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MOLECULAR EPIDEMIOLOGY OF CARBAPENEMASE AND EXTENDED-SPECTRUM BETA-LACTAMASE GENES IN MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* AND *KLEBSIELLA PNEUMONIAE* ISOLATED FROM NOSOCOMIAL WOUND INFECTIONS

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Abstract

The global spread of carbapenemase and extended-spectrum beta-lactamase (ESBL) genes in Pseudomonas aeruginosa and Klebsiella pneumoniae poses a significant threat to healthcare systems. This study aimed to characterize the molecular epidemiology of these resistance determinants in wound infection isolates. Over six months (March-August 2023), 112 non-duplicate Gram-negative isolates (65 P. aeruginosa and 47 K. pneumoniae) were collected from wound infections at a tertiary care hospital. Antimicrobial susceptibility was determined by disk diffusion and broth microdilution. Carbapenemase production was confirmed by the modified carbapenem inactivation method (mCIM). Genotypic characterization of resistance genes (blaNDM-1, blaKPC-2, blaOXA-48, blaVIM, blaIMP, blaTEM, blaSHV, blaOXA-1) was performed via multiplex PCR. Carbapenem resistance was found in 69.6% of isolates, with K. pneumoniae exhibiting higher resistance (76.6%) than P. aeruginosa (64.6%). The blaNDM-1 gene was predominant (47.4%), particularly in K. pneumoniae (58.3%). ESBL genes, especially blaTEM (71.8%) and blaSHV (44.9%), were also prevalent. Notably, 52.6% of carbapenem-resistant isolates co-harbored multiple resistance genes, with blaNDM-1 + blaTEM being the most common combination (25.6%). The study highlights the alarming prevalence of carbapenemase and ESBL genes in nosocomial wound isolates, with blaNDM-1 emerging as the dominant resistance mechanism. The high rate of genetic co-carriage underlines the emergence of pan-drug-resistant phenotypes, emphasizing the urgent need for molecular surveillance and enhanced infection control in hospital settings.

Keywords: *Antimicrobial resistance, Carbapenemase, ESBL, MDR, Molecular epidemiology, NDM-1, OXA-48, Wound infections*

INTRODUCTION

The relentless escalation of antimicrobial resistance (AMR) constitutes one of the most pressing global health challenges of the 21st century (1,2). Among Gram-negative bacteria, the rapid proliferation of carbapenem-resistant Enterobacteriaceae (CRE) and *Pseudomonas aeruginosa* has created a therapeutic crisis, particularly in healthcare-associated infections (3,4). Carbapenems, once considered the last line of defense against multidrug-resistant (MDR) Gram-negative infections, are increasingly being compromised by the emergence and global spread of carbapenemases (5). Increasing reports of multidrug-resistant Gram-negative pathogens across clinical, community, and animal environments further highlight the accelerating spread of AMR and the urgency for strengthened surveillance systems (6, 7).

Carbapenemases belong to three main molecular classes based on their Ambler classification: Class A (e.g., KPC), Class B (metallo-β-lactamases, MBLs, e.g., NDM, VIM, IMP), and Class D (OXA-48-like) (8). The New Delhi metallo-β-lactamase (NDM-1), first identified in 2008, has demonstrated remarkable transmissibility across bacterial species and continents, primarily through plasmid-mediated horizontal gene transfer (9). Similarly, OXA-48-like carbapenemases have become endemic in many regions, often exhibiting stealthy dissemination due to their weaker hydrolytic activity against carbapenems, which can lead to underestimation in routine phenotypic tests (10).

Concurrently, the persistence and evolution of extended-spectrum beta-lactamases (ESBLs), particularly TEM-, SHV-, and OXA-type enzymes, continue to compromise the efficacy of broad-spectrum cephalosporins. The convergence of ESBL production with carbapenemase activity creates pan-drug resistant (PDR) phenotypes, leaving clinicians with few, if any, effective therapeutic options (11, 12).
P. aeruginosa and *K. pneumoniae* are of particular concern in the context of wound and burn infections, where they frequently cause persistent, difficult-to-eradicate infections associated with significant morbidity and mortality (13, 14). The molecular characterization of resistance mechanisms in these pathogens is crucial for informing infection control practices, guiding empirical therapy, and understanding local epidemiology.

