Antimicrobial Resistance, Phenotypic Characteristics, and Biofilm Production in *Citrobacter freundii* Isolates Obtained from Carbapenem-Resistant Strains

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Abstract

Background: *Citrobacter freundii* is an emerging nosocomial pathogen increasingly associated with carbapenem resistance, rendering infections difficult to treat. The co- occurrence of biofilm production, multidrug resistance (MDR), and carbapenemase- encoding genes in clinical isolates poses a grave public health concern.

Objective: To evaluate the association between phenotypic traits, antimicrobial resistance patterns, and biofilm-producing capacity in clinical isolates of *Citrobacter freundii* (*C. freundii*), with a focus on carbapenem-resistant strains.

Methods: A total of 27 *Citrobacter* spp. isolates recovered from various clinical specimens

Isolates including urine, blood, and respiratory samples were subjected to colony morphology characterization, biochemical profiling, antibiotic susceptibility testing against 19–21 antimicrobial agents, biofilm detection by the Congo Red Agar (CRA) method, carbapenemase phenotypic detection, and molecular identification of resistance genes (OXA-48, NDM, KPC, SIM, VIM) by PCR.

Results: Of the 27 total *Citrobacter* spp. isolates, 8 (29.6%) were identified as carbapenem- resistant *Citrobacter* spp. (CRCR), of which 4 were confirmed *Citrobacter freundii* carbapenem-resistant (CFCMDR). Blood culture isolates accounted for 100% CFCMDR prevalence. All 8 carbapenem-resistant isolates demonstrated resistance to 19 antimicrobial classes. OXA-48 gene was detected in 50% of blood-derived isolates, and NDM gene in 50% of respiratory isolates. Biofilm formation was positively associated with carbapenemase production in the co-producing isolates.

Conclusion: This study confirms a significant correlation between phenotypic characteristics, multidrug antimicrobial resistance, and biofilm production in *C. freundii*, underscoring the need for rigorous phenotypic and genotypic surveillance to guide infection control policies.

Keywords: *Citrobacter freundii*; Carbapenem resistance; Biofilm; OXA-48; NDM; Antimicrobial resistance; Phenotypic characterization.

Introduction

Citrobacter freundii is a Gram-negative, facultative anaerobic bacillus that belongs to a family called

enterobacteriaceae. Historically considered a commensal organism of the human gastrointestinal tract, C.freundii has become an important opportunistic organism causing a wide range of nosocomial infections, such as urinary tract infections (UTIs), bacteremia, pneumonia, meningitis, and wound infections, especially in immunocompromised, neonatal and critically ill patients [1-3]. The clinical importance of this pathogen has increased substantially over the last 20 years due to its inherent ability to acquire and transmit antimicrobial resistance mechanisms.

Carbapenems, which include imipenem, meropenem, and ertapenem, represent the cornerstone of empirical therapy for severe infections caused by multidrug-resistant (MDR) Gram-negative bacteria. The emergence of carbapenem-resistant Enterobacteriaceae (CRE), particularly *C. freundii*, has been attributed to the acquisition of carbapenemase- encoding genes most notably OXA-48, New Delhi Metallo-β-lactamase (NDM), Klebsiella pneumoniae Carbapenemase (KPC), Verona Integron-encoded Metallo-β- lactamase (VIM), and Seoul Imipenemase (SIM) as well as the overexpression of chromosomal AmpC β-lactamases combined with outer membrane protein (OMP) loss.[4–6] These mechanisms collectively confer high-level resistance to virtually all β-lactam antibiotics, severely limiting therapeutic options.

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(VIM), and Seoul Imipenemase (SIM) are the most common - and overexpression of the chromosomal AmpC beta-lactamases concomitant with loss of outer membrane proteins (OMP) [4-6].

Biofilm formation is another important virulence mechanism for Citrobacter spp. Biofilms are organized communities of microorganisms embedded inside a self-produced extracellular polymeric matrix, which provides resistance to immune host responses, antimicrobial agents, and environmental pressures [7-9]. The coexistence of carbapenem resistance and biofilm production in C. freundii isolates increases their virulence and clinical intractability. Despite this, there have been very few systematic data correlating antimicrobial resistance, colony phenotype and biofilm production in clinical isolates of C. libera. freundii, especially in the South Asian context.

