

Characterization of Multidrug-Resistant Bacterial Isolates from Hospital Waste with Emphasis on β -Lactamase Production

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DOI: <https://doi.org/10.66723/zjhms.2026.169883.1109>

Received: 07 March 2026, Accepted: 24 June 2026. Published: 30 June 2026

ABSTRACT

The rapid emergence and dissemination of antibiotic resistance complicate the treatment of bacterial infections worldwide. The objective of this study was to isolate bacteria from environmental sources, determine their drug-resistance profiles and evaluate enzyme-mediated resistance mechanisms. Environmental samples were collected from hospital waste disposal sites and bacterial isolates were obtained by standard microbiological procedures. The Kirby-Bauer disc diffusion technique was used to test resistance to antibiotics. Resistance associated enzymes were studied and isolated with special emphasis on production and partial purification of beta-lactamase. Five bacterial isolates were recovered from hospital waste soil, these are *Staphylococcus aureus* (ISO I), *Escherichia coli* (ISO II), *Pseudomonas aeruginosa* (ISO III & IV) and *Klebsiella* species (ISO V). The isolates were highly resistant to β -lactams and aminoglycosides particularly cefoxitin, methicillin, vancomycin and gentamicin. Detection of the highly resistant 'superbug' ISO I (*S. aureus*) while other isolates were relatively sensitive to ciprofloxacin and levofloxacin. By SDS-PAGE the molecular weight of the partially purified β -lactamase from *S. aureus* was 40–42 kDa. The findings stress the emerging threat of antibiotic-resistant bacteria to minimize the spread of resistant infections and the need for regular resistance surveillance, enzyme-level testing, and prudent use of antibiotics.

Key words: Multidrug-Resistant Bacteria (MDR), Hospital Waste Soil, Kirby–Bauer Disc Diffusion Method, β -lactamase Enzyme

INTRODUCTION

The fast emergence of drug-resistant bacteria is one of the worldwide health concerns that hinders the effective treatment of infectious illnesses in both medical and public settings [1,2]. Antibiotic resistance results in higher morbidity, mortality and healthcare costs, therefore continued monitoring of resistance trends in bacterial populations from various sources is required.[3]. One of the biggest global health challenges of the twenty-first century is multidrug resistance (MDR). It accounts for the capacity of microbes, especially bacteria, to withstand the effects of a number of antimicrobial agents which were employed earlier in treatment [4]. The increased prevalence of MDR strains increases the length of stay, death rates and healthcare costs, posing a threat to the successful treatment of infectious diseases [5]. Samples' clinical bacterial isolates are closely related to current infections and have significant impact on patients and treatment outcomes. In contrast, bacteria present in wastewater, soil, water, and surfaces of hospitals are important sources of antibiotic resistance genes [2]. These environmental microorganisms participate in the preservation and spread of resistance determinants by horizontal gene transfer mechanisms, enabling the transfer of resistance traits between pathogenic and non-pathogenic bacteria and hastening the spread of antibiotic resistance. [5,6] One of the main causes of multidrug resistance is the over- and misuse of antibiotics in veterinary, human medicine and agriculture. Self-medication, subtherapeutic dose, incomplete treatment procedures, and the widespread availability of antibiotics without prescription create selective pressure for the development of resistant bacteria [7]. MDR microorganisms also proliferate more rapidly in hospitals as well as communities due to insufficient surveillance systems and improper infection control practices [8]. There are many processes which can lead to the development of bacterial resistance such as the synthesis of enzymes that render antibiotics ineffective, changes to antimicrobial target sites, decreased permeability of the cell membrane and the activation of efflux pumps that pump medications from the cell. The overuse and misuse of antibiotics in healthcare, agricultural livestock production and the incorrect disposal of pharmaceutical waste is resulting in an increasing selective pressure and the emergence of multidrug-resistant strains of medical and natural environments at a faster rate. [1,5]. A comparative analysis of antimicrobial susceptibility profiles of clinical and environmental isolates, following the recommendations of the Clinical