This study aims to address this gap by investigating the prevalence and distribution of carbapenemase and ESBL genes in *P. aeruginosa* and *K. pneumoniae* isolates from nosocomial wound infections. The study will characterize the molecular epidemiology of key resistance genes (*blaNDM-1*, *blaKPC-2*, *blaOXA-48*, *blaVIM*, *blaIMP*, *blaTEM*, *blaSHV*, *blaOXA-1*) and explore their genetic co-carriage, assessing the correlation with multidrug resistance profiles to inform better infection control and treatment strategies.

METHODOLOGY

STUDY DESIGN, SETTING, AND BACTERIAL ISOLATES

A prospective, cross-sectional study was conducted from March to August 2023 at a 1200-bed tertiary care teaching hospital, Hayatabad Medical Complex (HMC), Peshawar, Pakistan. A total of 112 non-duplicate, consecutive Gram-negative isolates (65 *P. aeruginosa* and 47 *K. pneumoniae*) were collected from purulent wound exudates, surgical site infections, and burn wound specimens from hospitalized patients. Only one isolate per patient was included to ensure clonal independence.

BACTERIAL IDENTIFICATION AND STORAGE

Initial isolation and identification were performed using standard microbiological techniques. Colonies were subcultured on MacConkey agar (HiMedia, India) and cetrimide agar (Sigma-Aldrich, USA) for selective isolation. Final identification was confirmed using the VITEK 2 Compact system (bioMérieux, France) with GN identification cards. All confirmed isolates were preserved in cryoprotective beads containing 15% glycerol at -80°C for subsequent analysis.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing was performed using both disk diffusion and broth microdilution methods, following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI 2023) (15). The following antibiotics were tested: piperacillin-tazobactam (100/10 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and tigecycline (15 µg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Multidrug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial categories, as per internationally accepted criteria (15).

PHENOTYPIC DETECTION OF CARBAPENEMASE PRODUCTION

All isolates exhibiting reduced susceptibility to any carbapenem (imipenem or meropenem) by disk diffusion were subjected to phenotypic confirmation using the modified carbapenem inactivation method

(mCIM) and EDTA-modified carbapenem inactivation method (eCIM) for differentiation of metallo-β-lactamases (16).

DNA EXTRACTION AND PCR AMPLIFICATION

Genomic DNA was extracted from fresh overnight cultures grown in Luria-Bertani broth using the QIAamp DNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions for bacterial DNA purification. The concentration and purity of extracted DNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Multiplex PCR assays were performed to detect carbapenemase genes (blaNDM-1, blaKPC-2, blaOXA-48, blaVIM, blaIMP) and ESBL genes (blaTEM, blaSHV, blaOXA-1) using previously published primers with modifications (17). The PCR reaction mixture (25 µL) contained 12.5 µL of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each primer (10 µM), 3 µL of DNA template, and nuclease-free water to volume. Amplification was carried out in a Bio-Rad T100 Thermal Cycler with the following conditions: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 50 seconds; followed by a final extension at 72°C for 7 minutes. Amplified products were separated by electrophoresis on a 1.8% agarose gel stained with ethidium bromide and visualized under UV light.

ETHICAL CONSIDERATIONS

The study protocol was reviewed and approved by the Institutional Review Board and Ethics Committee of Khyber Medical University (Ref: Dir/KMU-EB/IV/000885). The requirement for individual patient consent was waived due to the anonymized nature of the microbiological data, in accordance with national regulations and the Declaration of Helsinki.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS Statistics version 28.0 (IBM Corp., USA). Categorical variables were presented as frequencies and percentages. Chi-square test or Fisher's exact test was used to compare proportions between groups. A p-value of < 0.05 was considered statistically significant for all analyses.

RESULTS

PREVALENCE AND DEMOGRAPHIC CHARACTERISTICS

During the six-month study period, 112 Gram-negative isolates were collected, with *P. aeruginosa* (58.0%) being more prevalent than *K. pneumoniae* (42.0%). The overall prevalence of carbapenem resistance (CR) was 69.6% (78/112), with a significantly higher proportion observed in *K. pneumoniae* (76.6%, 36/47) compared to *P. aeruginosa* (64.6%, 42/65) ($p = 0.048$).

