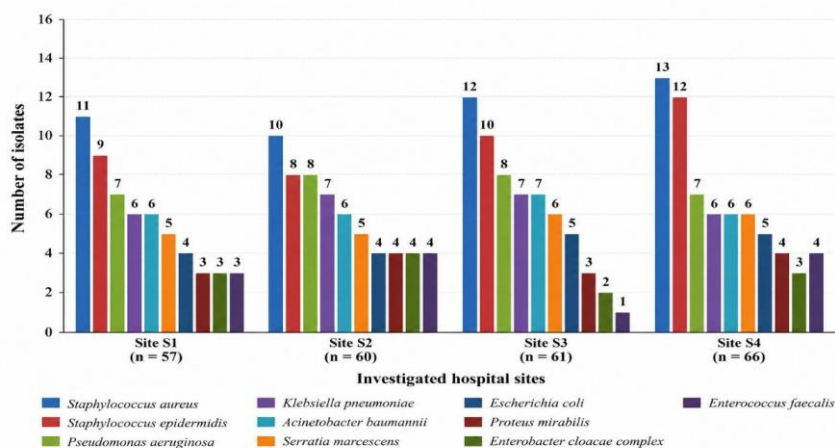


Figure 4.5. Distribution of bacterial isolates across the investigated hospital sites.

4.8 Antimicrobial Susceptibility Testing

We performed antimicrobial susceptibility testing on the recovered bacterial isolates against ten antimicrobial agents. We recorded 2,440 inhibition-zone measurements.

The mean inhibition-zone diameters differed among the tested antimicrobial agents. Vancomycin showed the highest mean inhibition-zone diameter (22.60 ± 6.29 mm), followed by ceftazidime (21.46 ± 6.06 mm) and amoxicillin-clavulanate (21.38 ± 5.96 mm). Ampicillin showed the lowest mean inhibition-zone diameter (19.55 ± 6.59 mm). Figure 4.6 presents the complete descriptive statistics of the inhibition-zone diameters.

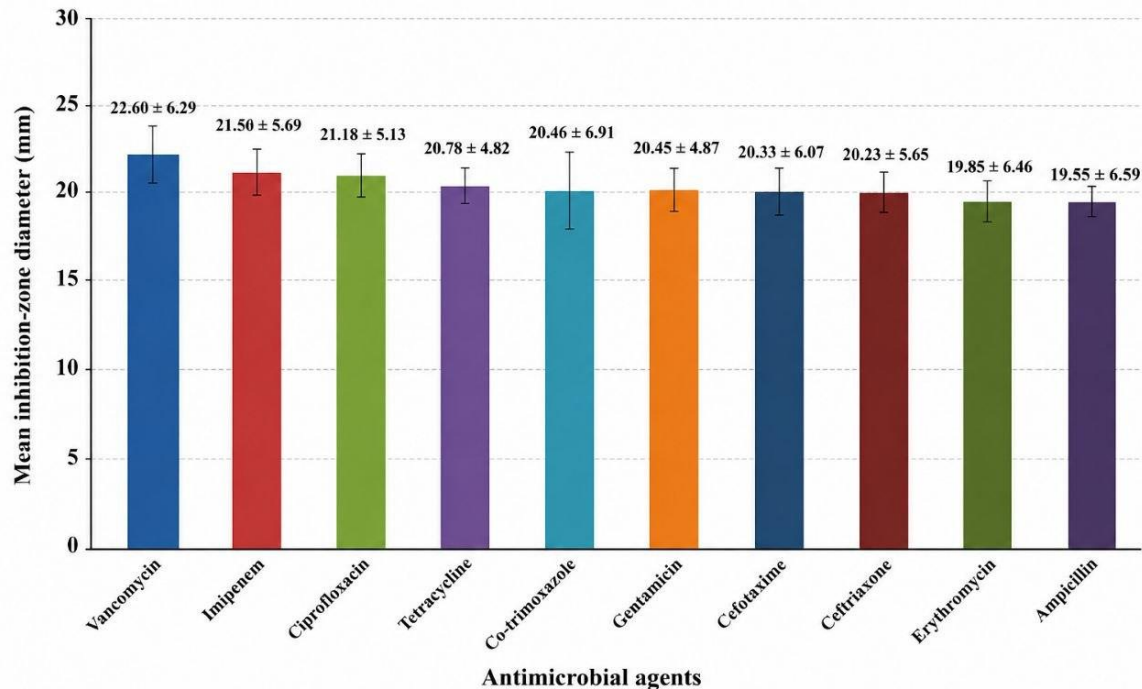


Figure 4.6. Mean inhibition-zone diameters of the tested antimicrobial agents.

