Vol. 5 No. 2 (2026)

Pakistan Journal of Medical & Cardiological Review

https://pakjmcr.com/index.php/1/about

Online ISSN 3007-2387

Print ISSN 3007-2379

valuation Of The Antimicrobial Efficacy Of Copper Solution Against Carbapenemase-Producing Gram-Negative Clinical Isolates

Zainab Ghaffar
Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

Muhammad Faisal Bashir
TML Labs & Diagnostics Health Care Network (Trustmed Lab), Lahore, Pakistan

Muhammad Waleed
Service Institute of Medical Sciences**,** Services Hospital Lahore, Pakistan

Saba Riaz*
Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan, Canli Institute of Emerging Health Technologies, Lahore, Pakistan &
TML Labs & Diagnostics Health Care Network (Trustmed Lab), Lahore, Pakistan
Email: saba.mmg@pu.edu.pk

Author Details

Keywords: Copper Sulfate, *E Coli*, Carbapenem Resistance, Multidrug Resistance, Minimum Inhibitory Concentration, Antimicrobial Activity

Received on 18 May 2026
Accepted on 17 Jun 2026
Published on 25 Jun 2026

Corresponding E-mail & Author*:

Saba Riaz
Email: saba.mmg@pu.edu.pk

Abstract

Urinary tract infections (UTIs) caused by multidrug-resistant uropathogenic Escherichia coli (MDR UPEC) present an increasing clinical challenge, particularly with the emergence of metallo-β-lactamases (MBLs) that confer resistance to carbapenems, the last-resort antibiotics used to manage severe Gram-negative infections. The declining effectiveness of conventional antimicrobial agents necessitates the urgent exploration of alternative therapeutic strategies. This study evaluated the in vitro antimicrobial activity of copper sulfate ($CuSO_4$) against carbapenem-resistant MDR UPEC isolates and characterized their resistance profiles at both phenotypic and molecular levels. A total of 70 urine samples were collected from patients with clinical features of UTIs, and 50 UPEC isolates were identified using standard biochemical methods. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method against a panel of 24 antibiotics, confirming multidrug resistance in all isolates. Phenotypic detection of carbapenem resistance was conducted using the Modified Hodge Test (MHT), the Modified Carbapenem Inactivation Method (mCIM), and the EDTA-modified Carbapenem Inactivation Method (eCIM). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of $CuSO_4$ were determined to quantify its antimicrobial efficacy. Additionally, genomic and plasmid DNA extraction was performed to support the molecular characterization of resistance determinants. The minimum inhibitory concentration (MIC) of $CuSO_4$ was 1600 µg/mL for 90% of the isolates, indicating consistent inhibitory activity across the group. Genomic DNA was successfully extracted from 40 out of 50 isolates, while plasmid DNA was recovered

from 30 out of 50 isolates. This success enabled downstream molecular analysis of resistance gene carriage. These findings demonstrate that $CuSO_4$ exhibits reliable in vitro antimicrobial activity against carbapenem-resistant, multidrug-resistant UPEC, supporting its potential as an alternative or adjunctive antimicrobial agent amid rising carbapenem resistance.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, affecting hundreds of millions of people each year across all age groups and healthcare settings (Flores-Mireles et al., 2015). The primary cause of these infections is Escherichia coli, which accounts for approximately 80-85% of community-acquired UTIs. It is also the most frequently isolated uropathogen in clinical microbiology specimens globally (Terlizzi et al., 2017). The strains responsible for these infections are known as uropathogenic Escherichia coli (UPEC). They are characterized by a range of virulence factors—including adhesins, pore-forming toxins, and systems for acquiring iron—that facilitate their colonization, persistence, and the progression of infection within the urinary tract (Subashchandrabose & Mobley, 2015). The clinical management of uropathogenic Escherichia coli (UPEC) infections has become significantly more challenging due to the rise and global spread of multidrug resistance (MDR). Strains that produce extended-spectrum β-lactamases (ESBLs) and carry the blaCTX-M genes have become increasingly common, making third-generation cephalosporins and most conventional β-lactam antibiotics ineffective for treatment (Paterson & Bonomo, 2005). As a result, there has been a growing reliance on carbapenems as the last-resort treatment option for severe UPEC infections. However, the widespread emergence of carbapenemase-producing Enterobacteriaceae (CPE), especially those carrying the blaNDM-1 and blaOXA-48 genes, has severely limited the effectiveness of this treatment option, creating a significant and concerning gap in available antimicrobial therapies (Logan & Weinstein, 2017; Heidari Soureshjani et al., 2020).The issue of antimicrobial resistance in Pakistan is particularly concerning. Clinical laboratories across the country have reported significantly high rates of ESBL-producing bacteria and carbapenem-resistant Enterobacteriaceae in both hospital and community settings (Aktas et al., 2016; Logan & Weinstein, 2017). These challenges are further exacerbated by the lack of effective national antimicrobial surveillance systems, the unrestricted availability of antibiotics over the counter, and the widespread practice of prescribing antibiotics empirically without microbiological guidance. These conditions contribute to the selection and spread of resistant strains. In light of this situation, finding novel and alternative antimicrobial strategies has become crucial for both clinical practice and public health.