Demographic analysis of patients with carbapenem-resistant infections revealed a male predominance (61.5%, 48/78). The majority of CR isolates were recovered from patients in the burn unit (57.7%, 45/78), followed by surgical wards (28.2%, 22/78) and diabetic foot clinics (14.1%, 11/78). The highest frequency of CR infections was observed in the 21-40 age group (41.0%, 32/78).

ANTIMICROBIAL SUSCEPTIBILITY PROFILES

The antibiotic resistance profiles of the 78 carbapenem-resistant isolates revealed alarming levels of multidrug resistance (Table I). All CR *K. pneumoniae* isolates were resistant to ceftriaxone, cefepime, and imipenem. The CR isolates exhibited high-level resistance across all major antibiotic classes, with 93.6% (73/78) meeting the criteria for MDR. Tigecycline remained the most active agent against *K. pneumoniae* (77.8% susceptibility), while colistin demonstrated good activity against both species (92.3% overall susceptibility).

Table I. Detailed antibiotic resistance profile of carbapenem-resistant isolates

Antibiotic Class	Antibiotic	*P. aeruginosa* (n=42) R (%)	*K. pneumoniae* (n=36) R (%)	Total (n=78) R (%)
Penicillins	TZP (piperacillin-tazobactam)	40 (95.2)	34 (94.4)	74 (94.9)
Cephalosporins	CRO (ceftriaxone)	42 (100)	36 (100)	78 (100)
	FEP (cefepime)	40 (95.2)	36 (100)	76 (97.4)
Carbapenems	IPM (imipenem)	42 (100)	36 (100)	78 (100)
	MEM (meropenem)	41 (97.6)	36 (100)	77 (98.7)
Aminoglycosides	AK (amikacin)	35 (83.3)	28 (77.8)	63 (80.8)
	GEN (gentamicin)	38 (90.5)	32 (88.9)	70 (89.7)
Fluoroquinolones	CIP (ciprofloxacin)	39 (92.9)	31 (86.1)	70 (89.7)
Glycylcyclines	TGC (tigecycline)	-	8 (22.2)	8 (22.2)*

*Tigecycline resistance reported only for *K. pneumoniae*; TZP: piperacillin-tazobactam; CRO: ceftriaxone; FEP: cefepime; IPM: imipenem; MEM: meropenem; AK: amikacin; GEN: gentamicin; CIP: ciprofloxacin; TGC: tigecycline

DISTRIBUTION OF CARBAPENEMASE GENES

Molecular characterization revealed a diverse array of carbapenemase genes among the 78 CR isolates (Table II). The blaNDM-1 gene was the predominant carbapenemase, detected in 47.4% (37/78) of isolates, with a significantly higher prevalence in *K. pneumoniae* (58.3%) compared to *P. aeruginosa* (38.1%) ($p = 0.032$). The blaOXA-48 gene was the second most common carbapenemase (28.2%, 22/78). Metallo-β-lactamase genes blaVIM and blaIMP were detected exclusively in *P. aeruginosa* isolates (23.8% and 9.5%, respectively). The blaKPC-2 gene was found only in *K. pneumoniae* isolates (16.7%). Molecular detection of antibiotic-resistant genes is shown in Figure 1.

