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Original article

Mutational Analysis of Carbapenem Resistance Genes in *Citrobacter Freundii* Isolated from Urinary Tract Infections (UTIs) Patients, Peshawar

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Abstract: In the current era, pathogens become more virulent due to the development of resistance against the major families of antibiotics. The most common infection which is both community-acquired and hospital-acquired is the Urinary tract infection (UTI). The current study is intended to determine the mutational analysis of carbapenem resistance in the isolates of *Citrobacter freundii* isolated from UTI patients. A total of 145 urine samples were collected from the UTI suspected patients at Khyber Teaching Hospital, (Peshawar, Pakistan). Out of 145 samples, 40 samples were detected as positive isolates of *C. freundii*. The phenotypic identification of isolates was done via API-10S kit. The carbapenem antibiotic resistance genes were detected via Polymerase Chain Reaction (PCR). The antibiotic susceptibility testing was done via Kirby Bauer method and the results revealed that the isolates were highly resistant towards cefepime (97.5%), amoxicillin (95%), and meropenem (92.5%). The molecular analysis revealed that the *VIM* gene was detected in 3 isolates, the *NDM-1* gene in 6 isolates, and the *OXA-48* gene was detected in 4 isolates of *C. freundii*. The result of Next generation sequencing revealed that no mutation was detected in the sequences of these carbapenem resistance genes. The output of the current study concluded that carbapenem resistance is emerging rapidly mainly in the uropathogens and the antibiotic resistance genes enhance the ability of uropathogens to combat against antibiotics.

Keywords: Citrobacter freundii, UTIs, *VIM* gene, *NDM*-1 gene, *OXA*-48 gene, phenotypic identification, API-10S kit, Kirby Bauer method.

Introduction

Urinary tract infections (UTIs) are among the most prevalent infections in clinical settings [1]. Globally, UTIs affect approximately 150 million individuals annually [2]. Members of the Gram-negative bacterial family significantly contribute to the incidence of UTIs, with *Citrobacter freundii*, *Escherichia coli*, and *Klebsiella pneumoniae* being the primary pathogens [3]. According to the World Health Organization, over the past two decades, uropathogens have demonstrated increased virulence due to the development of resistance against commonly available antibiotics, posing a substantial threat to global