Copper and copper-based compounds have been recognized for their broad-spectrum antimicrobial properties for a long time. The bactericidal mechanism of copper ions is complex, involving the disruption of bacterial cell membranes, generation of reactive oxygen species (ROS), inactivation of critical enzymatic pathways, and degradation of nucleic acids. This combination of actions makes it difficult for bacteria to develop effective resistance mechanisms (Lemire et al., 2013). Copper sulfate ($CuSO_4$), an inexpensive, chemically stable, and widely available inorganic salt, has gained particular interest as an antimicrobial agent due to its strong ionic activity and well-documented bactericidal effectiveness (Lemire et al., 2013; Vincent et al., 2016). While there is a growing body of evidence supporting the antimicrobial potential of $CuSO_4$ against various pathogenic organisms, its specific activity against carbapenem-resistant multidrug-resistant UPEC is still not well characterized, especially in the context of Pakistani clinical settings. This study was designed to address a specific gap in understanding. The objectives were to isolate and confirm the presence of Uropathogenic Escherichia coli (UPEC) from clinical urine samples of patients with

symptomatic urinary tract infections (UTIs). Additionally, we aimed to determine the antimicrobial susceptibility and phenotypic profiles of carbapenem resistance in these confirmed isolates. Finally, we evaluated the in vitro antimicrobial activity of Copper(II) sulfate ($CuSO_4$) by determining its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), assessing its potential as an alternative therapeutic agent against carbapenem-resistant multidrug-resistant (MDR) UPEC.

Materials and Methods

Sample Collection and Processing

A total of 70 midstream clean-catch urine samples were collected from patients with clinical features of urinary tract infection (UTI) in both the outpatient department (OPD) and the inpatient wards of the hospital. Before sample collection, patients were instructed to perform careful perineal hygiene. The samples were collected in sterile, wide-mouthed, screw-capped urine containers, each labelled with a unique patient identification number and the date and time of collection. All specimens were transported immediately to the microbiology laboratory and processed within 2 hours of collection to prevent bacterial overgrowth and ensure sample integrity.

Each urine sample was inoculated onto Cystine Lactose Electrolyte-Deficient (CLED) agar using a calibrated standard loop (0.001 mL) and incubated aerobically at 37°C for 24–48 hours. CLED agar was chosen as the primary isolation medium because it supports the growth of all urinary pathogens while inhibiting swarming by Proteus species. This characteristic facilitates clear assessment of colonial morphology. E. coli colonies on CLED agar were identified as yellow, opaque colonies with a diameter of 1–2 mm, consistent with established morphological criteria.

Biochemical Confirmation of *E. coli*

Presumptive E. coli colonies underwent a standard series of confirmatory biochemical tests, which included Triple Sugar Iron (TSI) agar, Simmons Citrate agar, the indole production test using Kovac's reagent, and Christensen's urea agar. Isolates that displayed a biochemical profile consistent with *E. coli*, specifically acid/acid with gas production on TSI, indole positivity, citrate negativity, and urease negativity, were confirmed as E. coli and retained for further analysis. Those meeting these criteria were subsequently classified as uropathogenic Escherichia coli (UPEC) based on their clinical origin from patients with symptomatic urinary tract infections (UTIs).

Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was conducted using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA), in strict accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. A panel of 24 antibiotics was evaluated, representing all major clinically relevant antibiotic classes, including penicillins, cephalosporins (first- to fourth-generation), carbapenems, fluoroquinolones, aminoglycosides, and polymyxins. Prior to inoculation, bacterial suspensions were standardized to a 0.5 McFarland turbidity standard. The diameters of the inhibition zones were measured and interpreted based on the current CLSI breakpoints. Multidrug resistance (MDR) was defined as the acquired non-susceptibility to at least one agent in three or more antimicrobial categories, in line with internationally recognized criteria for MDR.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Copper Sulfate

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was dissolved in sterile distilled water to prepare a stock solution at a concentration of 10,000 µg/mL, which was then filter-sterilised using a 0.22 µm membrane filter. Working concentrations of 200, 400, 800,

and 1600 µg/mL were prepared by performing serial two-fold dilutions in Mueller-Hinton broth (MHB). The minimum inhibitory concentration (MIC) was determined using the broth microdilution method in sterile 96-well microplates, following CLSI guidelines. Bacterial inocula were standardized to the 0.5 McFarland standard and diluted to a final concentration of 5×10^5 CFU/mL before being introduced into each well. The plates were incubated aerobically at 37°C for 18 to 24 hours. The MIC was defined as the lowest concentration of $CuSO_4$ at which no visible bacterial growth was observed. Growth controls (wells containing broth and bacteria without $CuSO_4$) and sterility controls (wells containing broth and $CuSO_4$ without bacteria) were included in each assay to ensure validity. All experiments were conducted in triplicate. The minimum bactericidal concentration (MBC) was subsequently determined by subculturing the contents of all wells that showed no visible growth onto Mueller-Hinton agar plates. The MBC was defined as the lowest concentration of $CuSO_4$ that resulted in a 99.9% reduction of the original bacterial inoculum after 18 to 24 hours of incubation at 37°C.