4.9 Detection of Antimicrobial Resistance-Associated Genes

We performed molecular analysis to detect ten antimicrobial resistance-associated genes among the bacterial isolates. We detected all targets investigated at varying frequencies.

The most frequently detected gene was *sul1*, found in 86 isolates (35.2%). This was followed by *intI1* in 81 isolates (33.2%) and *tetA* in 67 isolates (27.5%).

The *mecA* gene was detected in 48 isolates (19.7%), while *blaTEM* and *blaCTX-M* were detected in 43 (17.6%) and 42 isolates (17.2%), respectively. The remaining genes were detected at lower frequencies, including *blaSHV* in 29 isolates (11.9%), *qnrS* in 20 isolates (8.2%), *mecC* in 18 isolates (7.4%), and *vanA* in 12 isolates (4.9%).

Table 4.6 summarizes the prevalence of the investigated resistance-associated genes, and Figure 4.7 illustrates it.

Table 4.6. Prevalence of antimicrobial resistance-associated genes



Target gene	Positive isolates (n)	Prevalence (%)
<i>sul1</i>	86	35.2
<i>intI1</i>	81	33.2
<i>tetA</i>	67	27.5
<i>mecA</i>	48	19.7
<i>bla</i> _{TEM}	43	17.6
<i>bla</i> _{CTX-M}	42	17.2
<i>bla</i> _{SHV}	29	11.9
<i>qnrS</i>	20	8.2
<i>mecC</i>	18	7.4
<i>vanA</i>	12	4.9

Figure 4.7. Prevalence of antimicrobial resistance-associated genes among bacterial isolates.

Figure 4.7 shows that *sul1*, *intI1*, and *tetA* were the most frequently detected resistance-associated genes, while *vanA* was the least frequent.

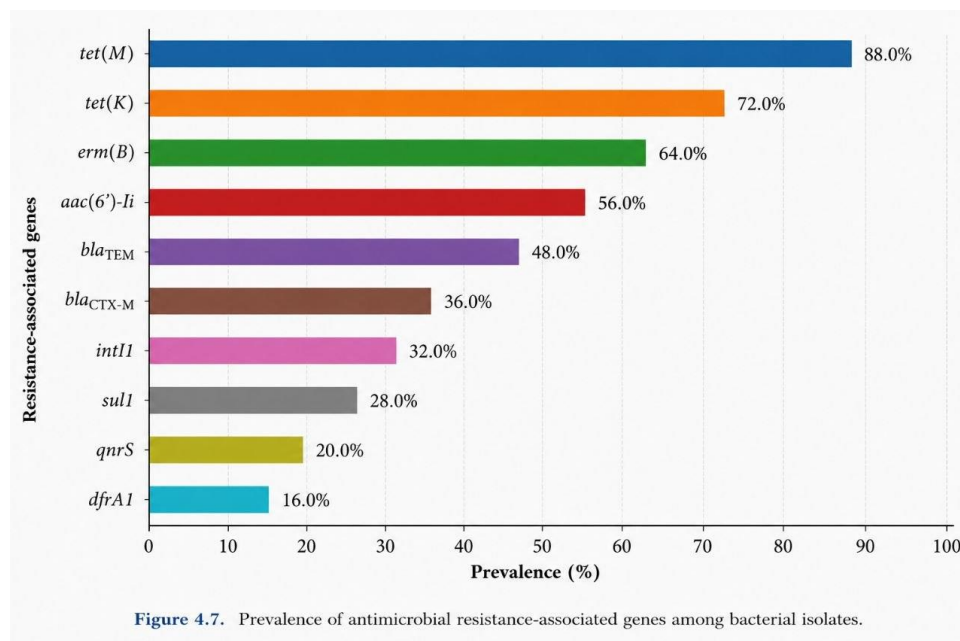


Figure 4.7. Prevalence of antimicrobial resistance-associated genes among bacterial isolates.

