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

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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:** Hospital environments support a multifaceted bacterial community that includes microorganisms and opportunistic bacteria carrying numerous antibiotic resistance determinants. The most common resistance genes include blaCTX-M and mcr-1, which are often linked to other genes and contribute to multidrug resistance, highlighting the role of the environment in shaping resistance and making it a global microbial resistance ecosystem. The influx of typical resistance determination, molecular cultures, and vector or legal examination and bodies and antibiotic resistance genes compels a much more significant characterization of the physical bomb and its resistance. Nonetheless, detecting a single resistance gene does not establish or confirm a direct transmission pathway to patients, and the results suggest that hospital environments may serve as 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.

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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]. This includes the significant roles of soil, water, artificial surfaces, and air pollution, which diversify microbial communities and create numerous complications in the global environment, with further implications for the global population [6]. Subsequently, the analysis of the One Health concept, with a particular focus on AMR, elucidates a framework linking animal and human health with environmental integration [7].

Clinical facilities like hospitals serve as remarkable field sites because they provide ideal ecological environments for AMR studies. This is because a large and diverse range of people in settings that use antimicrobial substances, facilitated by microbial niches, creates a complex mix of ongoing medical and environmental contamination.[8]. Environmental reservoirs, including soil, water, and air, support bacterial growth; as such, surfaces remain contaminated for longer periods [9]. 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 [10]. Microbial studies in clinical settings show that hospitals are primary sources of the bacterial environment, which is shaped by microbial communities that colonize specific 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].

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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.

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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, we designed the present experimental study 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.

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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 developed a protocol-logical perspective by examining bacterial distribution, revealing resistance features in the hospital environment, and enabling a thorough examination of discrepancies across Environmental niches.

Furthermore, the study investigated a panel of 10 resistance-associated genes using molecular characterization, including blaTEM, blaSHV, mecA, blaKPC, blaNDM, blaVIM, blaOXA-48, and tetA. The last of these niches, inti1, is an indicator associated with rapid resistance determinants, which includes alternative genes representing subsequent resistance mechanisms, enabling the identification of bacterial resistance phenotypes through the functioning of Environmental resistance for stock; hence, a significant contribution to the generic integration of phenotypic and genotypic Resources comes into the picture, as thorough comparison with the presence of specific genes and microbial resistance profiles enables an indelible examination of environmental antimicrobial resistance, thus identifying loopholes between phenotypic resistance expression and underlying genetic determinants.

Furthermore, this study addresses an important research gap by elucidating the relationship between environmental settings, antimicrobial resistance, and the hospital and environment as a whole, which potentially determines an ecological Reservoir rather than a container for bacterial contamination. This comprehensive analysis of bacterial diversity, antimicrobial sustainability, resistance genes, and environmental distribution of prototypes provides a practical molecular protocol framework for advancing a niche and significant understanding of mechanisms that support antimicrobial resistance through the environment and undermine infection prevention through surveillance strategies.

3. Materials and Methods

3.1 Study Design and Sampling from the Hospital Environment

We used a descriptive cross-sectional design and conducted a comprehensive examination of Environmental microbiology to characterize the hospital's clinical environment by evaluating the bacterial microbiome. This research focused on molecularly investigating selected antibiotic resistance genes by detecting opportunistic bacteria and assessing resistance patterns to antimicrobial agents. Consequently, the study framework combined susceptibility testing with molecular

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phenotyping methods that assess bacterial resistance. Overall, we collected environmental samples