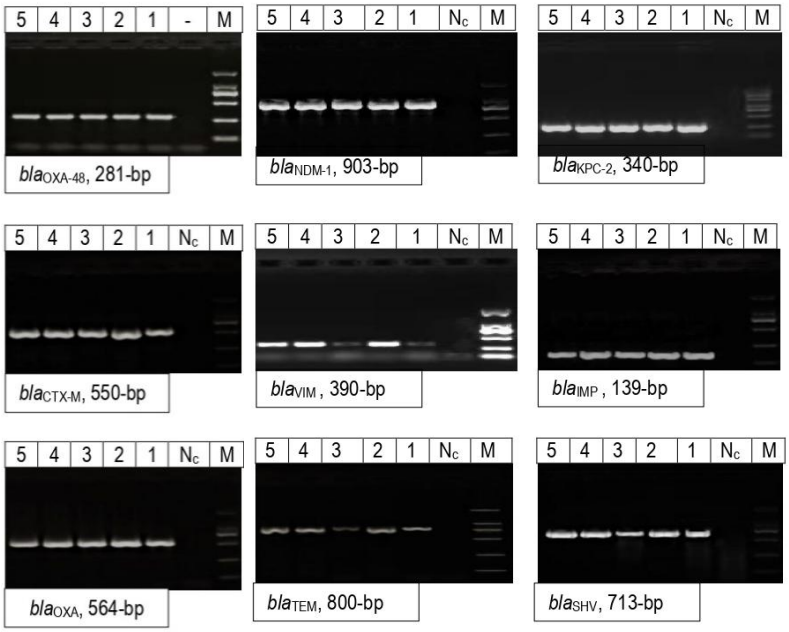

Fig. 1. Molecular detection of resistance genes in *K. pneumoniae* and *P. aeruginosa*

Table II. Distribution of carbapenemase genes among carbapenem-resistant isolates

Gene	*P. aeruginosa* (n=42)	*K. pneumoniae* (n=36)	Total (n=78)	p-value
blaNDM-1	16 (38.1%)	21 (58.3%)	37 (47.4%)	0.032
blaKPC-2	0 (0%)	6 (16.7%)	6 (7.7%)	0.008
blaOXA-48	10 (23.8%)	12 (33.3%)	22 (28.2%)	0.221
blaVIM	10 (23.8%)	0 (0%)	10 (12.8%)	0.001
blaIMP	4 (9.5%)	0 (0%)	4 (5.1%)	0.124
Total (%)	71.4%	88.9%	78.2%	

PREVALENCE OF ESBL GENES AND GENETIC CO-CARRIAGE

ESBL genes were highly prevalent among the CR isolates (Table III). The blaTEM gene was the most common (71.8%, 56/78), followed by blaSHV (44.9%, 35/78) and blaOXA-1 (35.9%, 28/78). A striking finding was the high frequency of genetic co-carriage: 52.6% (41/78) of CR isolates harbored at least one

carbapenemase and one ESBL gene simultaneously. The most common combination was blaNDM-1 + blaTEM, detected in 25.6% (20/78) of isolates. Additionally, 16.7% (13/78) of isolates carried multiple carbapenemase genes, with blaNDM-1 + blaOXA-48 being the predominant combination (9.0%, 7/78).

Table III. Distribution of ESBL Genes and Co-carriage Patterns

Genetic Profile	*P. aeruginosa* (n=42)	*K. pneumoniae* (n=36)	Total (n=78)
ESBL Genes			
blaTEM	28 (66.7%)	28 (77.8%)	56 (71.8%)
blaSHV	15 (35.7%)	20 (55.6%)	35 (44.9%)
blaOXA-1	13 (31.0%)	15 (41.7%)	28 (35.9%)
Co-carriage Patterns			
Carbapenemase + ESBL	19 (45.2%)	22 (61.1%)	41 (52.6%)
Multiple Carbapenemases	5 (11.9%)	8 (22.2%)	13 (16.7%)
Most Common Combinations			
blaNDM-1 + blaTEM	7 (16.7%)	13 (36.1%)	20 (25.6%)
blaOXA-48 + blaTEM	5 (11.9%)	6 (16.7%)	11 (14.1%)
blaNDM-1 + blaOXA-48	2 (4.8%)	5 (13.9%)	7 (9.0%)
Total (%)	-	-	52.6%

DISCUSSION

The global rise in antimicrobial resistance (AMR) is driven by the increasing prevalence of multidrug-resistant pathogens, particularly those producing carbapenemases and extended-spectrum beta-lactamases (ESBLs). Carbapenem-resistant organisms, especially *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, are emerging as critical threats to public health, as they are associated with high morbidity and mortality rates in healthcare settings (18-21).

This comprehensive molecular epidemiological study provides disturbing insights into the resistance landscape of Gram-negative pathogens isolated from nosocomial wound infections in our healthcare setting. The high overall prevalence of carbapenem resistance (69.6%) significantly exceeds rates reported in recent surveillance studies from other regions, suggesting a localized epidemic that demands immediate intervention (22, 23).