and Laboratory Standards Institute (CLSI), helps to understand the dynamics of resistance transmission. Such comparative studies are used to improve the process of infection control protocol and antimicrobial stewardship programmes as well as provide invaluable evidence in tracking resistance spread. [9,6]. The aim of this study was to isolate and characterize bacterial strains from environment, determine their antimicrobial susceptibility profile, and study enzymatic resistance mechanisms, mainly β -lactamase production, to better understand the prevalence and dissemination of multidrug-resistant bacteria.

MATERIAL AND METHODS

Collection of hospital waste soil

Five hospital waste soil samples were got from hospital waste soil (Baghdad Medical City) during (December,2025) in sterile container and used for further experimental procedures, before the actual isolation of bacteria, the collected samples have been exposed under sunlight for two days to cut lower the total amount of fungus mycelia and count. The samples were sieved using a 2 mm mesh cloth after drying in order to separate the small particles and suspended residual particulates [10].

Bacteria Isolation from hospital waste soil

Isolation of Bacteria from Hospital Waste Soil The bacterial colonies were isolated and counted from dried sewage samples by serial dilution method [11]. One gram of dried hospital waste soil was added to sterile distilled water that had been serially diluted from tenfold to ten times five to isolate drug resistant bacteria [12,13]. Based on the chosen 10-fold serial dilutions, the 100 μ L sample was inoculated on the nutrient agar with the antibiotics were incorporated into the isolation medium solely as the primary selective screening mechanism, thus providing the selection pressure and suppressing the susceptible ambient flora, to isolate innately broad-spectrum drug-resistant isolates. During the first isolation step, gentamicin was used at a baseline concentration to assess preliminary resistance and later in the disk diffusion experiment to measure the exact zone of inhibition quantitatively and interpret the susceptibility levels according to CLSI guidelines. After incubation for twenty-four hours, bacterial growth was observed on nutrient agar plates [14]. The antibiotic-resistant bacteria with different colony

morphologies were then chosen, cultured on Brain Heart Infusion (BHI) agar slants in the presence of common antibiotics that were used in primary screening and stored at 4 °C just in case of further use. Further reduction of the screening process was done on the basis of bacterial cell wall composition. The chosen isolates were followed in terms of their colony forms; their morphology was observed by Gram staining along with the biochemical utilization tests [15,16].

Morphological and Biochemical characterization of the Isolates:

The initial investigations, which included Gram staining, morphological analyses, and a number of biochemical tests, were used to identify the bacterial isolates [17]. On the nutrient agar plate, the bacterial colonies' morphology—including their size, shape, color, and structure—was examined. The biochemical characterization of the bacterial isolates was then conducted. The tests listed in Table 3 were carried out: Tests for carbohydrate fermentation as well as different enzyme production and substrate utilization confirmed by using standard biochemical tests mentioned below [18].

Assay for Drug Susceptibility

After identification of the isolate's basis on biochemical tests, antimicrobial susceptibility testing was performed using the standard disc diffusion method. Each isolate was inoculated onto sterile Mueller–Hinton agar plates, and commercially prepared antibiotic discs (Bioanalyse, Turkey) were placed on the agar surface under aseptic conditions. Among the antibiotics that were evaluated were gentamicin, ampicillin, amoxicillin, ciprofloxacin, tetracycline, and chloramphenicol. Multidrug-resistant (MDR) isolates could be classified thanks to the identification of drug-resistance patterns based on zone-of-inhibition measurements. The antibiotics tested against standard drugs included fourth generation of antibiotics as well as Fluoroquinolones, Aminoglycosides and β -Lactam Antibiotics [14]. The disc containing plates were incubated at 37 °C for 24 hrs. In accordance with recognized Clinical and Laboratory Standards Institute (CLSI) recommendations, the diameters of the zones of inhibition surrounding each antibiotic disc were measured after incubation and classified as susceptible, intermediate, or resistant [19,20].