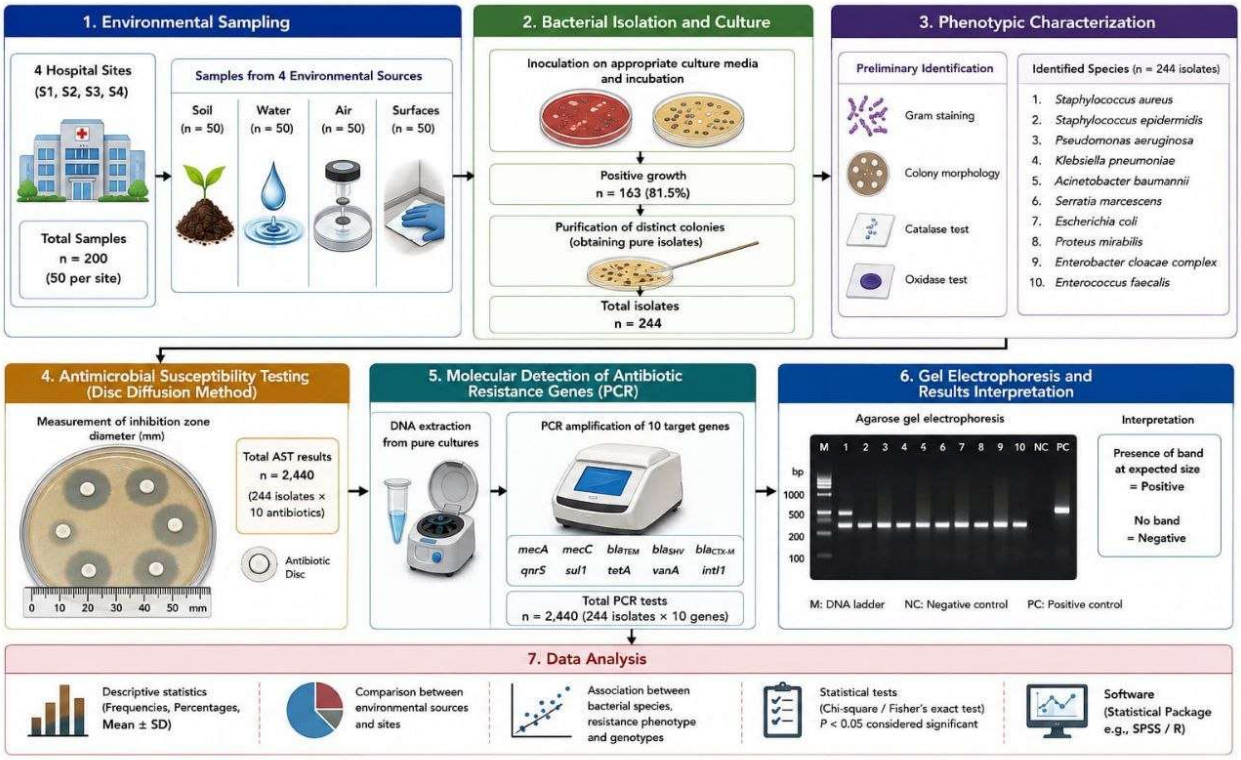

Figure 2.1. Study design and methodological workflow for the molecular-ecological characterization of the hospital environmental microbiome.

at a Hospital investigation site and analyzed them by coordinating the four sites as S1, S2, S3, and S4. Each clinical site contributed 50 samples, collected by representatives across four main environmental compartments: water (50), hospital services (50), and soil (50). We used sterile materials to minimize external contamination and cross-contamination during collection of chord samples from four different facilities. By assigning a unique identification number to each received sample, we linked the investigation side sample number to the environmental source, which depended on the climatic conditions of that particular Hospital. Post-collection, all samples were transported to the microbiological laboratory with due diligence and appropriate care, where they were processed within the stipulated time. Such sampling strategy enabled a comprehensive evaluation of subsequent and environmental compartments related to

3.2 Processing 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

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microorganisms possible and enabled a representative microbiological examination. We carried out all sample processing and microbiological examination steps under aseptic conditions.

