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Original Article

Microbiology Section

Extended Spectrum Beta Lactamase Profile of *Citrobacter* Species Isolated from Various Samples in the North East Coast of Tamil Nadu, India: A Cross-sectional Study

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ABSTRACT

Introduction: *Citrobacter* species play a significant role in causing hospital-acquired infections, especially those affecting the urinary and respiratory systems, and they are commonly present in faeces. Healthcare systems are facing a major challenge due to the rise of antibiotic-resistant Gram-negative infections resistant to antibiotics.

Aim: To identify the genotypic characterisation of the resistant gene bla_{TEM-1} in *Citrobacter* spp. obtained from a range of clinical samples. This investigation seeks to provide insights into the patterns of antibiotic resistance exhibited by these bacteria.

Materials and Methods: This cross-sectional study was conducted between August 2021 and February 2022. Preliminary examinations included screening as well as confirmatory tests for Extended Spectrum Beta Lactamase (ESBL) production, which were confirmed by the combination disk method and double disc synergy test. Genotypic detection of the bla_{TEM-1} gene was done using the Polymerase Chain Reaction (PCR) technique. Demographic parameters like gender and age were assessed. Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) Software version 21.0.

Results: *Citrobacter koseri* (66 isolates, 54.5%) was found to be the predominant species among the *Citrobacter* spp., followed by *Citrobacter freundii* (55 isolates, 45.5%). Urine samples were the primary source of *Citrobacter* spp. isolates, comprising 52 isolates (43%) of the total. In this study, the prevalence of ESBL producers was reported to be 49 isolates (77.8%) identified as ESBL producers. Tigecycline demonstrated a 100% effectiveness rate, followed by Piperacillin-Tazobactam (96% sensitivity) and Amikacin (95% sensitivity). Out of the 20 *Citrobacter* spp. isolates that tested positive for ESBL, 15 isolates (75%) were found to be positive for bla_{TEM-1}.

Conclusion: The results of this study provide insight into the changing patterns of antibiotic resistance in *Citrobacter* spp., with particular emphasis on ESBL profiles. Thus, it is imperative to implement intervention techniques to reduce the ongoing selection and spread of these more resistant bacteria, as well as efficient infection-control measures to manage epidemics. These results advance the comprehension of antibiotic resistance patterns and guide initiatives to combat the increasing threat of antibiotic resistance.

Keywords: Antimicrobial resistance, *Citrobacter freundii*, *Citrobacter koseri*, Extended spectrum beta lactamase producers

INTRODUCTION

Citrobacter species is a Gram-negative, aerobic bacillus that belongs to the Enterobacterales family. It can be found extensively in food, soil, water, and the digestive systems of both humans and animals [1]. The *Citrobacter* genus is becoming more harmful, since it is known to cause a number of illnesses, including Urinary Tract Infections (UTIs), meningitis in newborns, septicaemia, brain abscesses, and gastroenteritis. There are eleven distinct types of *Citrobacter*, including *C. koseri, C. freundii, C. braakii, C. farmerii, C. gillenii, C. sedlakii, C. werkmanii, C. amalonaticus, C. youngae, C. murliniae*, and *C. rodentium* [2]. *C. freundii* is the species most commonly linked to opportunistic infections related to healthcare. A growing number of illnesses, including those affecting the respiratory, circulatory, and urinary tracts, are associated with *C. freundii* [3].

According to a systematic surveillance study conducted in American medical institutions, *Citrobacter* infections made up 0.8% of Gram-negative infections and represents 3 to 6% of all Enterobacterales isolates in hospital environments [4]. Infections with *Citrobacter* are frequent in newborns and can manifest as sepsis, meningitis, or even cerebral abscesses. Individuals who are elderly, disabled, or immunocompromised are also susceptible to *Citrobacter* infections [4,5]. Furthermore, *Citrobacter* spp. are becoming more and more significant as clinical multidrug-resistant pathogens that cause infections acquired in both the community and nosocomial infections [6].

Due to the presence of plasmid-encoded resistance genes, *Citrobacter* spp. are often resistant to many antibiotics [7]. *Citrobacter* is now acknowledged as a serious nosocomial pathogen, and there is an increasing global trend of reported isolates that are multidrug-resistant. Healthcare systems are facing a major problem as a result of the growth of Gram-negative infections which are resistant to antibiotics [8]. According to data from the National Nosocomial Infections Surveillance System of the Centers for Disease Control and Prevention (CDC), there is an increasing prevalence of hospital-acquired infections caused by antibiotic-resistant Gram-negative organisms [9].

This research specifically focuses on identifying the bla_{TEM-1} gene, which harbours ESBL genes that may significantly contribute to the spread of antibiotic resistance. The presence of bla_{TEM-1} confers resistance to beta-lactam antibiotics, such as cephalosporins and penicillins. Because these antibiotics are often used to treat bacterial infections, managing *Citrobacter* spp. infections might become noticeably challenging as a result of this resistance. Consequently, the discovery of bla_{TEM-1} underscores the importance of implementing infection control procedures in place in order to stop the spread of antibiotic-resistant organisms in hospital settings.

Adherence to strict infection control measures, including hand hygiene, isolation precautions, and complete environmental cleaning, is essential in mitigating the spread of *Citrobacter* spp. strains that contain *bla*$_{TEM-1}$. Therefore, the present study aims to characterise ESBL profile and the genotypic characterisation of the resistant gene *bla*$_{TEM-1}$ in *Citrobacter* spp. from various clinical samples, providing insights into antibiotic resistance patterns and informing strategies for effective infection control and treatment.