public health [4]. *Citrobacter freundii* is a facultative anaerobic, non-capsulated, motile, Gram-negative rod belonging to the family *Enterobacteriaceae* and the genus *Citrobacter* [5]. This bacterium is commonly found in soil, sewage, water, and the intestinal tracts of animals and humans. Under immunocompromised conditions, *C. freundii* acts as an opportunistic pathogen, leading to various infections in humans [6]. The clinical manifestations of *C. freundii* include UTIs [7], respiratory tract infections [8], neonatal meningitis [9], gastrointestinal infections [10], and sepsis [11]. Among the causative agents of UTIs, *C. freundii* ranks as the third most prevalent uropathogen after *E. coli* and *Klebsiella* species, accounting for 5–12% of UTI cases. The emergence of multidrug-resistant strains of *C. freundii* worldwide has further exacerbated the prevalence and severity of UTIs associated with this pathogen [12]. The emergence of multidrug-resistant (MDR) strains of Citrobacter freundii is attributed to multiple mechanisms that confer resistance to available antibiotics [13]. Major classes of antibiotics targeted by these mechanisms include inhibitors of protein synthesis [14], nucleic acid synthesis [15], peptidoglycan synthesis [16], cell membrane synthesis [17], and folic acid synthesis [18]. The bacterial genome exhibits remarkable flexibility, enabling it to adapt to environmental changes and modify cellular processes to withstand stress, including antibiotic pressure [19]. The primary mechanisms by which bacteria develop resistance include the production of β-lactamases, alteration of antibiotic target sites, and reduced cell membrane permeability [20]. Among these, the production of β-lactamases is regarded as the most significant and widespread mechanism within the *Enterobacteriaceae* family. β-lactamases are enzymes that hydrolyze β-lactam antibiotics, rendering them ineffective. These enzymes are categorized into two main groups based on their structural characteristics: serine β-lactamases and metallo-β-lactamases [21]. Serine β-lactamases, characterized by the presence of a serine residue at their active site, are further subdivided into three subclasses: Class A β-lactamases, which include *Temoniera Escherichia coli* mutant (*bla-TEM*), sulfhydryl variable (*bla-SHV*), cefotaxime-Munich β-lactamase (*bla-CTX-M*), and *Klebsiella pneumoniae* Carbapenemase (*bla-KPC*) [22]. This adaptability and diversity in resistance mechanisms highlight the urgent need for novel therapeutic strategies and a deeper understanding of bacterial genomic plasticity to combat MDR pathogens effectively. Class C β-lactamases, such as ampicillin resistance genes (AmpC), and Class D β-lactamases, including the oxacillinase gene (bla-*OXA-48*-like), are other significant contributors to antibiotic resistance in C. freundii [23, 24]. Metallo-β-lactamases (MBLs), characterized by their dependence on zinc ions for catalytic activity, are classified into Class B and include genes such as imipenem's (*bla-IMP*), New Delhi metallo-β-lactamase (*bla-NDM*-1), and Verona integrons-mediated metallo-β-lactamase (*bla-VIM*) [25]. In recent years, *C. freundii* has emerged as a prevalent uropathogen, displaying advanced resistance mechanisms against a broad spectrum of antimicrobial agents. Determining antibiotic susceptibility patterns is critical for guiding effective and targeted treatment and for managing infections caused by *C. freundii*. Regular monitoring of Carbapenemase expression in *C. freundii* isolates is essential, as it significantly contributes to antimicrobial resistance. This study investigates the prevalence of *C. freundii* and analyzes its antibiotic susceptibility patterns against major classes of antibiotics. Emphasis is placed on detecting β-lactamase production and evaluating its impact on the rates of morbidity and mortality. These findings aim to enhance understanding of resistance mechanisms and inform strategies for mitigating the clinical burden of *C. freundii*-associated infections.