3.3 Bacterial Cultivation and Isolation

We reduced cross-contamination risks through reliable bacteriological investigations and individual environmental systems. We determined isolation and bacterial cultivation by inoculating selective and general microbial culture media. The inoculated environmental samples enabled successful stability testing, cultivation, and identification of various bacterial populations; the researchers then plated and incubated them under conditions that supported environmental and clinical growth of relevant bacteria. Post incubation, the examination of microbial growth took place through the culture plates which handed samples were classified based on culture positivity and visible bacterial colonies nonetheless if there was no growth of bacteria observe under the blood cultural conditions the classification of such samples was declared culture negative first vectorial colonies were thus selected depending on their morphological stereotypes which helped refine the investigation as the establishment of characteristic features of nomenclature which were subjected to hemolytic activity, elevation, shape, colony size, pigmentation and the most importantly surface. The purification process involved repeated subculturing of selected colonies on appropriate nutrient media until we obtained pure bacterial cultures 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 and used them 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

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- 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 applied the suspension uniformly to the agar medium to create a bacterial lawn as evenly 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.

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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, qurS, su1L, MECC, MECA, TETA, Int1 and vanA

Henceforth, we used the primary PCR pair for each target gene 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,

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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.

To clearly visualize a DNA band, we used a PCR-positive sample at the expected position of the targeted Gene. This process is quick, ensures quality, and reduces the risk of false-positive results; therefore, we processed a negative control and appropriate positive controls in the PCR analysis.

3.11 Quality Assurance

In such an experiment, the researchers applied quality assurance measures appropriate to molecular biological and microbiological investigation procedures. Using bacterial cultures for DNA extraction and phenotypic investigations, along with sterile controls, detected bacterial contamination during cultivation and sample processing. The researchers used standardized steps for antimicrobial susceptibility testing, including preparing the bacterial suspension, placing antibiotic disks on the agar medium, incubating, and measuring inhibition zone diameters. This approach used appropriate negative and positive controls for PCR investigations, particularly to detect possible contamination of reaction mixtures or PCR reagents; positive controls confirmed the basic functionality of the subsequent PCR system, enabling amplification of the target gene.

3.12 Data Management and Statistical Analysis

All microbiological, molecular biology, and antimicrobial results were recorded in a structured digital database linking isolate codes with subsequent symptom observations, enabling traceability among molecular findings, antimicrobial susceptibility results, bacterial isolates, and the environmental sources of all four facilities (S1, S2, S3, and S4).

We then included bacterial species from isolated cultural positives in environmental samples based on Gram staining results, and we detected antibiotic resistance genes using inhibition diameters, which we presented descriptively.

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.

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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 assessed bacterial growth by microbiological culture, followed by bacterial identification, nucleic acid detection of selected resistance-associated genes, and antimicrobial susceptibility testing. We presented the results chronologically, beginning with basic environmental sample characteristics, followed by culture findings, 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 ± 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.

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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 ± 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 culture-positive and culture-negative 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.

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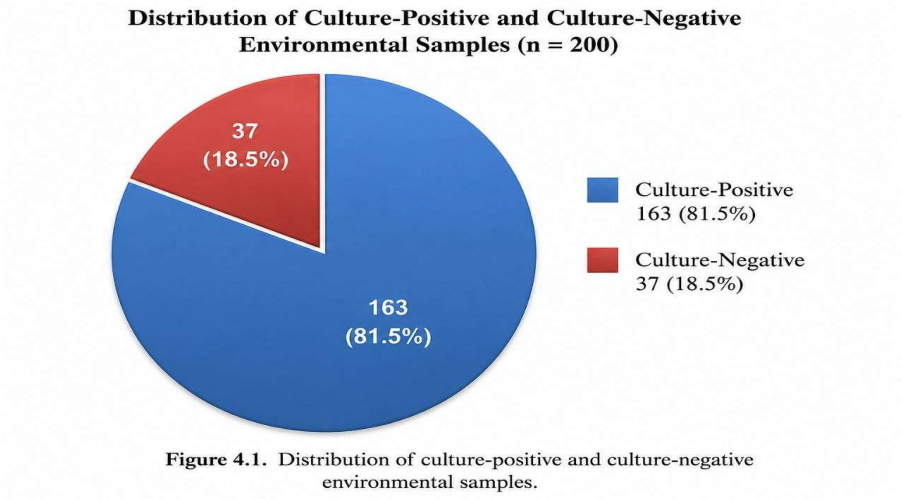

Figure 4.1. Distribution of culture-positive and culture-negative environmental samples.