Colony morphology and biochemical phenotyping remain as an indispensable part of conventional microbiological identification and has been linked to virulence potential in some Enterobacteriaceae. Larger colony-forming strains have been associated with increased capsule production and pathogenicity [10]. The phenotypic determination of resistance of clinical isolates could be used to aid the rapid identification of high-risk strains before molecular confirmation.

The present study was therefore undertaken to characterise phenotypic characteristics, antimicrobial resistance profiles and biofilm producing capability of C. freundii isolates obtained from laboratory confirmed clinical specimen and assess the molecular basis of carbapenem resistance by gene amplification.

Materials and Methods

Study Design and Sample Collection

This was a descriptive cross-sectional laboratory-based study. A total of 27 *Citrobacter* spp. isolates were collected from clinical samples — including urine, blood, and respiratory specimens (sputum, endotracheal secretions, bronchoalveolar aspirate) — submitted to the Microbiology Laboratory during the study period. All isolates were obtained from laboratory-confirmed culture reports. The study was conducted after obtaining approval from the Institutional Ethics Committee.

Bacterial Identification

All isolates were subjected to standard microbiological identification. Initial characterization was performed by streaking clinical specimens on Nutrient Agar, MacConkey Agar, Blood Agar, Chocolate Agar, and Chromogenic Agar, followed by incubation at 37°C for 24–48 hours. Colony morphology was recorded for each medium. Biochemical characterization was performed using a panel of 15 standard tests, including carbohydrate fermentation reactions, Triple Sugar Iron (TSI) agar reactions, Indole production, Methyl Red (MR), Voges-Proskauer (VP), Citrate utilization (IMViC), Urease, Catalase, Oxidase, and Motility tests. Gram staining was performed to confirm morphology and staining characteristics.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing (AST) was performed on all carbapenem-resistant isolates (n = 8) by the Kirby-Bauer disc diffusion method on Mueller Hinton Agar in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The following 19–
21 antimicrobial agents were tested: Ampicillin/Sulbactam, Tobramycin, Piperacillin- Tazobactam (PC), Fosfomycin (FOR), Cefepime (CPM), Amikacin (AMK), Ofloxacin (OF), Levofloxacin (LEV), Ciprofloxacin (CIF), Doxycycline (DO), Co-trimoxazole (CO), Gentamicin (G), Aztreonam (ZO), Moxifloxacin (MGX), Imipenem (IM), Meropenem (MR), Tigecycline (TGC), Polymyxin B (PB), Colistin (COL), Nitrofurantoin (NF), and Norfloxacin (NOR). Results were interpreted as sensitive, intermediate, or resistant.

2.4 Phenotypic Detection of Carbapenemase Production

Carbapenemase production was detected phenotypically using the modified Hodge Test (MHT) and/or the Carba NP test, as per standard protocols. Each isolate was classified as carbapenemase-positive or negative based on the test result.

2.5 Biofilm Detection

Biofilm production was assessed using the Congo Red Agar (CRA) method. Isolates were inoculated onto Brain Heart Infusion Agar supplemented with Congo Red dye and sucrose, then incubated at 37°C for 24 hours under aerobic conditions. Black crystalline colonies were considered biofilm-positive, while red non-crystalline colonies were considered biofilm-negative. Results were confirmed by the tissue culture plate (TCP) method where indicated.

Molecular Detection of Carbapenemase Genes

DNA was extracted from all 27 *Citrobacter freundii* isolates (CFCR-1 to CFCR-27) using the standard boiling method. PCR amplification was performed targeting five major carbapenemase genes: OXA-48, NDM, KPC, SIM, and VIM. Gene-specific primers were used under standard thermocycling conditions. Amplified products were visualized by agarose gel electrophoresis under UV illumination. Isolates were designated positive when a band of the expected molecular weight was identified.

Statistical Analysis

Descriptive statistics were used to express frequencies and percentages. Associations between biofilm production and carbapenemase production were assessed by cross- tabulation.