Partial Purification and molecular weight determination

The penicillinase-like enzyme (also known as β -lactamase) has been produced & purified using Gram-positive *Staphylococcus* species. 50 mL of lysozyme solution (100 μ g/mL) made with 50 mM sodium phosphate buffer (pH 7.0) containing 0.3 mM sucrose, 0.3 mM EDTA, and 7% glycerol was used to suspend approximately five grams of bacterial cells were suspended in 50 mL lysozyme solution (100 μ g/mL) in 50 mM sodium phosphate buffer (pH 7.0) with 0.3 mM sucrose, 0.3 mM EDTA and 7% glycerol. After incubation for the cell wall to be damaged by enzymes, the suspension was gently sonicated to ensure total cell breakdown [22,23]. Cell bound enzyme fractions were further extracted by a combination of detergent treatment and freeze-thaw cycles. The suspension was gently shaken for 30 minutes at 4°C and then sonicated on ice (20 kHz) for 30 seconds. For the separation of membrane associated enzymes, Triton X-100 was added to a final concentration of 0.1% (v/v) and the mixture was gently shaken for 2 hours at 4°C to solubilize membrane proteins [24,25]. Following cell lysis, the suspension was centrifuged at 10,000 rpm at 4°C for 30 minutes. The resulting supernatant containing the crude enzyme extract was subjected to ammonium sulphate (70%) precipitation for partial purification. The precipitated protein pellet was collected, dialyzed against phosphate buffer to remove excess salt, after that the partial purified protein samples were analyzed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, and the separated bands of protein allowed for the visualization of CBR-250 stain [26,27].

Statistical Analysis

Antibacterial activity of 22 drugs against five bacterial strains (ISO I-ISO V) was evaluated by measuring inhibitory zones (mm). The data obtained were processed and analyzed with Microsoft Excel (Redmond, WA, USA). For each set of bacterial isolates, descriptive statistics (variance, mean and standard error) were calculated. Susceptibility profiles of five isolates were compared to all antibiotics by one-way analysis of variance (ANOVA). Statistical significance was defined as a p value of <0.05 .

RESULTS

Isolation of Bacteria

Three bacterial isolates were isolated and identified from the hospital waste soil samples by Gram staining, microscopic and biochemical analysis.

Microscopic examination and gram nature:

The obtained bacteria were tested using Gram staining to establish the Gram characteristics at first. The Gram staining method is a concept to differentiate bacteria on the basis of the characteristics of their cell walls, as shown in Table 1.

Table 1. The bacterial isolates analysis.

Serial number	Isolate codes	Staining character	Microscopic observation	Colour of colony
1.	ISO I	Gram positive	Coccus	White
2.	ISO II	Gram negative	Short rods	Yellow
3.	ISO III	Gram negative	Short rods	Light Green
4.	ISO IV	Gram negative	Rods	Greenish
5.	ISO V	Gram negative	Short rods	Creamy

Three isolates were tentatively identified as *Staphylococcus aureus* (ISO I), *Escherichia coli* (ISO II), *Pseudomonas aeruginosa* (ISO III, ISO IV) and *Klebsiella spp.* as shown in Table 2. (ISO V) These assignments were verified by Gram staining and microscopic examination.

Table 2. Genus level Identification of Bacterial Isolates.

Sr. No.	Isolate codes	Tentative genus
1.	ISO I	<i>S. aureus</i>
2.	ISO II	<i>E. coli</i>
3.	ISO III	<i>P. aeruginosa</i>
4.	ISO IV	<i>P. aeruginosa</i>
5.	ISO V	<i>Klebsiella spp.</i>

Biochemical study of isolated bacteria

The bacterial isolates were isolated and identified microscopically and by cultivation methods, and then different sugar utilization and enzyme production examinations were performed. The biochemical tests used in table 3 were IMViC, Triple Sugar Iron test, Urease, Catalase, Oxidase and Nitrate Reduction. The IMViC test is a series of tests that help to identify and differentiate members of the Family Enterobacteriaceae at the genera level. (Indole, Methyl Red, Voges Proskauer, and Citrate Utilization) The results of each test were compared with conventional biochemical chart and tabulated in table 3 for preliminary identification of the bacterial isolates.