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.

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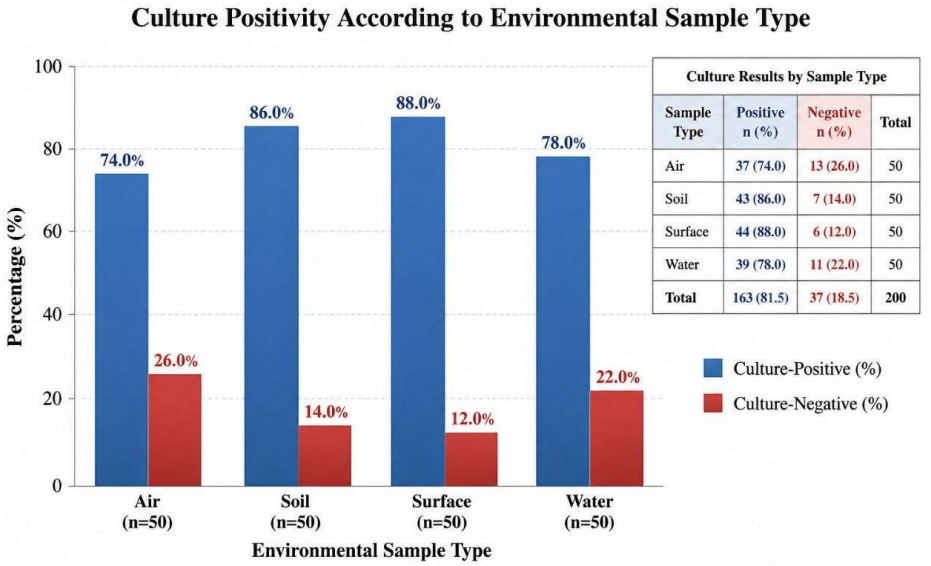

Figure 4.2. Culture positivity according to environmental sample type.

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 ± SD**	**Median**	**Minimum**	**Maximum**
Air	212.08±228.16	180.0	0	640
Soil	202.52±243. 42	200.5	0	649
Surface	198.11±307.62	333.5	0	645
Water	200.82±232.72	198.0	0	621

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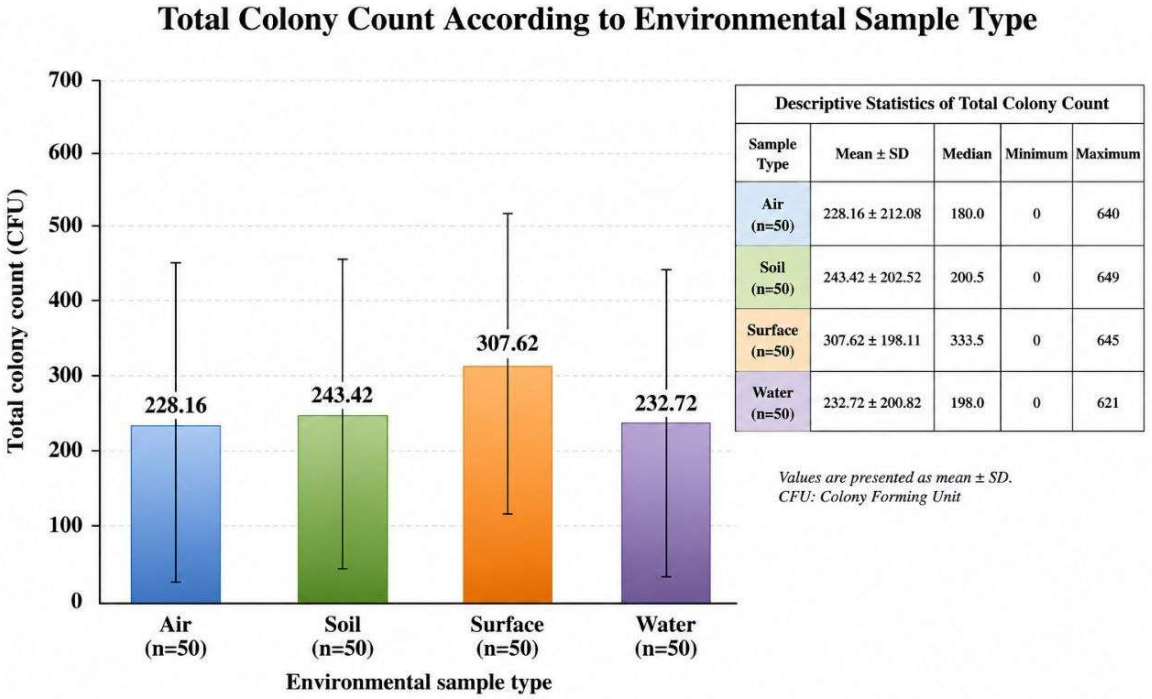