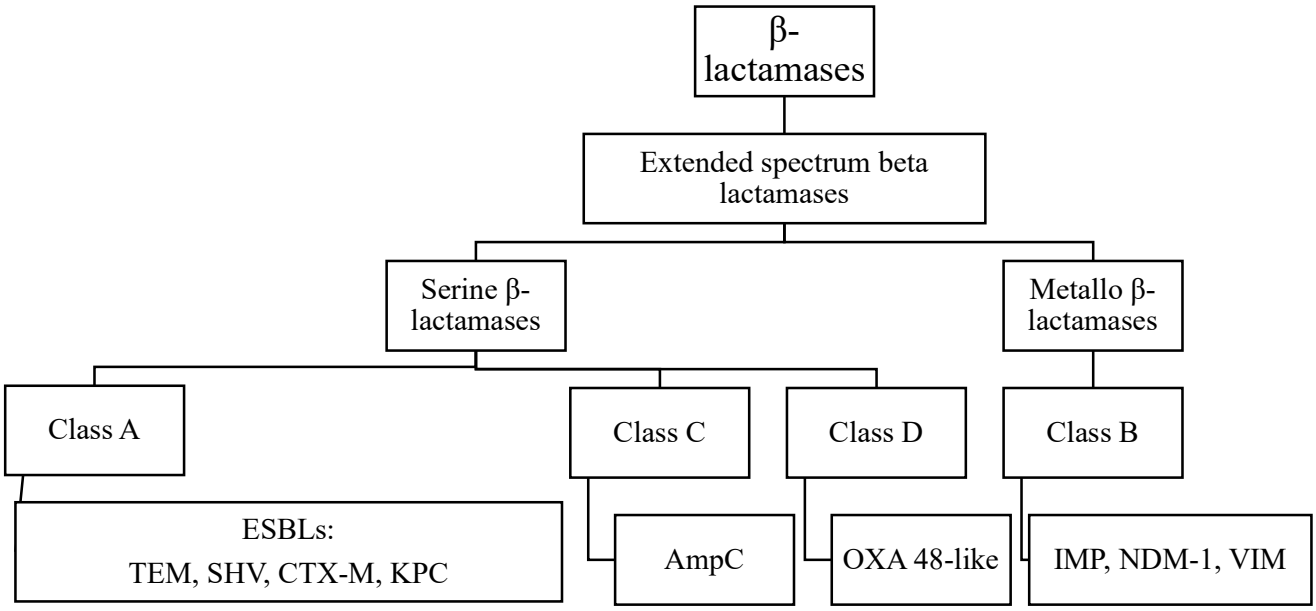

Figure 1: Classification of β-lactamases

Material And Methods

This study was conducted at the Molecular and Microbiology Laboratory, Centre of Biotechnology and Microbiology, University of Peshawar (UOP), Peshawar, Pakistan. Urine samples were collected from patients diagnosed with urinary tract infections (UTIs) at the Pathology and Microbiology Laboratory of Khyber Teaching Hospital (KTH), Peshawar. Selective media, including Cysteine Lysine Electrolytes Deficient (CLED) agar and MacConkey agar, were used for culturing the urine samples [26]. Following incubation, bacterial colonies were subjected to macroscopic analysis to examine their physical appearance, microscopic analysis using Gram staining, and biochemical characterization via the API 10S kit (Analytical Profile Index) [27]. Identified bacterial isolates underwent antibiotic susceptibility testing using the disc diffusion method. Mueller-Hinton agar was employed as the testing medium, with antibiotic discs placed on the agar surface. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured and interpreted as sensitive, intermediate, or resistant based on the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) 2020 [28]. For DNA extraction, 24-hour broth cultures of the bacterial isolates were used. Genomic DNA was extracted using the GJC® DNA Purification Kit, and the quality of the extracted DNA was assessed by electrophoresis on a 1.5% agarose gel. The gel was visualized using a Bio-Rad Molecular Imager® Gel Doc™ system to confirm the presence of DNA. The amplification of carbapenem resistance genes (VIM, NDM-1, and OXA-48) was performed using a Thermal Cycler PCR system. PCR conditions were optimized for each gene using specific primers listed in Table 1. The PCR reaction mixture (25 µL) consisted of 12.5 µL GoTaq® Green Master Mix, 1 µL forward primer, 1 µL reverse primer, 1 µL DNA sample, and 11.5 µL PCR-grade water. The amplified PCR products were resolved on a 1.5% agarose gel alongside a 100 bp DNA ladder and visualized under a Bio-Rad Molecular Imager® Gel Doc™ system [29]. The PCR products corresponding to the carbapenem resistance genes were subsequently subjected to sequencing. The sequencing process utilized the Next-

Generation Sequencing (NGS) approach for high-throughput analysis. Alignment of the carbapenem resistance gene sequences was performed using BioEdit (v7.2) software. The aligned sequences were compared with reference sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) [29]. Numerical data generated during the study were analyzed using Microsoft Excel 2013 (Version 15.0). Statistical analysis was also performed using Excel, while graphical representations of the results were created with OriginPro 8.5. Additionally, mutational analysis of the gene sequences was conducted using BioEdit (v7.2) and I-Mutant (v3.0).

Table 1: Primers of carbapenem resistance genes and PCR-optimized conditions

	Gene	Primer	Product size (bp)	Annealing temperature	No of cycles
1	*VIM*	F: GTTTGGTCGCATATCGCAAC R: AATGCGCACGACCAGGATAG	389	52℃ for 60 second	35
2	*NDM-1*	F: ATTGCCCAATATTATGCACCC R: GGAATGGCTCATCACGATCAT	724	54℃ for 30 second	35
3	*OXA-48*	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	438	56℃ for 30 second	35