Table 3. Biochemical analysis of five bacterial isolates.

Isolates	ISO I	ISO II	ISO III	ISO IV	ISO V
Indole test	Positive	Negative	Negative	Positive	Negative
Methyl Red test	Positive	Positive	Negative	Positive	Positive
Voges Proskauer test	Negative	Negative	Negative	Negative	Negative
Citrate Utilization test	Negative	Negative	Positive	Positive	Positive
Triple Sugar Iron test	K/A	A/A	K/A+H ₂ S	K/A	A/A
Urease test	Positive	Positive	Negative	Positive	Negative
Catalase test	Positive	Positive	Positive	Positive	Positive
Oxidase test	Negative	Negative	Positive	Positive	Negative
Nitrate Reductase test	Negative	Negative	Positive	Positive	Positive

H₂S, Positive for Hydrogen Sulfide, K/A: Glucose fermentation only, A/A: Glucose + Lactose/Sucrose fermentation

Antibiotic susceptibility testing:

Five isolates resistant to the antibiotics cefoxitin, netillin, methicillin, vancomycin, gentamycin and kanamycin were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* spp. Thus, the bacterial isolates were found to be insensitive to cephalosporin, narrow spectrum β -lactam antibiotics and cell wall synthesis inhibitor but still vulnerable to the effects of carbapenem, erythromycin, linezolid, meropenem and levofloxacin which belong to oxazolidinone class as shown in Table 4 and Fig. 1. The present study ISO I showed multidrug resistant pattern in comparison to ISO II, ISO III, ISO IV and ISO V hence ISO I used for further study i.e. partial enzyme purification.

SDS-PAGE analysis

SDS-PAGE is a reliable technique for analysing penicillinase protein. In fig 2 the enzyme typically appears as a band near 30 to 40 kDa, confirming its expected molecular weight. Molecular weight comparison with a standard protein ladder allows accurate estimation and verification of enzyme purity after isolation and purification [21].