Descriptive Statistics of Total Colony Count

Sample Type	Mean ± SD	Median	Minimum	Maximum
Air (n=50)	228.16 ± 212.08	180.0	0	640
Soil (n=50)	243.42 ± 202.52	200.5	0	649
Surface (n=50)	307.62 ± 198.11	333.5	0	645
Water (n=50)	232.72 ± 200.82	198.0	0	621

Values are presented as mean ± SD.
CFU: Colony Forming Unit

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.

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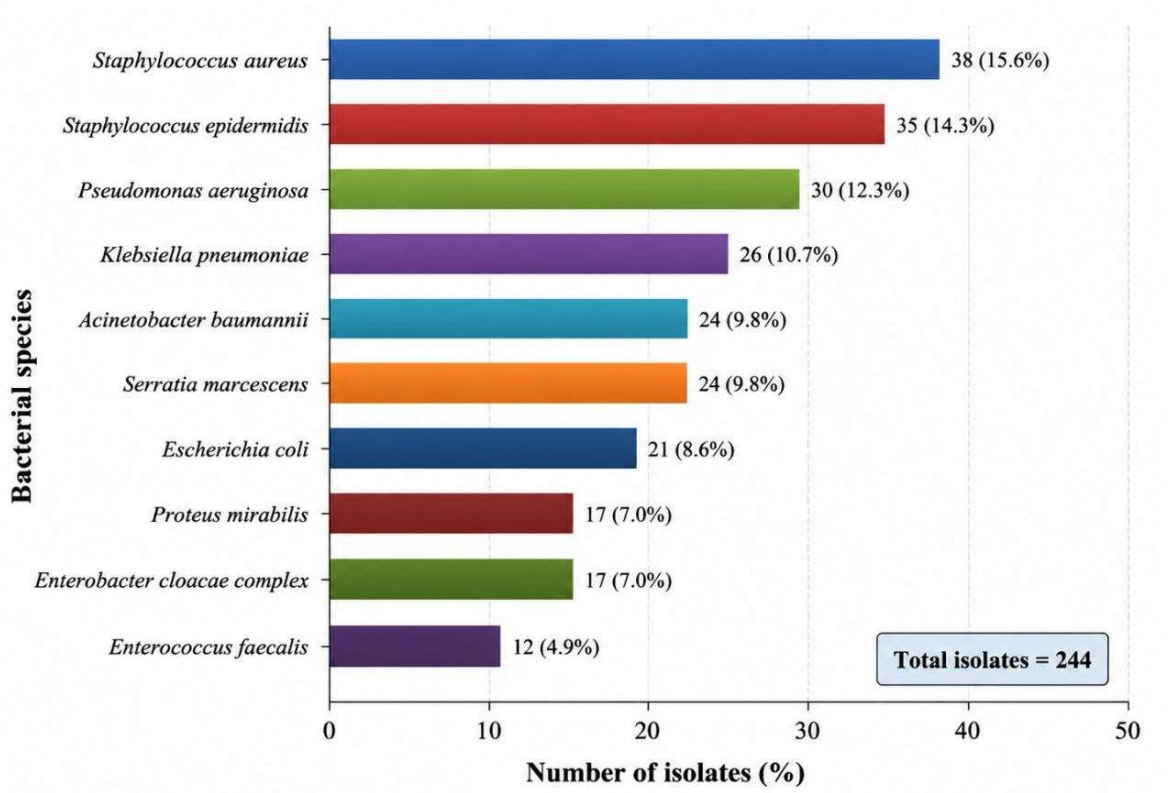