Agar Well Diffusion Assay

The antimicrobial activity of $CuSO_4$ was evaluated using the agar well diffusion method. Mueller-Hinton agar plates were inoculated with standardized bacterial suspensions (0.5 McFarland standard) using sterile swabs. Wells with a diameter of 6 mm were created in the agar using a sterile cork borer, and 20 µL of $CuSO_4$ solution at five different concentrations (100, 50, 25, 12.5, and 6.25 mg/mL) was added to each well. The plates were allowed to stand at room temperature for 30 minutes to enable pre-diffusion of the compound, then incubated at 37°C for 24 hours. The diameters of the inhibition zones were measured in millimetres, and the results were expressed as mean ± standard deviation (SD) from three independent experiments, each conducted in triplicate.

Genomic DNA Extraction

Genomic DNA was extracted from all 50 confirmed multidrug-resistant (MDR) UPEC isolates using the sodium acetate precipitation method. In brief, bacterial cells were lysed with sodium dodecyl sulfate (SDS) and proteinase K. This was followed by phenol-chloroform extraction and sodium acetate-ethanol precipitation. The purity of the DNA was assessed spectrophotometrically by measuring the absorbance ratio A260/A280, and the integrity of the DNA was confirmed by agarose gel electrophoresis. Plasmid DNA was extracted from the same 50 MDR UPEC isolates using the alkaline lysis method. Bacterial cells were lysed with a NaOH/SDS lysis buffer, neutralized with potassium acetate buffer, and the plasmid DNA was precipitated with isopropanol. The integrity of the plasmid DNA was visualized using agarose gel electrophoresis. Genomic DNA was successfully extracted from 40 of the 50 isolates, and plasmid DNA from 30 of the isolates.

Phenotypic Detection of Carbapenem Resistance

Modified Hodge Test (MHT)

The Modified Hodge Test (MHT) was performed on all isolates in accordance with CLSI standards to screen for carbapenemase production. Mueller-Hinton agar plates were inoculated with a 1:10 dilution of a 0.5 McFarland suspension of the indicator strain, E. coli ATCC 25922. A meropenem disc (10 µg) was placed at the centre of each plate, and the test isolates were streaked radially from the edge of the disc towards the periphery of the plate. The plates were then incubated at 37°C for 16 to 24 hours. A positive result for the MHT was characterized by the presence of a cloverleaf-shaped indentation or distortion in the inhibition zone at the intersection of the test streak, indicating carbapenemase activity.

Modified Carbapenem Inactivation Method (mCIM)

The mCIM (modified Carbapenem Inactivation Method) was performed to phenotypically confirm carbapenemase production. A 10 µg meropenem disc was incubated with a suspension of the test isolate in 2 mL of tryptic soy broth (TSB) at 37°C for four hours. After incubation, the disc was retrieved and placed on a Mueller-Hinton agar plate inoculated with a 0.5 McFarland suspension of E. coli ATCC 25922, which served as the indicator organism. The plates were then incubated at 37°C for 16 to 24 hours. Finally, the diameters of the inhibition zones were measured and interpreted according to the CLSI mCIM criteria.

EDTA-Modified Carbapenem Inactivation Method (eCIM)

The eCIM was performed concurrently with the mCIM to distinguish metallo-β-lactamase (MBL)-producing isolates from those that produce serine carbapenemases. The procedure was identical to that of the mCIM, except that 20 µL of 0.5 M EDTA was added to the TSB incubation mixture. EDTA acts by chelating the zinc ions that are essential for MBL activity, thereby inhibiting the hydrolysis of carbapenems by MBL enzymes. A significant increase in the diameter of the meropenem inhibition zone in the eCIM compared to the mCIM indicates MBL production, while similar zone diameters in both tests suggest the presence of serine carbapenemase activity.