The predominance of the blaNDM-1 gene among our carbapenem-resistant isolates, particularly in *K. pneumoniae* (58.3%), aligns with its established reputation as a rapidly disseminating global resistance threat (24, 25). The genetic flexibility of blaNDM-1, often located on broad-host-range plasmids with efficient conjugation systems, facilitates its interspecies transmission and explains its dominance in our clinical isolates (26). The significantly higher prevalence of blaNDM-1 in *K. pneumoniae* compared to *P. aeruginosa* may reflect species-specific differences in plasmid compatibility and fitness costs associated with gene carriage.

The substantial prevalence of blaOXA-48 (28.2%) is equally concerning. OXA-48-like carbapenemases are particularly problematic due to their often subtle phenotypic expression, which can lead to underestimation in routine laboratory testing and subsequent failure in implementing appropriate infection control measures (27). The concurrent detection of blaVIM and blaIMP exclusively in *P. aeruginosa* reflects the species-specific distribution patterns of certain metallo-β-lactamases, possibly related to their chromosomal integration or association with specific mobile genetic elements in this pathogen (28).

The high frequency of ESBL genes, particularly blaTEM (71.8%) and blaSHV (44.9%), creates a genetic background where the addition of a carbapenemase gene results in pan-drug resistant phenotypes. This genetic convergence effectively dismantles the entire beta-lactam armamentarium, leaving only a few last-resort options like tigecycline and colistin, both of which have significant limitations in terms of efficacy, toxicity, and pharmacokinetics (29).

Perhaps the most alarming finding of our study is the high rate of genetic co-carriage, with 52.6% of isolates harboring both carbapenemase and ESBL genes. This genetic "perfect storm" creates bacterial clones resistant to virtually all beta-lactam antibiotics and significantly increases the risk of treatment failure (30). The blaNDM-1 + blaTEM combination, detected in 25.6% of isolates, represents a particularly successful genetic partnership that warrants close monitoring.

The overrepresentation of carbapenem-resistant isolates from burn units (57.7%) highlights this patient population as a critical reservoir for these superbugs. The compromised skin barrier, frequent antibiotic exposure, and prolonged hospitalization characteristic of burn patients create an ideal environment for the selection and persistence of MDR pathogens (31).

In terms of infection control implications, the findings from this study highlight the urgent need for stringent infection prevention measures in hospital settings, particularly in high-risk units such as burn wards. Active surveillance and rapid molecular diagnostics for carbapenemase and ESBL genes should be incorporated into routine clinical practice to enable early detection and containment of resistant pathogens. Furthermore, molecular surveillance programs must be expanded to monitor the emergence of new resistance mechanisms, such as novel carbapenemases or ESBL variants, to inform treatment strategies and minimize the spread of resistant strains.

CONCLUSION

This detailed molecular epidemiological investigation reveals a dire situation regarding carbapenem and extended-spectrum beta-lactam resistance in our healthcare setting. The high prevalence and diversity of transferable resistance genes, particularly the dominance of *blaNDM-1* and the alarming frequency of genetic co-carriage, create a therapeutic landscape of unprecedented challenge. The findings highlight the critical need for molecular surveillance and stringent infection control measures to combat the rise of multidrug-resistant pathogens, particularly in high-risk clinical settings like burn wards.

Limitations:

This study has several limitations. Samples were collected from a single location, limiting the geographic representation of the findings and potentially affecting the generalizability of the results to other regions or healthcare settings. The absence of molecular typing methods (such as PFGE or MLST) prevents us from determining the clonal relatedness of isolates and understanding the contribution of clonal spread versus horizontal gene transfer to the resistance epidemiology. Additionally, our PCR-based approach targeted only the most common resistance genes, potentially missing novel or rare variants.

Author contributions:

RUK & AA. Conceived the study and designed the research plan; RUK Performed data analysis and drafted the manuscript; MU, AG, AB, TG, RN, NF, RUK & FK Contributed to sample collection, laboratory work, and data curation. AT Provided clinical coordination and assisted with manuscript revision; HU Contributed to methodology, data interpretation, and manuscript editing. AA Provided overall supervision and critically reviewed the final manuscript. All authors contributed to the study and approved the final manuscript.

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