Results

Colony Morphology on Different Culture Media

All 27 *Citrobacter* spp. isolates were characterized on five different culture media. Distinct and consistent morphological features were observed. Nutrient Agar shows (Smooth, convex, moist, greyish translucent to opaque colonies), MacConkey agar,(Lactose-fermenting pink or pale pink colonies), Blood Agar (Smooth, moist, and shiny colonies), chocolate Agar (Smooth, moist, and grey colonies) ,Chromogenic Agar (Green- blue Colonies) (Himedia, Mumbai, India) and incubated aerobically at 37°C for 24 h. After the incubation period, the culture plates were examined for size colony morophology .

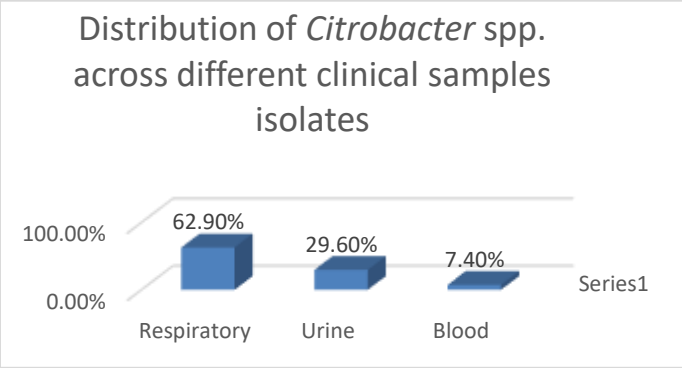
Biochemical Characterization

Biochemical profiling confirmed the *Citrobacter* spp. identity across all isolates. Key discriminatory findings included positivity for glucose, sucrose, and lactose fermentation, citrate utilization, urease, catalase, and motility, while indole production, Voges-Proskauer, oxidase, and mannitol/fructose fermentation were negative. TSI showed acid/acid (AA) reaction with H_2S and gas production. Gram staining revealed Gram-negative rod-shaped morphology .

Distribution of *Citrobacter* spp. Across Clinical Samples isolates :

Of the 27 isolates, the majority isolates were recovered from respiratory specimens (62.9%, n = 17), followed by urine (29.6%, n = 8), and blood (0.07%, n = 2). No isolates were obtained from pus/wound or fluid samples (**Table 1, Fig .1**).

Table 1 & Fig 1 . Distribution of *Citrobacter* spp. across different clinical sample isolates (n = 27)



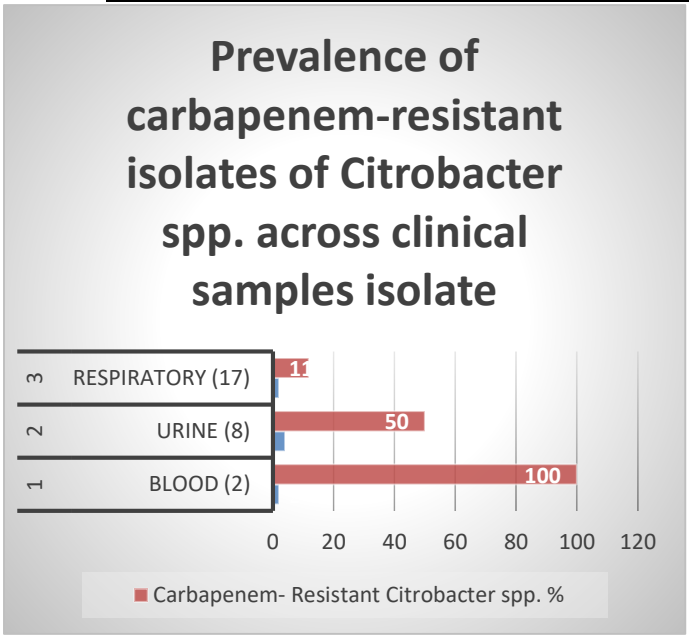
S.No	Sample Type	No of isolate	%
1	Respiratory	17	62.90%
2	Urine	8	29.60%
3	Blood	2	7.40%

3.4 Prevalence of Carbapenem-Resistant Isolates

Of the 27 *Citrobacter* spp. isolates, 8 (29.6%) were carbapenem-resistant. Blood isolates demonstrated the highest carbapenem resistance rate (100%, 2/2), followed by urine (50%, 4/8) and respiratory specimens (11.7%, 2/17). All 4 confirmed *Citrobacter freundii* carbapenem-resistant (CFCMDR) isolates were derived from blood (n = 2) and respiratory(n = 2) cultures (**Table 2 & Fig .2**).