Results

Isolation of UPEC and Study Population

A total of 70 midstream clean-catch urine samples were processed from patients presenting with clinical features of urinary tract infection (UTI). Among these, 50 isolates (71.4%) were confirmed as Escherichia coli based on characteristic colonial morphology on Cystine Lactose Electrolyte-Deficient (CLED) agar and supported by a comprehensive biochemical profile. The remaining 20 samples either yielded no significant growth, showed mixed flora indicative of contamination, or grew organisms other than E. coli, and were therefore excluded from further analysis. On CLED agar, the presumptive E. coli colonies appeared as yellow, opaque, smooth, and convex colonies measuring 1–2 mm in diameter after 24–48 hours of aerobic incubation at 37°C. The yellow coloration is characteristic of lactose fermentation by E. coli on this medium. Additionally, the electrolyte-deficient composition of CLED agar prevented the swarming of any Proteus species present, ensuring clear differentiation of colonies. All 50 presumptive isolates displayed colonial morphologies fully consistent with established E. coli characteristics on this medium. Biochemical confirmation was then performed on all presumptive colonies using a standardized panel of differential tests. All 50 isolates demonstrated a biochemical profile that unequivocally identified them as *Escherichia coli,* (Table 1). All isolates exhibited an acid/acid reaction with gas production on Triple Sugar Iron (TSI) agar, indicating the fermentation of glucose and lactose, along with the release of carbon dioxide (CO_2) and hydrogen (H_2). These reactions are indicative of E. coli metabolism. Additionally, all isolates tested positive for indole production with Kovac's reagent, confirming the presence of tryptophanase activity. This enzyme cleaves tryptophan into indole, a biochemical characteristic that is highly specific to E. coli within the Enterobacteriaceae family.

Simmons Citrate agar yielded uniformly negative results for all isolates, confirming their inability to utilize citrate as a sole carbon source, thereby excluding Klebsiella and Enterobacter species. Similarly, Christensen's urea agar was negative for all isolates, ruling out urease-producing organisms such as Proteus mirabilis and Klebsiella pneumoniae. No isolate produced hydrogen sulfide (H_2S) on TSI agar, further solidifying the identification of E. coli and excluding Salmonella and Proteus species. The complete concordance of colonial morphology and biochemical results across all 50 isolates provided robust confirmation of Escherichia coli identification,

eliminating the need for additional automated or molecular identification methods at this stage. All 50 confirmed isolates were subsequently designated as uropathogenic Escherichia coli (UPEC) based on their clinical origin from patients with symptomatic UTIs, in accordance with established UPEC classification criteria (Flores-Mireles et al., 2015). These isolates were retained for further analysis, including antimicrobial susceptibility testing, phenotypic characterization of carbapenem resistance, assessment of copper sulfate activity, and molecular analysis as described in the following sections.

Table 1: Demographic Information of Study Participants

Variable	Frequency (n)	Percentages (%)
Gender		
Male	20	40%
Female	30	60%
Age group		
≤20 years	5	10%
21- 40 years	14	28%
41- 60 years	19	38%
More than 60 years	12	24%
Ward		
KCOPD	12	24%
OPD	8	16%
ICU	6	12%
MED	4	8%
OTHERS	20	40%

Antibiotic Susceptibility Profiling and Multidrug Resistance Characterisation

Antimicrobial susceptibility testing of all 50 confirmed UPEC isolates revealed an alarmingly high prevalence of multidrug resistance (MDR). All isolates met the internationally standardized MDR definition, indicating acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012) (Figure 1). Resistance rates were particularly elevated among β-lactam agents and antifolate compounds; every isolate demonstrated complete resistance to ceftazidime-avibactam (CZA), cefepime (FEP), and trimethoprim-sulfamethoxazole (SXT). Additionally, the presence of carbapenem-resistant UPEC was confirmed by meropenem (MEM) resistance, a finding of serious clinical significance, given that carbapenems are considered last-resort treatments for severe MDR Gram-negative infections. Nitrofurantoin (NIT), commonly used as first-line empirical therapy for uncomplicated UTIs, also showed significantly high rates of resistance. In contrast, amikacin (AK), gentamicin (CN), tigecycline (TGC), and colistin (COL) retained some activity against a portion of the isolates. However, reduced susceptibility to colistin and tigecycline in certain isolates is especially concerning, as resistance to these agents represents the outermost boundary of available antimicrobial therapies for carbapenem-resistant Enterobacteriaceae. The overall resistance pattern, characterized by limited susceptibility to only one or two antimicrobial categories in a significant proportion of isolates, indicates an extensively drug-resistant (XDR) phenotype and highlights the urgent need for alternative antimicrobial strategies in this clinical setting.

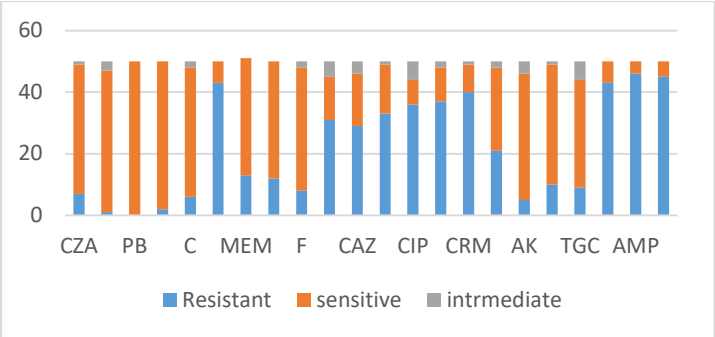

Figure 1: Antibiotic susceptibility patterns of 50 bacterial strains, indicating their responses as sensitive, intermediate, or resistant to various antibiotics. The data reveals variability among the strains; certain antibiotics produced zones of inhibition, indicating sensitivity, while others exhibited reduced or no inhibition, signifying intermediate or resistant responses