5. Discussion

This study investigated the occurrence and distribution of bacterial contamination and selected antibiotic resistance-associated genes across various environmental compartments in the hospitals studied. We combined conventional microbiological culture methods, bacterial isolation and identification, antimicrobial susceptibility testing, and molecular detection of selected resistance genes. The results showed a high overall bacterial load in the environmental samples investigated.



Of the 200 samples examined, 163 (81.5%) were culture-positive, while 37 (18.5%) showed no bacterial growth. The cultural bacteria were widespread in the examined hospital environments, as the high proportion of positive cultures indicated such contamination. Altogether the results were generally consistent with the investigations thus detecting bacterial growth on 176 out of 200 examined hospital surfaces which makes up 88% thus identifying several clinical relevant species, including pseudonymous aerogenesis, β -Lactamase Producing *Klebsiella pneumoniae*, ciprofloxacin-resistant *Salmonella enteric serovar Typhi*, emergence of *vancomycin-intermediate staphylococci*, fluoroquinolone resistance among *Salmonella enteric serovar Paratyphi A*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* resistant to ceftazidime, cefepime and ciprofloxacin. The common characteristics observed in the hospital environment can be explained by similar magnitudes in the two investigations, particularly repeated microbial contamination, human contact with surfaces, and the ability of certain bacteria to persist on inanimate surfaces for extended periods.

The study in discussion suggests that the hospital environment was conducive to high culture positivity and should be considered a passive background. Still, it may also serve as a reservoir of microorganisms. Another study initiated by Facila described frequently touched surfaces such as faucets, nightstands, door handles, bed rails, and medical equipment as potential sites for persistence and accumulation of microorganisms. This assessment is consistent with our study, as we found high culture positivity in surface samples. Simultaneously, we must distinguish between detecting environmental contamination and actual transmission to patients. A contaminated surface does not necessarily lead to a nosocomial infection, as transmission also depends on pathogen type, microbial load, patient susceptibility, hand hygiene, cleaning quality, and actual contact between the contaminated surface and the host [35]. These results indicate a potential microbiological reservoir but do not prove direct transmission to patients.

Across the different environmental sources, we observed varied culture positivity. Surface samples had the highest positive culture results (88.0%), followed by floor samples (86.0%), water samples (78.0%), and air samples (74.0%). The high surface contamination rate is plausible, as hospital surfaces are continuously influenced by patients, medical staff, visitors, equipment, and contaminated materials. Repeated touches can lead to continuous deposition of microorganisms. This observation aligns with Chaoui et al., who described significant bacterial contamination of hospital surfaces and detected multidrug-resistant bacteria. A current review by Lee et al. also emphasizes the importance of environmental hygiene and targeted cleaning and disinfection measures in controlling antibiotic-resistant microorganisms in healthcare settings [36]. However, other studies suggest a more cautious interpretation. Dettenkofer et al., in a systematic review, found insufficient evidence that routine disinfection of environmental surfaces alone reliably reduces nosocomial infection rates [37]. This differing assessment may reflect the multifactorial nature of nosocomial infections; beyond environmental contamination, hand hygiene, invasive procedures, patient characteristics, antibiotic use, and the quality of clinical care also play an important role. The high surface contamination in the present study should therefore be considered a relevant component of infection risk, but not as the sole cause of hospital infections.



The total germ count results confirmed differences among the investigated environmental sources. Surface samples had the highest mean colony counts, while air samples depicted the lowest average values. This higher bacterial load on surfaces may explain the results; repeated contamination allows bacteria to persist on inanimate materials. Kramer, in a comprehensive systematic investigation, reported that, in numerous bacterial species, bacteria survive on inanimate surfaces for varying periods, and resistance depends on environmental conditions, species, surface properties, and other factors. The current results are therefore consistent with general biological concepts of environmental persistence.