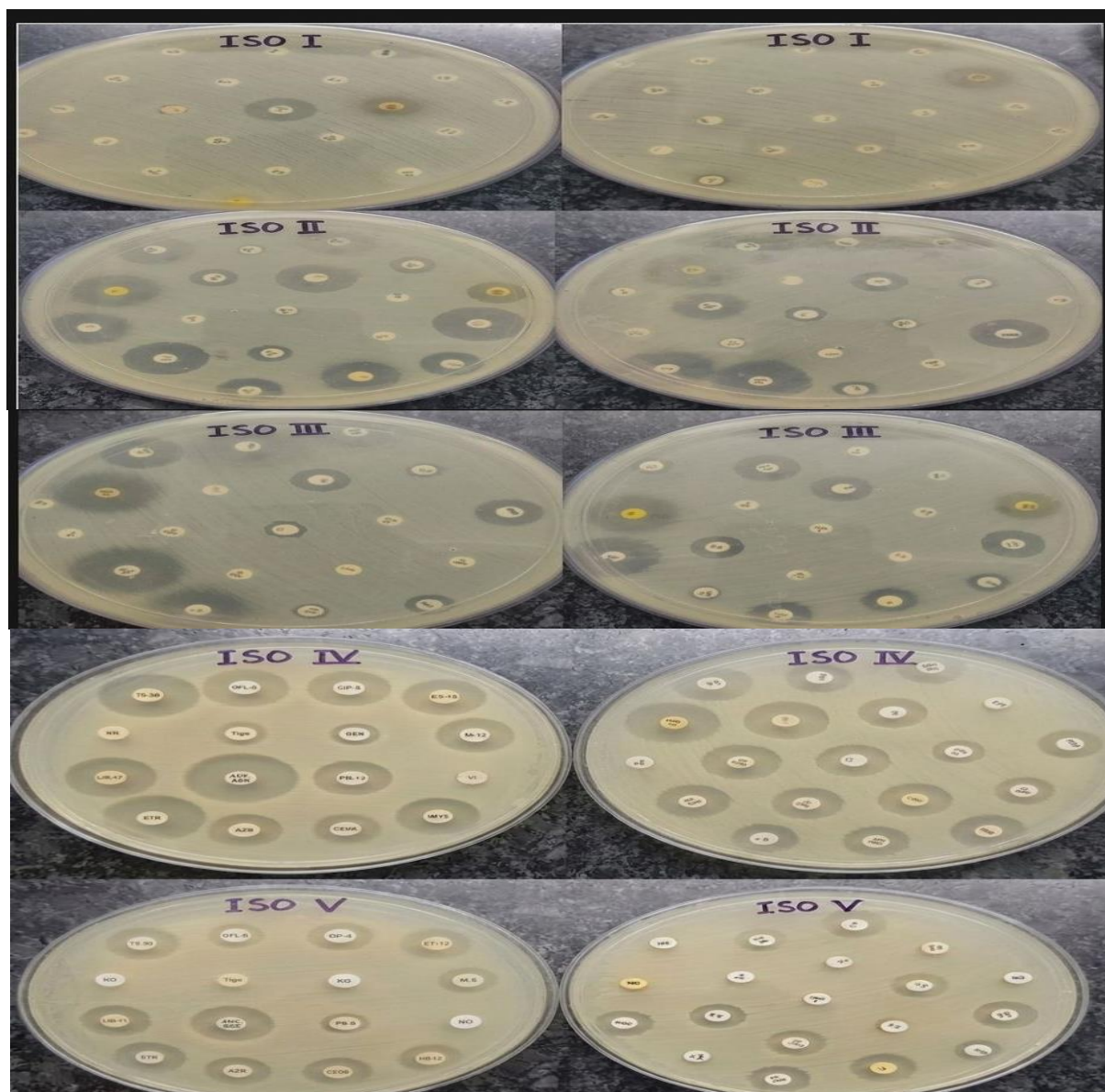
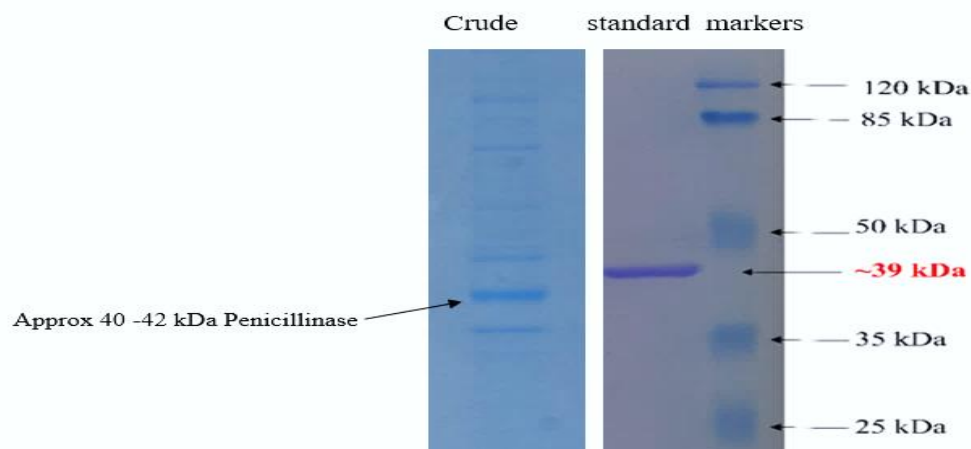


Figure1. Disc Diffusion method for antimicrobial susceptibility using common antibiotics discs.