Figure 4.4. Distribution of Bacterial Species Isolated from Environmental Samples (n = 244).

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
Acinetobacter baumannii	6	4	8	6	24
Enterobacter cloacae complex	6	2	6	3	17
Enterococcus faecalis	2	3	2	5	12
Escherichia coli	5	5	5	6	21
Klebsiella pneumoniae	3	9	7	7	26
Proteus mirabilis	7	1	6	3	17

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Pseudomonas aeruginosa	6	8	8	8	30
Serratia marcescens	6	8	3	7	24
Staphylococcus aureus	8	10	9	11	38
Staphylococcus epidermidis	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.

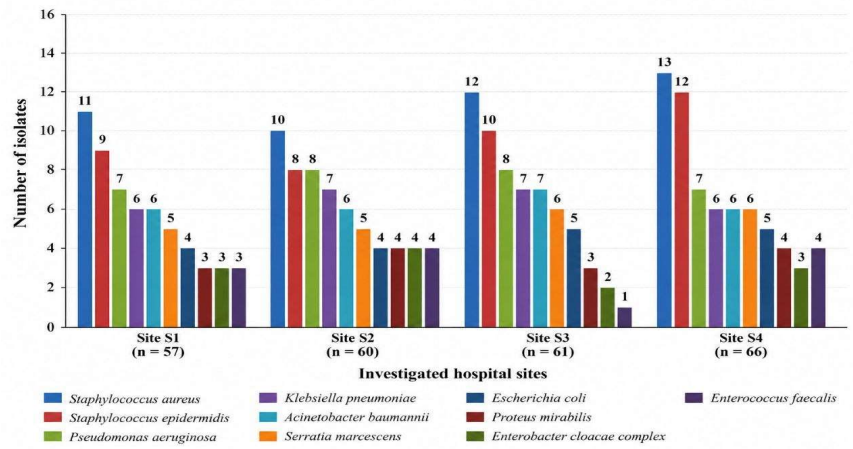

Figure 4.5. Distribution of bacterial isolates across the investigated hospital sites.

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.

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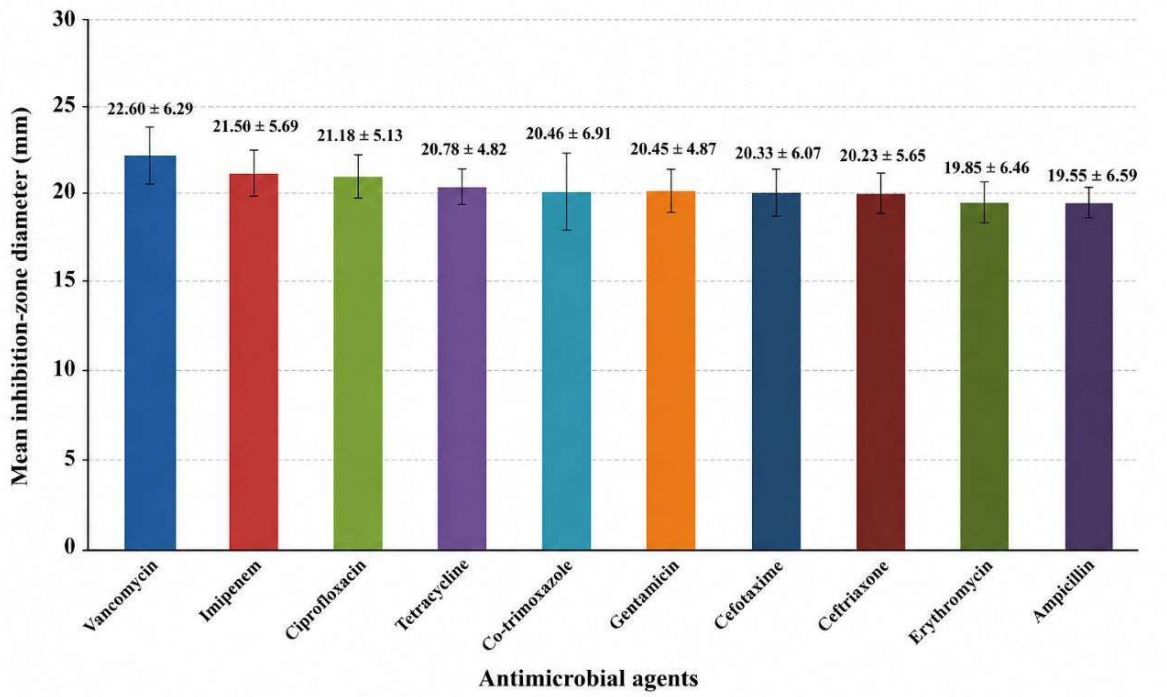