S. No	Sample	Carbapenem-Resistant *Citrobacter* spp.		Carbapenem-Resistant *C. freundii*	
		Number	%	Number	%
1	Urine	4	50 %	0	0
2	Blood	2	100%	2	100
3	Respiratory	2	11.7%	2	100

4	Pus/Wound	0	0	0	0
5	Fluids	0	0	0	0
	Total	**8**		**4**	



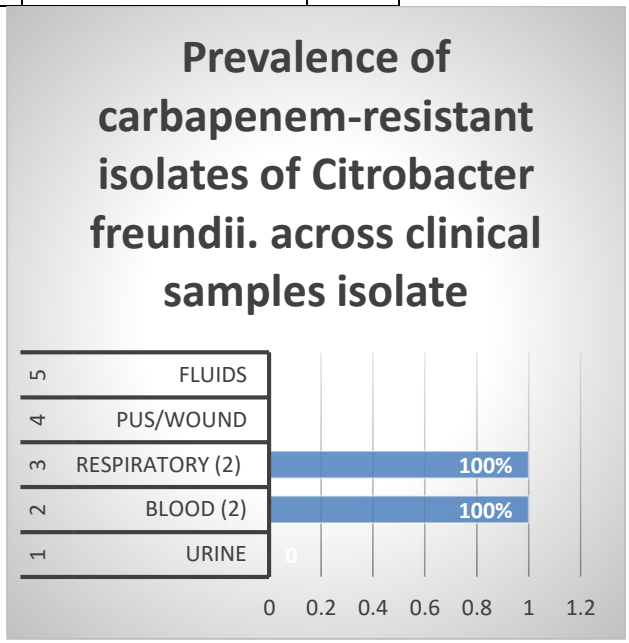


Fig .2 Prevalence of Carbapenem-Resistant *Citrobacter spp & Citrobacter freundii* Isolates

Antibiotic Resistance Profiles of Carbapenem-Resistant Isolates

All 8 carbapenem-resistant *Citrobacter* spp. isolates demonstrated resistance to all 19 tested antimicrobial agents from sputum, blood, and urine sources isolates , confirming a pan-drug- resistant (PDR) profile. Notably, Tigecycline (TGC) and Colistin (COL) showed sensitivity in urine-derived isolates (0% resistance), while remaining resistant in respiratory and blood isolates .

Biofilm Production and Carbapenemase Detection in Individual Isolates

Of the 27 *C. freundii* isolates (CFCR-1 to CFCR-27), biofilm formation was detected in 8 isolates, and carbapenemase production was detected in 7 isolates phenotypically (**Table 3**). Notably, isolates CFCR-1, CFCR-3, CFCR-11, and CFCR-13 co-expressed both biofilm production and carbapenemase activity, representing the highest-risk phenotypic profile.

Table 3. Biofilm formation and carbapenemase production among individual *Citrobacter freundii* isolates

S. No	Isolate	Biofilm	Carbapenemase
1	CFCR-1	+	+
2	CFCR-2	–	+
3	CFCR-3	+	+
4	CFCR-4	–	–
5	CFCR-5	–	–
6	CFCR-6	–	+
7	CFCR-7	+	–
8	CFCR-8	–	–
9	CFCR-9	–	–
10	CFCR-10	+	–
11	CFCR-11	+	+
12	CFCR-12	–	–
13	CFCR-13	+	+
14	CFCR-14	–	+
15	CFCR-15	–	–
16	CFCR-16	–	–
17	CFCR-17	–	–
18	CFCR-18	–	–
19	CFCR-19	–	–
20	CFCR-20	+	–
21	CFCR-21	–	+
22	CFCR-22	–	–
23	CFCR-23	–	–
24	CFCR-24	–	–
25	CFCR-25	+	–
26	CFCR-26	–	–
27	CFCR-27	+	–

(+ = positive; − = negative; Highlighted isolates: CFCR-1, -3, -11, -13 showed co- expression of both traits).