Determination of MIC and MBC

The antimicrobial activity of $CuSO_4$ was evaluated on all 50 UPEC isolates at concentrations of 200, 400, 800, and 1600 µg/mL. At a concentration of 1600 µg/mL, the minimum inhibitory concentration (MIC) was observed in 45 isolates (90%), while only 5 isolates (10%) showed minimum bactericidal concentration (MBC) activity. At 800 µg/mL, the MIC was detected in 39 isolates (78%), but the MBC was present in only 3 isolates (6%), with no inhibition observed in 8 isolates (16%). At 400 µg/mL, the MIC activity was noted in 10 isolates (20%), with no MBC activity recorded, and 40 isolates (80%) were found to be non-inhibitory. No antimicrobial activity was detected at 200 µg/mL, with only 5 isolates (10%) exhibiting MIC activity. These results indicate that 1600 µg/mL was the most effective concentration, inhibiting 90% of the isolates tested.

Table 2: Distribution of MIC, MBC, and MAT among isolates at different concentrations of copper sulfate.

CONC.	MIC	MBC	MAT	TOTAL
	N (%)	N (%)	N (%)	
1600	45(90)	5(10)	0	50
800	39(78)	3(6)	8(16)	50
400	10(20)	0	40(80)	50
200	5(10)	0	45(90)	50

Microtiter Plate Broth Microdilution Assay

The in vitro antimicrobial activity of $CuSO_4$ was assessed against four representative multidrug-resistant (MDR) UPEC isolates using the broth microdilution method. The minimum inhibitory concentration (MIC) values recorded for strains A2, A1, A4, and A3 were 0.003, 0.056, 0.089, and 0.143 mg/mL, respectively (see Table 4). Strain A2 demonstrated the highest susceptibility, with the lowest MIC of 0.003 mg/mL, while strain A3 exhibited the highest tolerance, with an MIC of 0.143 mg/mL. This indicates inter-strain variability in copper susceptibility among MDR UPEC isolates. Despite this variability, the narrow MIC range observed suggests a broadly consistent inhibitory effect of $CuSO_4$ at sub-milligram concentrations, supporting its potential as a reproducible antimicrobial agent against carbapenem-resistant UPEC.

Agar Well Diffusion

The antimicrobial activity of $CuSO_4$ was further evaluated against five MDR UPEC isolates using agar well diffusion across a two-fold concentration range of 6.25 to 100 mg/mL. A clear and consistent concentration-dependent inhibitory pattern was observed across all isolates tested. The largest inhibition zones were recorded at 100 mg/mL, with a mean diameter of 8 mm. As the concentration decreased, zone diameters progressively diminished, ranging from 4 to 6 mm at 50 mg/mL, 3 to 5 mm at 25 mg/mL, and 0 to 3 mm at 12.5 mg/mL. No inhibition zone was observed at the lowest concentration of 6.25 mg/mL for any isolate, indicating a complete absence of antibacterial activity below this threshold. These findings establish 12.5 mg/mL as the approximate minimum concentration at which activity can be detected, confirming that the antimicrobial efficacy of $CuSO_4$ is strictly concentration-dependent, with reliable inhibitory activity only demonstrable at or above 25 mg/mL under agar diffusion conditions.

DNA Isolation

Genomic DNA was successfully extracted from 40 out of 50 MDR UPEC isolates (80%) using the sodium acetate precipitation method. Successful extraction was confirmed by the presence of high-molecular-weight bands of the expected size on agarose gel electrophoresis. The remaining ten isolates (20%) yielded insufficient or degraded DNA, likely due to incomplete cell lysis during processing or excessive nuclease activity, rendering these samples unsuitable for downstream PCR amplification. Plasmid DNA was recovered from 30 out of 50 isolates (60%) using the alkaline lysis method, with distinct plasmid bands visualised by agarose gel electrophoresis, confirming successful extraction. The absence of detectable plasmid DNA in the remaining 20 isolates may reflect either a genuine lack of plasmid content in these strains or the loss of plasmid material during extraction. The recovery of plasmid DNA from 60% of the MDR UPEC isolates is clinically significant, suggesting that plasmid-mediated horizontal gene transfer may be a major contributing mechanism underlying the MDR phenotype observed in this cohort. This finding warrants further molecular characterization of plasmid-borne resistance determinants in subsequent analyses.