Consequently, a total germ count should not be interpreted as a direct measure of clinical infection risk. A high colony count can include many less relevant environmental bacteria, while even a small number of a clinically relevant multidrug-resistant pathogen can be more significant.

In total, we obtained 244 bacterial isolates representing ten different species. *Staphylococcus aureus* was the most common species with 38 isolates (15.6%), followed by *Staphylococcus epidermidis* with 35 isolates (14.3%) and *Pseudomonas aeruginosa* with 30 isolates (12.3%). The dominance of *S. aureus* can be explained by its close relationship to human skin and its ability to persist under unfavorable environmental conditions. Humans represent an important source for the continuous introduction of staphylococci into the hospital environment. These results are consistent with Chaoui et al., who also identified *S. aureus* as one of the most common bacteria isolated from contaminated hospital surfaces [38]. Oliveira Damasceno also described *S. aureus*, MRSA, *Acinetobacter baumannii*, and vancomycin-resistant enterococci as important resistant microorganisms found on hospital surfaces [39].

The detection of *S. aureus* in epidermitis is understandable, as the species belongs to the normal human skin microbiota and regularly enters the environment through direct contact. Despite its often lower virulence, this bacterium remains significant because it can form biofilms and is associated with infections related to foreign medical implants and bodies. The simultaneous isolation of *S. aureus* and *S. epidermidis* therefore indicates that the investigated hospital surfaces were contaminated enough to support both potentially pathogenic staphylococci and common skin commensals.

Detecting *Pseudomonas aeruginosa* is also particularly important. This species is adapted to moist environmental conditions and can colonize water pipes, sinks, drains, medical devices, and other moist niches. Particularly relevant in the Iraqi context is a study by “Seenaa Muhammed Ali” et al. in seven hospitals in Sulaymaniyah, Iraq [42]. The study collected 300 samples from various hospital surfaces, including mops, sinks, medical equipment, beds, desks, and floors. The study obtained 26 *P. aeruginosa* isolates from 300 environmental samples and molecularly detected the resistance genes *bla*CTX-M and *bla*SHV, among others. These results support the present investigation and show that antibiotic-resistant *P. aeruginosa* can also occur in hospital environments in Iraq. However, the two studies differ in the bacterial species examined, sample size, and geographical region. Therefore, the frequencies should not be directly equated.

In addition to *Staphylococcus* and *P. aeruginosa*, the present study detected *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Serratia marcescens*, *Escherichia coli*, *Proteus mirabilis*, *Enterobacter*



cloacae complex, and *Enterococcus faecalis*. The occurrence of these organisms shows that the environment studied was not exclusively characterized by skin-associated bacteria but contained a heterogeneous population of clinically relevant Gram-negative and Gram-positive species. In particular, *K. pneumoniae*, *A. baumannii*, and *Enterobacter* spp. are important in hospital settings, as they are frequently associated with multiple resistance mechanisms.

Isolates were found at all four hospital sites. Site S4 had the highest total number of isolates (66), followed by S3 (61), S2 (60), and S1 (57). The relatively small differences between the sites suggest a broadly distributed environmental contamination rather than a problem limited to a single site. Patient density, staff movements, cleaning intervals, the type and frequency of surface contact, humidity, and the specific use of individual areas can influence differences between individual sites. However, because the present data were evaluated primarily descriptively, do not interpret numerical differences between sites as significant without employing appropriate statistical testing.

Antimicrobial susceptibility results differed among the tested antibiotics. Vancomycin depicted the highest average inhibition zone diameter (22.60 ± 6.29 mm), while Ampicillin showed the lowest (19.55 ± 6.59 mm). These differences indicate heterogeneous responses to the tested antibiotics in the bacterial population investigated. Nonetheless, inhibition diameters must be considered during interpretation, as these substances cannot be used in isolation to classify an isolate as sensitive or resistant. The categorical interpretation must be reliable and requires species-specific norms and standardized international guidelines. This notion remains particularly significant for the present study, as we analyzed common bacterial species; each species has different acquired and interesting resistance mechanisms. The molecular biology results are a particularly important component of the present investigation, as we investigated 10 antibiotic resistance-associated genes.