Sample-Wise Distribution of Biofilm and Carbapenemase Production

Biofilm formation was documented in 100% of blood (2/2) and sputum (2/2) isolates tested. Carbapenemase production was observed in 100% of isolates across urine (8/8), blood (2/2), and sputum (2/2 of 17) samples tested. No biofilm or carbapenemase activity was recorded in pus, wound, fluid, bronchial aspirate, or ET secretion isolates (**Table 4**).

Table 4. Sample-wise distribution of biofilm formation and carbapenemase production

S. No	Sample	Biofilm 4 No. of Isolates	Biofilm 4 No. Positive	Biofilm %	Carbapenemase 27 No. of Isolates	Carbapenemase — No. Positive	Carbapenemase %
1	Urine	–	–	–	8	8	100%
2	Blood	2	2	100%	2	2	100%
3	Sputum	2	2	11.7%*	17	17	100%
4	BR Aspirate	–	–	–	–	–	–
5	ET Secretion	–	–	–	–	–	–
6	Pus	–	–	–	–	–	–
7	Wound	–	–	–	–	–	–
8	Fluid	–	–	–	–	–	–

*(*Percentage relative to total respiratory isolates = 17)*

Association Between Biofilm and Carbapenemase Production

Cross-tabulation revealed that among the 8 carbapenem-resistant isolates (n = 8 Carba), 4 were biofilm-positive and 4 were biofilm-negative. Among biofilm-positive isolates (n = 9 total), 4 were carbapenemase-positive and 5 were carbapenemase-negative. The total biofilm-negative group comprised 8 isolates, equally split between carbapenemase-positive and -negative (**Table 5 & Fig 3**).

Table 5. Association between biofilm formation and carbapenemase production in *Citrobacter freundii* isolates

S. No	Biofilm Status	Carbapenemase Positive	Carbapenemase Negative	Total
1	Biofilm Positive	4	5	9
2	Biofilm Negative	4	14	18
	Total	**8**	**19**	**27**



Molecular Detection of Carbapenemase Genes by PCR

Molecular analysis of the 27 CFCR isolates revealed the following gene distribution: OXA- 48 was identified in isolates CFCR-1, CFCR-3, CFCR-7, CFCR-11, CFCR-13, CFCR-20, CFCR-25, and CFCR-27. NDM was detected in CFCR-1, CFCR-3, CFCR-11, and CFCR-
13. KPC was identified in CFCR-2 and CFCR-14. VIM was detected in CFCR-6 and CFCR-21. No SIM gene was detected in any isolate. Notably, isolates CFCR-1, CFCR-3, CFCR-11, and CFCR-13 carried co-expressed OXA-48 and NDM genes (**Table 6**).

Table 6. Molecular detection of carbapenemase genes (OXA-48, NDM, KPC, SIM, VIM) in *Citrobacter freundii* isolates (CFCR-1 to CFCR-

S. No	Isolate	OXA-48	NDM	KPC	SIM	VIM
1	CFCR-1	+	+	–	–	–
2	CFCR-2	–	–	+	–	–
3	CFCR-3	+	+	–	–	–
4	CFCR-4	–	–	–	–	–
5	CFCR-5	–	–	–	–	–
6	CFCR-6	–	–	–	–	+
7	CFCR-7	+	–	–	–	–
8	CFCR-8	–	–	–	–	–
9	CFCR-9	–	–	–	–	–
10	CFCR-10	–	–	–	–	–
11	CFCR-11	+	+	–	–	–

12	CFCR-12	–	–	–	–	–
13	CFCR-13	+	+	–	–	–
14	CFCR-14	–	–	+	–	–
15	CFCR-15	–	–	–	–	–
16	CFCR-16	–	–	–	–	–
17	CFCR-17	–	–	–	–	–
18	CFCR-18	–	–	–	–	–
19	CFCR-19	–	–	–	–	–
20	CFCR-20	+	–	–	–	–
21	CFCR-21	–	–	–	–	+
22	CFCR-22	–	–	–	–	–
23	CFCR-23	–	–	–	–	–
24	CFCR-24	–	–	–	–	–
25	CFCR-25	+	–	–	–	–
26	CFCR-26	–	–	–	–	–
27	CFCR-27	+	–	–	–	–

(+ = gene detected; − = gene not detected; Bold/highlighted: CFCR-1, -3, -11, -13 co- express OXA-48 and NDM)

Discussion:

In the present investigation we systematically characterised the phenotypic characteristics, antibiotic resistance profiles and biofilm producing capacity of twenty seven clinical isolates of Citrobacter spp. 29.6% were confirmed as carbapenem resistant. This prevalence is in line with emerging data from South-Asian tertiary care centres, in which CRE rates have shown a steep rise in the past decade, with the main mechanism of gene transfer being horizontal gene transfer of carbapenemase encoding plasmids [11-13].