Figure 4.6. Mean inhibition-zone diameters of the tested antimicrobial agents.

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

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Target gene	Positive isolates (n)	Prevalence (%)
sul1	86	35.2
intI1	81	33.2
tetA	67	27.5
mecA	48	19.7
blaTEM	43	17.6
blaCTX-M	42	17.2
blaSHV	29	11.9
qnrS	20	8.2
mecC	18	7.4
vanA	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.

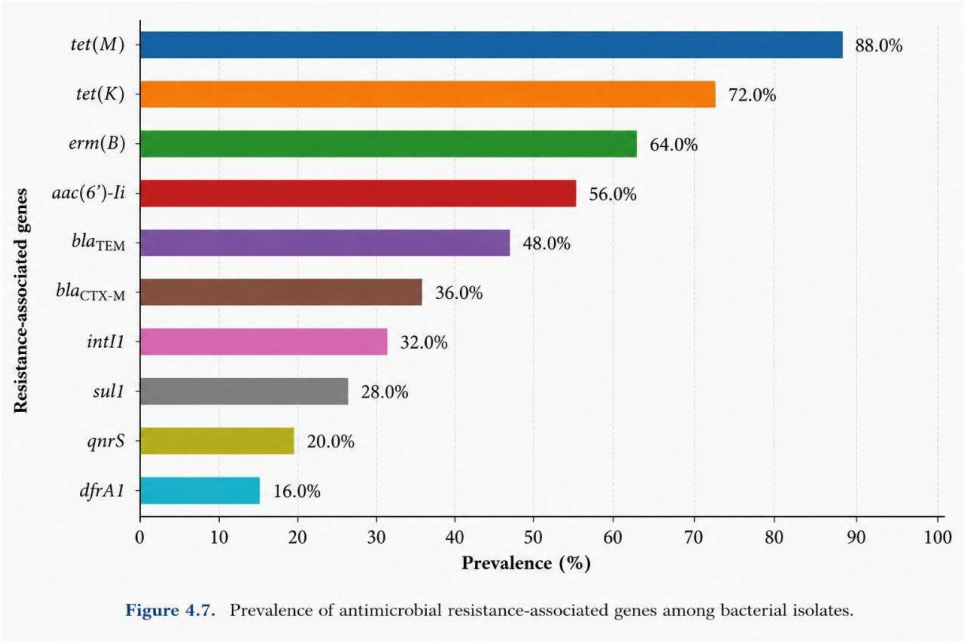

Figure 4.7. Prevalence of antimicrobial resistance-associated genes among bacterial isolates.

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.