Phenotypic Detection of Carbapenem Resistance

Modified Hodge Test (MHT)

The Modified Hodge Test was performed on nine multidrug-resistant (MDR) UPEC isolates selected for their demonstrated meropenem resistance during antimicrobial susceptibility testing. Among these isolates, six (66.7%) tested positive, as indicated by the characteristic clover leaf-shaped indentation observed at the intersection of the test streak and the inhibition zone of the indicator strain *E. coli* ATCC 25922, which is consistent with active carbapenemase production. The remaining three isolates (33.3%) tested negative for MHT, showing no distortion of the inhibition zone. The high positivity rate of 66.7% among the phenotypically meropenem-resistant isolates suggests a significant prevalence of carbapenemase-producing *E. coli* within this clinical cohort, aligning with broader epidemiological trends of carbapenemase dissemination among uropathogenic Enterobacteriaceae reported in South Asian clinical settings.

Modified Carbapenem Inactivation Method (mCIM)

The mCIM was performed concurrently on the same nine isolates, demonstrating carbapenemase production in all samples, yielding a 100% positivity rate. After four hours of pre-incubation of the meropenem disc with each test isolate in tryptic soy broth (TSB), no inhibition zone was observed around the meropenem disc on the indicator lawn of *E. coli* ATCC 25922. This indicates complete enzymatic

inactivation of the carbapenem substrate by all isolates. The universal mCIM positivity confirms the presence of functionally active carbapenemase enzymes, consistent with the high-level meropenem resistance observed during disk diffusion testing. The higher sensitivity of mCIM than MHT for detecting carbapenemase production in this cohort aligns with previous studies identifying mCIM as a more reliable phenotypic screening method, particularly for metallo-β-lactamase (MBL)-producing organisms (van der Zwaluw et al., 2015).

EDTA-Modified Carbapenem Inactivation Method (eCIM)

The eCIM was performed simultaneously with the mCIM to differentiate between MBL producers and serine carbapenemase producers. This test confirmed the production of metallo-β-lactamases (MBLs) in all tested isolates. A significant increase in the diameter of the meropenem inhibition zone was consistently observed in the eCIM compared to the corresponding mCIM results across all isolates. This finding indicates that EDTA effectively chelated the zinc ions essential for MBL catalytic activity, neutralizing carbapenemase function and restoring susceptibility to meropenem. The restoration of the inhibition zone diameter in the presence of EDTA is a phenotypic hallmark of MBL production and distinctly differentiates MBL-mediated carbapenem resistance from that conferred by serine carbapenemases, such as KPC and OXA-type enzymes, which are insensitive to EDTA. The confirmation of MBL production in all carbapenemase-positive isolates is of considerable clinical and epidemiological significance, as MBLs, particularly NDM-type enzymes, are frequently plasmid-encoded, enabling rapid horizontal dissemination among Enterobacteriaceae in both hospital and community settings. Collectively, these findings establish MBL-mediated carbapenem resistance as the predominant phenotypic resistance mechanism in the carbapenem-resistant UPEC isolates characterized in this study.

Discussion

The current study reveals significant in vitro antimicrobial activity of copper sulfate ($CuSO_4$) against clinical isolates of multidrug-resistant (MDR) uropathogenic Escherichia coli (UPEC), including carbapenem-resistant strains. These findings have important clinical implications, especially given the rapidly worsening situation of antimicrobial resistance in Pakistan. The spread of carbapenemase-producing Enterobacteriaceae (CPE) in both healthcare and community settings has become a critical public health threat, both nationally and globally. This situation is leaving clinicians with an increasingly limited set of effective treatment options (Heidari Soureshjani et al., 2020; Logan & Weinstein, 2017; Aktas et al., 2016). The isolation rate of uropathogenic Escherichia coli (UPEC) from clinical urine samples in this study aligns with previously reported rates in similar clinical settings, confirming E. coli as the dominant uropathogen in this population (Terlizzi et al., 2017; Hannan et al., 2012; Yang et al., 2022). The finding that 100% of confirmed isolates exhibited multidrug resistance (MDR) underscores a significant burden of resistance in Pakistani clinical laboratories. This situation is exacerbated by the unrestricted over-the-counter availability of antibiotics, widespread empirical prescribing practices, and the lack of effective national antimicrobial stewardship programs, all of which promote the emergence and spread of MDR phenotypes (Zawawi et al., 2015).The antimicrobial susceptibility profile of the cohort showed alarmingly high resistance rates to meropenem, ceftazidime-avibactam, fosfomycin, and trimethoprim-sulfamethoxazole. This pattern suggests the presence of multiple concurrent resistance mechanisms within individual isolates. Notably, a subpopulation of isolates maintained relative susceptibility to tigecycline and colistin, consistent with international reports that recognize these agents as some of the last remaining therapeutic options against extensively drug-resistant (XDR) Enterobacteriaceae.

However, the emerging resistance to both agents in this cohort underscores the precariousness of even these last-resort treatments (Magiorakos et al., 2012).