Respiratory specimens were the most common source of total isolates (62.9%), which is consistent with the well documented pathogenic role of C. freundii in ventilator associated pneumonia and lower respiratory tract infections in intensive care settings. Blood culture isolates, though in a lower absolute number, showed the highest carbapenem resistance rate (100% respectively), thus highlighting the importance of bloodstream infections due to this organism.

Biochemical characterisation in this study revealed the classical biochemistry of the Citrobacter spp. - positive for citrate utilisation, positive for urease and H_2S production and negative for indole, consistent with known identification criteria [14]. The gram-negative rod morphology along with motility provided further support of the genus Identification. These findings support the validity of classical profiling methods of biochemical analysis in resource - limited contexts where molecular tools may not be readily available.

All eight carbapenem - resistant isolates showed resistance to all nineteen antimicrobial agents that were tested, suggesting a pan - drug - resistant (PDR) phenotype. This pattern included fluoroquinolones, aminoglycosides, combination of beta-lactam/beta-lactamase inhibitors, tetracyclines and polymixins. Of note, tigecycline and colistin demonstrated partial susceptibility only in urine isolates, which suggests the presence of residual in-vitro activity possibly to be used to guide directed therapy in uncomplicated UTIs, although efficacy in-vivo needs to be validated [15].

Molecular analysis identified OXA-48 as the most common carbapenemase gene and it was found in several isolates, followed by NDM, KPC, and VIM genes. Of particular alarming finding is the co-existence of OXA-48 and NDM in single isolates (CFCR-1, CFCR-3,

CFCR-11, CFCR-13). Dual gene carriage has been reported in CRE isolates from India and from other low middle income countries and has been linked with higher MICs, treatment failure and mortality [16-18]. The fact that none of this cohort had the SIM gene is in keeping with the rarity of the gene outside clinical settings in East Asia.

Biofilm formation was identified in eight out of twenty-seven (29.6-fold) isolates and was co-expressed with production of carbapenemase enzymes in four isolates, that is, the most dangerous phenotypic combination in clinical terms. Biofilm-forming carbapenem resistant organisms have been reported to be tolerant to much more antibiotics than planktonic cells with tolerance increased from one hundred to one thousand fold [19]. The simultaneous expression of both traits in isolates of C. freundii from both blood and respiratory specimens raises important issues concerning the role of persistent bacteremia and catheter-associated infections in critical care units.

Colony morphology analysis resulted in consistent phenotypic characteristics for media. The lower prevalence of large sized colony forming strains in the drug resistant groups than in pan susceptible isolates suggests the possible presence of a phenotypic trade off between the acquisition of resistance and the colony size as has been indicated in other Enterobacteriaceae [20]. Chromogenic agar confirmed unique green-blue with red colonies which potentially can be used as a rapid screening tool for preliminary citrobacter identification in high throughput laboratory environments.

- Conclusion

This study shows a significant relationship of phenotypic characteristics, multidrug antimicrobial resistance, and biofilm production in clinical isolates of Citrobacter freundii. The high prevalence of carbapenem resistance (29.6%) and pan-drug resistance to nineteen antimicrobials, the co-carriage of OXA-48 and NDM genes and the capacity of biofilm formation, highlight the changing threat from this pathogen in healthcare settings. The detection of isolates co-harbouring more than one carbapenemase gene and biofilm forming ability requires urgent introduction of active microbiological surveillance, stringent infection control regimens and rational use of last-resort antimicrobials. Future studies using whole genome sequencing of a larger multicentric cohort are warranted to establish the molecular epidemiology and transmission dynamics of these high risk clones.

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