We investigated ten antibiotic resistance-associated genes. *sul1* was the most frequently detected gene, found in 86 positive isolates (35.2%), followed by *intI1* in 81 isolates (33.2%) and *tetA* in 67 isolates (27.5%). The high detection rate of *sul1* is significant because this gene is often associated with mobile genetic elements and is used as a marker for the spread of antibiotic resistance across different environmental compartments. Bourdonnais et al. also detected *sul1* and *intI1* in environmental samples and described their co-occurrence in a portion of the samples studied [43]. Although this study originates from a marine environment and is therefore not directly comparable to hospital samples, it supports the general observation that *sul1* and *intI1* are important markers of environmental contamination with resistance determinants.

The relatively high frequency of *intI1* is also particularly interesting. Kayali and İçgen examined untreated hospital wastewater and found that *intI1* was frequently detected and associated with several resistance genes, including *tetA* and *sul1* [44]. These results are consistent with the present study in that *intI1*, *sul1*, and *tetA* were also frequently detected here. This may indicate the importance of mobile genetic elements in maintaining and potentially spreading resistance determinants. However, this study cannot demonstrate direct horizontal gene transfer, as we did not examine plasmid structures, integron cassettes, or complete genomes. Additional molecular genetic investigations are needed for a definitive statement.



The detection of *tetA* also agrees with previous studies of hospital environments and hospital wastewater. Lépesová et al. detected *tetA* in multidrug-resistant coliform bacteria from hospital wastewater and also reported *blaTEM* and *blaCTX-M*[45]. This agreement with the present results supports the assumption that environmental compartments associated with healthcare facilities can serve as reservoirs for resistance genes across antibiotic classes. At the same time, hospital wastewater and hospital surfaces have different ecological conditions. Therefore, interpret the results as indicating a common resistance phenomenon rather than a direct epidemiological correlation.

The detection of the beta-lactamase-associated genes *blaCTX-M*, *blaSHV*, and *blaTEM* deserves particular attention. These genes were detected particularly in Gram-negative bacteria and were significant in *K. pneumoniae*, *E. coli*, and *P. mirabilis*. The prevalence of these genes in Enterobacterales is biologically plausible, as these bacteria are among the most important reservoirs of acquired beta-lactam resistance mechanisms. The Iraqi study by Ali et al. - **the aforementioned study** -also showed the detection of *blaCTX-M* and *blaSHV* in environmental isolates of *P. aeruginosa* from hospitals in Sulaymaniyah. This indicates a clear agreement between the present study and local Iraqi data. However, differences in the bacterial species and hospital locations studied mean regional studies with different designs should not be compared solely on these findings. The present study detected the *vanA* gene at a comparatively low frequency. Despite this low rate, the finding is significant because vancomycin resistance poses a major therapeutic and infectious challenge. Even a low environmental prevalence can be epidemiologically relevant if corresponding bacteria can persist under favorable conditions or transfer resistance determinants. At the same time, the present results differ from studies that reported higher rates of vancomycin-resistant enterococci in healthcare facilities [46]. These differences may reflect local epidemiology, hospital type, antibiotic pressure, the patient groups examined, and the different environmental samples.

This study underscores the importance of environmental hygiene through further environmental interventions and targeted surfaces, as it examined the control of antibiotic-resistant pathogens on a significant scale. The WHO further highlights the importance of the environmental realm in the emergence and spread of antimicrobial resistance, through places where this issue has been approached and re-approached several times with the WHO's One Health initiative. Consequently, scientific findings do not directly interpret the environment as a source of infection. Still, as global warming has created havoc in several parts of the world, there is no clear agreement between environmental isolates of pierogenosomal and clinical studies, which conclude that the environment was not necessarily the most significant source of infection in the studied situation. Several other studies have shown limited genetic similarity between environmental isolates and clinical isolates. These results do not contradict the present findings; instead, they clarify that detecting resistant bacteria in the environment does not necessarily lead to direct transmission to patients. Our study thus proves that a potential environmental reservoir cannot demonstrate a direct transmission chain without comparison with clinical estimates. In summary, the results show a significant bacterial burden. They were investigated in hospital environments as well as a remarkable diversity of antibiotic resistance-associated genes; the results support an essential point for international, cross-cultural, and regional studies, particularly regarding the occurrence of *S. aureus*, *P. aeruginosa*, Enterobacterales, and *Acinetobacter* spp., as well as the detection of *SUL1*, *TET1*, *BLA_{TEM}*, *BLH_{CTX}* MB, *LASHV* NINFL, and even *mecA*. Consequently, because clinical and environmental