The concentration-dependent antimicrobial activity of $CuSO_4$ observed in this study is marked by 90% inhibition of isolates at 1600 µg/mL and 78% inhibition at 800 µg/mL. These results are consistent with previously published data regarding the bactericidal activity of copper ions against Gram-negative bacteria. The multitarget mechanism of action of copper ions is particularly significant in this context. Unlike conventional antibiotics, which typically act on a single molecular target, copper ions exert simultaneous bactericidal effects. This occurs through the disruption of the bacterial cell membrane, inhibition of critical enzymatic pathways, generation of reactive oxygen species (ROS), and degradation of nucleic acids. This multimodal mechanism makes it considerably more challenging for bacteria to develop effective resistance compared to single-target antimicrobials (Lemire et al., 2013; Grass et al., 2011; Lemire & Turner, 2017).

Importantly, the mechanisms underlying β-lactam resistance—such as the production of carbapenemases like NDM-1 and OXA-48 are entirely different from those that confer resistance to copper ions, as these enzymes do not act on inorganic metal ions. This difference is responsible for the sustained susceptibility to copper observed in carbapenem-resistant isolates in this study, positioning $CuSO_4$ as a rational alternative or supplementary agent against carbapenemase-producing Enterobacteriaceae (CPE) (Heidari Soureshjani et al., 2020; van Duin & Doi, 2017). The minimum bactericidal concentration (MBC) data further support the bactericidal nature of $CuSO_4$, as five isolates (10%) showed MBC values at 1600 µg/mL, which aligns with previously reported bactericidal thresholds for copper compounds against clinical Gram-negative pathogens (Vincent et al., 2016; Macomber & Imlay, 2009).Genomic DNA was extracted successfully from 80% of the isolates, while plasmid DNA was extracted from 60%. This provides a molecular foundation for the future characterization of resistance determinants. The detection of plasmid DNA in most multidrug-resistant (MDR) isolates aligns with the established role of plasmid-mediated horizontal gene transfer in the dissemination of clinically important resistance genes such as blaNDM-1, blaOXA-48, and blaCTX-M—among clinical Enterobacteriaceae (Jamal et al., 2016; Khan et al., 2017; Wang et al., 2020). The absence of detectable plasmid bands in the remaining 20 isolates could be due to chromosomal integration of resistance genes or the presence of large or low-copy-number plasmids that are difficult to recover using alkaline lysis methods, or it might be attributed to technical variability inherent in conventional plasmid extraction protocols (Bondarczuk & Piotrowska-Seget, 2013). Definitive molecular characterization of resistance gene presence in these isolates will require PCR-based detection or whole-genome sequencing in future studies. Several limitations of this study should be acknowledged. The in vitro experimental design, while suitable for an initial assessment of antimicrobial activity, does not fully replicate the complex biological environment of the human urinary tract. Factors such as urine composition, pH variability, host immune interactions, and biofilm formation must be considered. Before any clinical application, rigorous evaluation of the cytotoxicity of $CuSO_4$ at bactericidal concentrations, along with its pharmacokinetic and bioavailability profile within the urinary tract and the potential for systemic copper accumulation, is essential. This will require cell-based cytotoxicity assays and in vivo animal model studies. Furthermore, genotypic confirmation of carbapenemase gene presence through multiplex PCR targeting blaNDM-1, blaOXA-48, and blaKPC, or by whole-genome sequencing, would significantly enhance the molecular characterization of resistance mechanisms in these isolates and provide a more comprehensive understanding of resistance gene epidemiology within this clinical cohort.

Conclusion

This study presents strong in vitro evidence of the antimicrobial efficacy of copper sulfate ($CuSO_4$) against multidrug- and carbapenem-resistant uropathogenic Escherichia coli. $CuSO_4$ showed consistent inhibitory activity, with minimum inhibitory concentration (MIC) values of 1600 µg/mL observed in 90% of the tested isolates, and bactericidal activity was confirmed at higher concentrations. Notably, the action of copper ions is independent of β-lactamase-mediated resistance pathways, including carbapenemase production, making $CuSO_4$ a viable candidate as an alternative or complementary antimicrobial agent for treating carbapenem-resistant Enterobacteriaceae (CPE), especially given the critical limitations of conventional therapeutic options. Additionally, the detection of plasmid DNA in 60% of multidrug-resistant isolates suggests that plasmid-mediated horizontal gene transfer significantly contributes to the multidrug-resistant phenotype in these clinical strains. Future research that includes in vivo efficacy studies, evaluations of cytotoxicity at therapeutic concentrations, and comprehensive molecular characterization of resistance gene carriage is necessary to enhance the clinical application of copper-based antimicrobial strategies in managing multidrug-resistant and carbapenem-resistant urinary tract infections.

References

Aktas, Z., Sahbudak Bal, Z., et al. (2016). The carbapenem-resistant Enterobacteriaceae threat is growing: NDM-1 epidemic at a training hospital in Turkey. Infection and Drug Resistance, 9, 1–10.

Al Sheikh, Y. A., Marie, M. A. M., John, J., Krishnappa, L. G., & Dabwab, K. H. M. (2023). Prevalence of carbapenem-resistant Enterobacteriaceae in western Saudi Arabia and increasing trends in antimicrobial resistance. Saudi Medical Journal, 44(3), 254–262.