RESULTS

A total of 145 urine samples were collected, of which 40 isolates were phenotypically identified as *Citrobacter freundii*. Among these, 27 isolates (67.5%) were obtained from male patients, while 13 isolates (32.5%) were from female patients. The prevalence of *C. freundii* was further analyzed across different age groups. The highest prevalence was observed in individuals aged 41–60 years (37.5%), followed by those aged 21–40 years (20%), 1–20 years (17.5%), and 61–80 years (17.5%). A lower prevalence was noted in infants aged <1 year (5%), and the lowest prevalence was found in individuals aged 81–90 years (2.5%) (Figure 2). Antibiotic susceptibility testing of the isolates was performed using the disc diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The antibiotics tested in this study included amoxicillin-clavulanate (AMC), meropenem (MEM), cefepime (FEP), ciprofloxacin (CIP), aztreonam (ATM), amikacin (AK), sulbactam-Cefoperazone (SCF), Fosfomycin (FOS), piperacillin-tazobactam (TZP), trimethoprim-sulfamethoxazole (SXT), and nitrofurantoin (F). The antibiogram results revealed that the majority of *C. freundii* isolates exhibited high levels of resistance to most antibiotics tested (Table 2).

Table 2: Antibiogram of *C. freundii*

S.no	Antibiotics	Symbols	Resistant	Sensitive
1	Amoxicillin	AMC	38 (95%)	2 (5%)
2	Meropenem	MEM	37 (92.5%)	3 (7.5%)
3	Cefepime	FEP	39 (97.5%)	1 (2.5%)
4	Ciprofloxacin	CIP	32 (80%)	6 (15%)
5	Aztreonam	ATM	36 (90%)	3 (7.5%)
6	Amikacin	AK	22 (55%)	18 (45%)
7	Cefoperazone-sulbactam	SCF	31 (77.5%0	9 (22.5%)
8	Fosfomycin	FOS	5 (12.5%)	34 (85%)
9	Piperacillin-tazobactam	TZP	34 (85%)	5 (12.5%)

10	Cotrimoxazole	SXT	37 (92.5%)	2 (5%)
11	Nitrofurantoin	F	31 (77.5%)	9 (22.5%)

The PCR analysis revealed that all Carbapenemase-positive C. freundii isolates exhibited high resistance to meropenem. Among the tested isolates, the NDM-1 carbapenem resistance gene was detected in 6 isolates (15%), the VIM gene in 3 isolates (7.5%), and the OXA-48 gene in 4 isolates (10%), as summarized in **Table 3**. Sequence alignment of the detected resistance genes (*NDM*-1, *VIM*, and *OXA*-48) showed no mutations, indicating that these genes maintained their original sequences responsible for conferring resistance to carbapenems.

Table3: Percentage of carbapenems resistance genes in carbapenem resistant isolates of C. freundii

S.no	Gene	Positive isolates	Percentage (%)
1	NDM-1	6	15
2	VIM	3	7.5
3	OXA-48	4	10