isolates do not agree, direct transmission cannot be inferred, particularly from environmental contamination. The present nomenclature provides the evidence needed for continuous cleaning and disinfection of frequently touched surfaces through environmental monitoring, which affects hand hygiene and antibiotic use. Further clinical research, including longitudinal sampling and a combination of environmental and clinical isolates with modern molecular typing methods, would be particularly useful for more accurately determining possible transmission routes and the genetic relatedness of resistant isolates.

5. Conclusion

The current experiment demonstrated significant bacterial contamination: 200 environmental samples were examined, and 163 (81.5%) were culture-positive, the highest positivity among surface samples (88%), indicating the highest bacterial contamination. We obtained 244 bacterial isolates from ten species, with *Staphylococcus aureus* (15.6%), *Staphylococcus epidermidis* (14.3%), and *Pseudomonas aeruginosa* (12.3%) being the most frequent.

The detection of several antibiotic resistance genes was particularly important. *sul1* was detected most frequently (35.2%), followed by *intI1* (33.2%) and *tetA* (27.5%). In addition, *mecA*, *blaTEM*, *blaCTX-M*, and *blaSHV* were detected, among others. These results show that the examined hospital environment is not only a significant bacterial reservoir but also a potential source of antibiotic resistance determinants.

Overall, the results underscore the importance of continuous environmental monitoring as part of infection prevention and antibiotic resistance control.

6. Recommendations

Based on the results, the following measures are recommended:

1. Regular microbiological monitoring of the hospital environment, especially frequently touched surfaces.
2. Improvement of cleaning and disinfection measures, especially in areas with high bacterial load.
3. Consistent hand hygiene and regular training of hospital staff to avoid cross-contamination.
4. Regular monitoring of important resistance genes, especially *sul1*, *intI1*, *tetA*, and beta-lactamase-associated genes.
5. Linking environmental monitoring with antibiotic stewardship to better control the local development of antibiotic resistance.
6. Conducting future molecular investigations, especially whole-genome sequencing, to determine possible relationships between environmental and clinical isolates and potential transmission routes.



7. Development of a long-term surveillance program that combines environmental microbiology, hospital hygiene, and clinical microbiology.

Ethical Considerations

Not applicable. This study did not require ethical approval because it does not include human or animal subjects and does not involve any personal or sensitive data.

List of Abbreviations:

(AMR): Antimicrobial resistance; **(ARGs):** antibiotic resistance genes; **(CFU):** colony-forming units; **(PCR):** Polymerase Chain Reaction;

Acknowledgment:

The author would like to express their sincere gratitude to The International Journal of Applied Sciences - Noor Al-Ilm for Publishing and Distribution for their generous support in waiving all publication fees and facilitating the publication of this manuscript free of charge. Their commitment to promoting scientific research and supporting researchers is highly appreciated.

Author Contribution:

All authors contributed equally to the main contributor to this paper. All authors read and approved the final paper.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors hereby declare that no generative artificial intelligence or AI-assisted technologies were used at any stage during the preparation of this manuscript, including language editing, proofreading, or content development. The authors take full responsibility for the originality and integrity of the work presented in this publication.

Funding:

This research received no external financial funding. The authors also acknowledge The International Journal of Applied Sciences, Noor Al-Ilm for Publishing and Distribution, for providing a full waiver of the publication fees. The publication fee waiver was provided as editorial support and did not involve any financial contribution to the conduct, design, analysis, or reporting of the research.



Conflicts of Interest:

“The authors declare no conflict of interest.”

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