Asadi Karam, M. R., Habibi, M., & Bouzari, S. (2019). Urinary tract infection: Pathogenicity, antibiotic resistance and development of effective vaccines against uropathogenic Escherichia coli. Molecular Immunology, 108, 56–67.

Bondarczuk, K., & Piotrowska-Seget, Z. (2013). Molecular basis of active copper resistance mechanisms in Gram-negative bacteria. Cell Biology and Toxicology, 29(6), 397–405.

Bunduki, G. K., et al. (2024). High rates of antimicrobial resistance and emergence of high-risk clones in community-acquired uropathogenic Escherichia coli. BMC Infectious Diseases, 24, 1–12.

Clinical and Laboratory Standards Institute. (2023). Performance standards for antimicrobial susceptibility testing (33rd ed., CLSI supplement M100). CLSI.

Flores-Mireles, A. L., Walker, J. N., Caparon, M., & Hultgren, S. J. (2015). Urinary tract infections: Epidemiology, mechanisms of infection and treatment options. Nature Reviews Microbiology, 13(5), 269–284.

Grass, G., Rensing, C., & Solioz, M. (2011). Metallic copper as an antimicrobial surface. Applied and Environmental Microbiology, 77(5), 1541–1547.

Hannan, T. J., Totsika, M., Mansfield, K. J., Moore, K. H., Schembri, M. A., & Hultgren, S. J. (2012). Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic Escherichia coli bladder infection. FEMS Microbiology Reviews, 36(3), 616–648.

Heidari Soureshjani, E., et al. (2020). Evaluation of phenotypic and genotypic characteristics of carbapenemase-producing Enterobacteriaceae and its prevalence in a referral hospital in Tehran City. Iranian Journal of Microbiology, 12(6), 537–545.

Jamal, W. Y., Albert, M. J., & Rotimi, V. O. (2016). High prevalence of New Delhi Metallo-β-lactamase-1 (NDM-1) producers among carbapenem-resistant Enterobacteriaceae in Kuwait. PLoS ONE, 11(3), e0152638.

Khan, A. U., Maryam, L., & Zarrilli, R. (2017). Structure, genetics and worldwide spread of New Delhi Metallo-β-lactamase (NDM): A threat to public health. BMC Microbiology, 17, 101.

Lemire, J. A., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: Mechanisms, molecular targets and applications. Nature Reviews Microbiology, 11(6), 371–384.

Lemire, J., & Turner, R. J. (2017). Mechanistic insights into the biocidal action of metals in microorganisms. In S. Das & H. R. Dash (Eds.), Handbook of metal-microbe interactions and bioremediation. CRC Press.

Logan, L. K., & Weinstein, R. A. (2017). The epidemiology of carbapenem-resistant Enterobacteriaceae: The impact and evolution of a global menace. Journal of Infectious Diseases, 215(Suppl. 1), S28–S36.

Macomber, L., & Imlay, J. A. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proceedings of the National Academy of Sciences, 106(20), 8344–8349.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: A clinical update. Clinical Microbiology Reviews, 18(4), 657–686.

Quaranta, D., Krans, T., Espirito Santo, C., Elowsky, C. G., Domaille, D. W., Chang, C. J., & Grass, G. (2011). Mechanisms of contact-mediated killing of yeast cells on dry metallic copper surfaces. Applied and Environmental Microbiology, 77(2), 416–426.

Subashchandrabose, S., & Mobley, H. L. T. (2015). Virulence and fitness determinants of uropathogenic Escherichia coli. Microbiology Spectrum, 3(4).

Terlizzi, M. E., Gribaudo, G., & Maffei, M. E. (2017). Uropathogenic Escherichia coli (UPEC) infections: Virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. Frontiers in Microbiology, 8, 1566.

van Duin, D., & Doi, Y. (2017). The global epidemiology of carbapenemase-producing Enterobacteriaceae. Virulence, 8(4), 460–469.

Vincent, M., Hartemann, P., & Engels-Deutsch, M. (2016). Antimicrobial applications of copper. International Journal of Hygiene and Environmental Health, 219(7), 585–591.

Wang, Q., Zhang, Y., Yao, X., et al. (2020). Dissemination of carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) among carbapenem-resistant Enterobacteriaceae isolated from adult and children's patients in China. Frontiers in Cellular and Infection Microbiology, 10, 314.

Yang, X., Chen, H., Zheng, Y., Qu, S., Wang, H., & Yi, F. (2022). Disease burden and long-term trends of urinary tract infections: A worldwide report. Frontiers in Public Health, 10, 888205.

Zawawi, H. M., Harris, P. N. A., Roberts, M. J., et al. (2015). The emerging threat of multidrug-resistant Gram-negative bacteria in urology. Nature Reviews Urology, 12(10), 570–584.