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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 by Facila described frequently touched surfaces such as faucets, nightstands, door handles, bed rails, and medical equipment as potential sites for microorganism persistence and accumulation. We found high culture positivity in surface samples, consistent with our assessment throughout the study. Consequently, we also distinguished between actual transmission of bacterial contamination to patients and the environmental prognosis of the surfaces for stock. Transmission does not necessarily depend on pathogen type; instead, it depends on patient susceptibility, microbial load, surface hygiene, hand hygiene, and real-time contact between the host and the contaminated surface. These results suggest potential direct transmission of patients through a microbiological Reservoir. Additionally, the environmental sources showed varied cultural positivity across different sources. Surface samples showed the highest positive cultural results (88%), followed by floor surface samples (86%), then air samples (74%), while water samples were 78%. The highest surface contamination rate is plausible because clinical sites like hospital surfaces are influenced by visitors, patients, and medical staff alike, along with contaminated materials from numerous high-technology equipment.[35]. Microorganisms are repeatedly deposited through this touch-feel nexus of contamination. This observation also aligns with another study by Chaoui, who described bacterial contamination of hospital surfaces as a significant catalyst for multidrug-resistant bacteria.[36]. Furthermore current review by Lee emphasized the significance of targeting cleaning and disinfection measures through environmental hygiene which controls microorganisms and antibiotic resistance in clinical setups for Stone however there are some contrasting studies which control Healthcare settings into antibiotic resistance microorganisms which are more cautious interpretation for example Dettenkofer, in the systematic review has explored that the routine disinfection of Environmental surfaces eludes to the corroborative evidence which rely describes the reliably reducing not so[37].

This differing assessment may reflect the multifactorial nature of nosocomial infections; beyond environmental contamination, hand hygiene, invasive procedures, patient characteristics, antibiotic

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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 lower virulence, the bacterium forms biofilms and is associated with infections related to foreign medical devices and implants. The congruent and concurrent isolation of S. epidermidis and S. aureus indicates that the hospital service surfaces investigated contained enough contamination to support both potentially pathogenic and common skin commensals. Furthermore, detecting Pseudomonas aeruginosa is particularly significant, as it thrives in hospital environments, colonizes medical devices and water pipes, and can colonize moist niches. In Iraq, the “Seenaa Muhammed Ali” Hospital in Suleimaniyah conducted a study of seven hospitals and collected 300 samples from hospital surfaces, including floors, desks, beds, medical equipment, sinks, and mops. This study obtained 26 isolates from these 300 environmental samples and molecularly detected the resistance genes blaCTX-M-2 and blaCTX-M-2, along with many others. The present investigation suggests that antibiotic resistance may be heterogeneous and may also occur in hospital environments in Iraq. However, the two studies are not in line, as they examined different bacterial species, geographical locations, and sample sizes, and these parameters are not comparable [42].

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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 environmental study now detects uncharacterized organisms, excluding skin-associated bacteria but including a few clinically relevant Gram-positive and Gram-negative species. In particular, Enterobacter spp., K. pneumoniae, and A. baumannii are important in hospital settings because 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]. The technical study of microbial resistance through tetA, sul1, and intI1 thoroughly detected these markers. The significance of mobile genetic elements indicates their potential to maintain and disseminate resistance determinants. Nonetheless, this study does not demonstrate direct horizontal gene transfer through integron cassettes; to examine this in plasmid structures and/or complete genomes, we need additional molecular genetic investigations for a definitive, scientifically evaluated statement. The subsequent detection of theta

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also agrees with prevalent studies on hospital wastewater and hospital environments. In one such study, researchers like Lépesová et al. detected the drug resistance gene blaCTX–M in hospital wastewater. [45]. This agreement is consistent with the current results. It supports the assumption that environmental compartments serve as reservoirs for resistance genes across antibiotic classes and are associated with health care facilities.

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 SULL, TETA, BLATEM, BLHCTX 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

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disinfection of frequently touched surfaces through environmental monitoring, which affects hand hygiene and antibiotic use. Further clinical research, including longitudinal sampling and combining 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.

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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;

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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.

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Conflicts of Interest:

"The authors declare no conflict of interest."

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