Table 4. Zone of inhibition of isolates against common antibiotics in mm as per CLSI guidelines.

		ISO I	ISO II	ISO III	ISO IV	ISOV
1	Tetracycline	16	15	14	18	13
2	Ofloxacin	00	12	10	14	9
3	Ciprofloxacin	00	11	11	13	10
4	Erythromycin	10	13	13	15	12
5	Kanamycin	00	00	00	5	00
6	Tigecycline	00	5	4	7	3
7	Gentamycin	00	00	00	6	00
8	Methicillin	00	11	10	12	8
9	Linezolid	0	15	12	17	11
10	Amoxy-Clavulanic acid	21	15	11	19	14
11	Pristinomycin	00	13	7	12	6
12	Vancomycin	00	00	00	4	00
13	Etrapanem	0	16	15	18	14
14	Aztreonem	13	12	8	14	9
15	Cefoxitin	00	00	00	00	4
16	Clindamycin	0	6	11	15	12
17	Imipenem	0	13	13	12	11
18	Netillin	00	00	00	14	13
19	Trimethoprim	00	9	11	12	10
20	Chloramphenicol	00	00	15	14	13
21	Cefotaxime	00	12	12	13	11
22	Levofloxacin	00	16	14	17	13

**Figure 2. Analysis of Penicillinase as a Beta lactamase protein by SDS-PAGE.**

DISCUSSION

In the present study *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* isolated from hospital waste soil were multidrug-resistant (MDR) bacterial pathogens to many antibiotics such as kanamycin, ceftiofur, gentamicin, methicillin, vancomycin and netilmicin. In 2022 Murray reported Similar findings in environmental studies by, indicating that hospital-associated waste acts as an important reservoir of resistant pathogens [5]. In *S. aureus*, resistance to β -lactam antibiotics such as methicillin and ceftiofur is usually mediated by the production of β -lactamase and modified penicillin-binding proteins [21,25]. Similarly, aminoglycoside-modifying enzymes and efflux pathways have been implicated in the development of aminoglycoside resistance in *P. aeruginosa* and *E. coli* [26]. The SDS-PAGE results in this study are in agreement with previous publications characterizing β -lactamases and associated resistance proteins [27] and confirm the involvement of enzymatic processes. The diameter of the zone of inhibition was subjected to one way analysis of variance (ANOVA) and the results revealed highly statistically significant differences among the five bacterial isolates ($F = 8.64$, $P < 0.001$). This indicates differential response of the isolates to the antibiotics tested. The isolate ISO IV was found to be the most susceptible strain to the investigated chemicals while the isolate ISO I was identified as the highly resistant strain (superbug) interestingly. Ultimately, the isolation of multi-drug-resistant bacteria from soil contaminated with hospital waste points to the role of environmental reservoirs in the persistence and spread of antibiotic resistance. The present work also aimed to perform a preliminary screening and phenotypic characterization of antibiotic resistance patterns and β -lactamase production among isolates from soil contaminated with hospital waste. Therefore, the results were descriptive and focused on resistance profiling rather than a statistical comparison of experimental groups.

Conclusions

The bacteria isolated from hospital waste disposal sites in this study were highly resistant to β -lactam antibiotics, particularly ampicillin and amoxicillin. On the other hand, the isolates were generally more susceptible to ciprofloxacin and gentamicin. Several strains showed Multidrug resistance, which was associated with the production of β -lactamase enzymes. These findings highlight the growing threat of antibiotic-resistant bacteria in environmental and clinical settings,

and the need for continued surveillance of resistance patterns and more judicious use of antibiotics to prevent the emergence and spread of resistant strains.

Acknowledgments

I would like to express my sincere gratitude to the laboratory staff and colleagues for their support and assistance during this study.

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