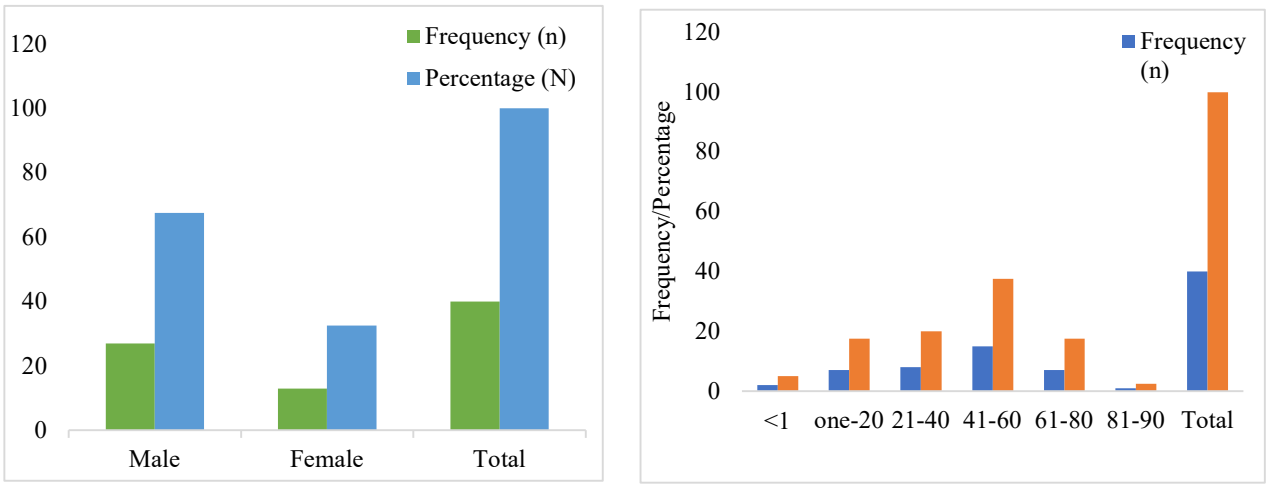

Figure 2: Distribution of *C. freundii* in different age groups and gender

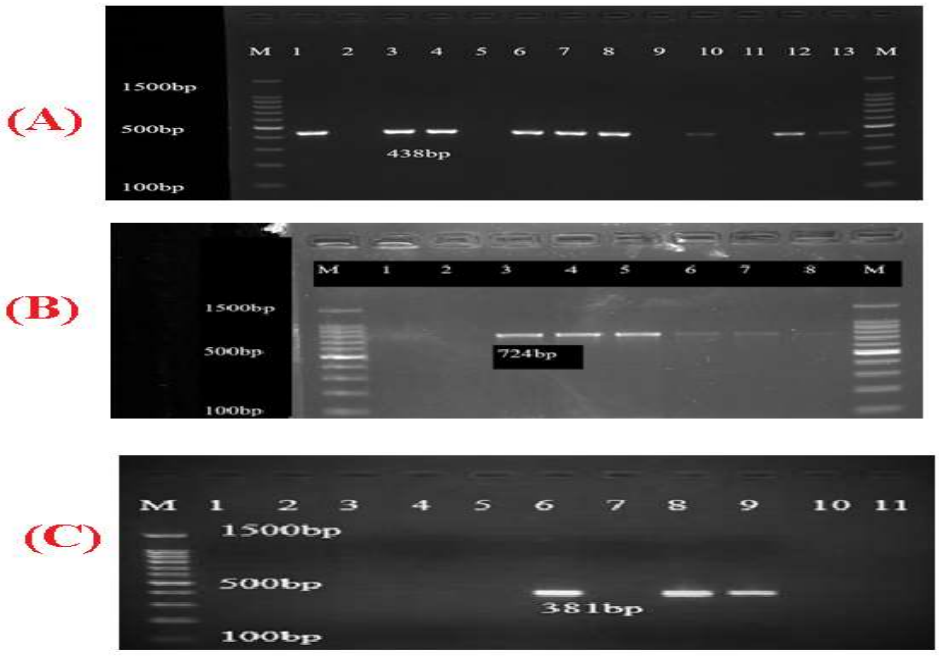

Figure 3: (A) Gel electrophoresis of *bla-NDM-1* gene: Lane M, molecular marker; Lane 1, negative control; Lane 2, negative *bla-NDM-1* isolates; Lanes 3-8, positive *bla-NDM-1* isolates. **(B)** Gel electrophoresis of *bla-VIM* gene: Lane M, molecular marker; Lanes 1-5, negative *bla-VIM* isolates; Lane 6, positive *bla-VIM* isolate; Lane 7, negative *bla-VIM* isolate; Lanes 8-9, positive *bla-VIM* isolates; Lanes 10-11, negative *bla-VIM* isolates. **(C)** Gel electrophoresis of *blaOXA-48* gene: Lane M, molecular marker; Lane 1, positive control; Lane 2, negative control; Lanes 3-4, positive *blaOXA-48* isolates; Lane 5, negative *blaOXA-48* isolate; Lanes 6-8, positive *bla-OXA-48* isolates; Lane 9, negative *bla-OXA-48* isolate; Lane 10, positive *bla-OXA-48* isolate; Lane 11, negative *bla-OXA-48* isolate; Lanes 12-13, positive *bla-OXA-48* isolates.

Discussion

Urinary tract infections (UTIs) are among the most frequent infections in both hospital and community settings, predominantly caused by Gram-negative bacteria such as *Citrobacter freundii*, *Escherichia coli*, and *Klebsiella pneumoniae*. A prospective study in Iran's Burn Unit Centre analyzed 733 samples and identified 124 isolates, with *Pseudomonas aeruginosa* (57.3%) as the most prevalent, followed by *Citrobacter* spp. (35.5%), of which 95.5% were identified as *C. freundii*[^30]. Similarly, a study in Bangladesh found that 33.33% of 150 UTI samples were positive, with *E. coli* (48%) being the most common pathogen, followed by *Enterobacter* spp. (18%), *P. aeruginosa* (10%), and *Citrobacter* spp. (6%) [31]. In Pakistan, a study conducted at Khyber Teaching Hospital (KTH) in Peshawar examined 2,950 clinical samples and identified 130 (4.4%) as *C. freundii*. The highest prevalence was observed in the 21–40 age group (43.08%). Antibiogram results showed high resistance to trimethoprim-sulfamethoxazole (78.57%), levofloxacin (71.42%), and ciprofloxacin (64.29%), with lower resistance to piperacillin-tazobactam (42.86%)[32]. These findings are consistent with the present study, which observed the highest prevalence in individuals aged 41–60 (37.5%) and significant resistance to cotrimoxazole (92.5%), ciprofloxacin (80%), and piperacillin-tazobactam (85%). The role of β-lactamases in antibiotic resistance has been extensively investigated. Molecular detection of *bla-SHV* and *bla-OXA* genes in Gram-negative rods (GNRs) from Jinnah Hospital, Lahore, revealed that 15% of

extended-spectrum β-lactamase (ESBL) producers were positive for *bla-SHV* and 43% for *bla-OXA*, with high resistance to aztreonam[33]. This aligns with the present study, where *bla-OXA* expression contributed to moderate resistance (10%). In Iraq, a study at Al-Najaf Central Hospital identified 30 (6.5%) *C. freundii* isolates among 461 samples, with 63.3% being ESBL producers[^34]. Molecular analysis from acute leukemia patients in Spain revealed co-expression of *blaOXA-48* and *blaVIM-1* genes in three *C. freundii* isolates, significantly enhancing carbapenem resistance[^35]. Similarly, *blaNDM-1* was detected in 12.5% of *C. freundii* isolates in a study of 116 blood samples from children in a tertiary care hospital in Lahore[^36]. Another study on meropenem-resistant isolates reported that 13 *C. freundii* isolates expressed the *blaNDM-1* gene[37]. These findings underscore the high prevalence of *C. freundii* as a uropathogen and its significant contribution to antimicrobial resistance, necessitating ongoing surveillance and targeted antibiotic stewardship strategies.

Conclusion

The primary objective of this study was to investigate the role of Carbapenemase-producing *Citrobacter freundii* strains in resistance to meropenem. *C. freundii* has emerged as a significant uropathogen responsible for both community-acquired and hospital-acquired urinary tract infections (UTIs). The study observed a high level of resistance to cefepime and meropenem. However, Fosfomycin demonstrated high efficacy, with most isolates being susceptible, making it a viable therapeutic option. Molecular analysis identified carbapenem-resistant *C. freundii* isolates carrying Carbapenemase genes, including *bla-VIM*, *blaNDM-1*, and *blaOXA-48*. These findings highlight the alarming emergence of carbapenem resistance mechanisms in *C. freundii*, posing a significant threat to public health. The results underscore the critical need for prudent antibiotic prescribing practices and stringent antimicrobial stewardship to mitigate the spread of resistance and preserve the efficacy of existing treatment options.

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