MATERIALS AND METHODS

This cross-sectional study was conducted at SRM Medical College Hospital and Research Centre, located in Kattankulathur, Chengalpattu district, Tamil Nadu, India, between August 2021 and February 2022. Ethical approval (2362/IEC/2021) was obtained from the Institute Ethical Committee (Human Studies). A total of 121 *Citrobacter* spp. were isolated from diverse clinical samples, like urine, pus, sputum, blood, ear swabs, wound swabs, tracheal aspirates, and tissue. As this was a time-bound study, all samples that were present in the study duration were included. Clinical data for each patient was collected, including demographics, underlying disorders, systemic antimicrobial therapy, surgeries, concomitant infections, and results.

Inclusion criteria: All *Citrobacter* spp. isolated and reported from clinical samples during the study period in the microbiology laboratory were included in the study.

Exclusion criteria: Other Gram-negative organisms isolated and reported during the study period were excluded. All duplicates of *Citrobacter* spp. isolated from various clinical samples were also excluded from the study.

Isolation and identification: *Citrobacter* spp. were identified using conventional techniques such as Gram staining, colony characteristics, and biochemical reactions. Antimicrobial sensitivity testing of the *Citrobacter* spp. isolates was conducted on Mueller-Hinton agar using the Kirby-Bauer method, and the results were assessed according to the criteria outlined in the 2022 guidelines from the Clinical Laboratory Standards Institute (CLSI) [10].

Combination disk diffusion method: The screening tests for ESBL production were performed on Mueller-Hinton agar as previously described [11]. A sterile swab was employed to create a uniform culture of the isolated *Citrobacter* spp. on Mueller-Hinton agar. Ceftazidime and a ceftazidime-clavulanic acid disc were positioned approximately 24 mm apart. The culture was then incubated at 37°C for 24 hours. After the incubation period, the zones of inhibition were measured with a ruler. A difference of 5 mm or more between these discs indicates an ESBL-positive isolate.

Double disc synergy test: In the confirmation test for ESBL production, two antibiotic discs were employed as previously described [11]: ceftazidime and ceftazidime with clavulanic acid. The ceftazidime-clavulanic acid was positioned in the center of the agar plate, while the ceftazidime disc was placed 1.5 cm apart from each other. The results were measured after 24 hours of incubation at 37°C, which helps indicate the presence of ESBL-producing organisms. A positive test result is determined by a combination of reduced susceptibility to cefotaxime and a distinct improvement in the inhibition zone of cefotaxime around the clavulanate-containing disc. This often leads to the formation of a recognizable zone with a characteristic shape, commonly referred to as a "champagne cork" or "keyhole."

PCR for *bla*$_{TEM-1}$ gene detection: PCR was conducted to identify the presence of the ESBL gene *bla*$_{TEM-1}$, as previously described by Monstein HJ [12]. The DNA extraction process involved the phenol-chloroform-isoamyl alcohol extraction method. The isolated bacterial DNA is ready for use in PCR and can be stored at -20°C. Specific primers for sequencing *bla*$_{TEM-1}$ were employed as described in [Table/Fig-1], and the PCR steps are described in [Table/Fig-2].

ESBL-*bla*$_{TEM-1}$-F	GCTATGTGGCGCGGTATTAT	424
ESBL-*bla*$_{TEM-1}$-R	AACTTTATCCGCCTCCATCC	

[Table/Fig-1]: Primer sequence.

Steps	No. of cycles	Duration of cycle	Temperature
Denaturation	1	5 minutes	95°C
Annealing	25	30 seconds	72°C
Extension	1	5 minutes	72°C

[Table/Fig-2]: PCR steps.

Agarose gel electrophoresis: The agarose solution was prepared and carefully poured into the gel tray, ensuring that the wells were fully covered. It was allowed to solidify completely for 30 minutes. Each DNA sample was mixed with an appropriate volume of gel-loading buffer. The DNA samples and a DNA ladder were carefully loaded into the wells using a micropipette or a gel-loading tip. The gel tray was placed into the electrophoresis chamber, connected to power supply and set to the desired voltage and running time. After the electrophoresis run was complete, the gel tray was removed from the chamber and placed in a gel imaging system or on a UV transilluminator.

STATISTICAL ANALYSIS

The statistical analyses were carried out using SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). A Chi-square test was performed, and a p-value <0.05 was considered statistically significant.

RESULTS

In the present study, a total of 121 *Citrobacter* spp. isolates were obtained. Among these, 76 (62.8%) were from male patients, and 45 (37.2%) were from female patients. The average age of all the patients was 30.25 years, with an age range spanning from three days to 80 years. *Citrobacter* spp. was predominantly isolated from urine samples, accounting for 52 (43%) of the isolates. The importance of separating *Citrobacter* spp. from a nasal swab relies on several variables, including the person's health, the likelihood of infection or colonisation, the existence of antibiotic resistance, and the history of environmental exposure. Since endogenous infections are extremely rare, it is crucial to detect nasal reservoir colonisation as a potential source of endogenous infections. Out of the 121 *Citrobacter* spp. isolates, 66 (54.5%) were identified as *Citrobacter koseri*, making it the most predominant isolate. Socio-demographic characteristics are listed in [Table/Fig-3].

Study characteristics	n (%)
Gender	
Male	76 (62.8)
Female	45 (37.2)
Age (in years)	
0-15	3 (2.5)
16-30	25 (20.7)
31-45	32 (26.4)
46-60	38 (31.4)
61-75	17 (14.0)
>76	6 (5)
Sample wise distribution	
Urine	52 (43)
Pus	33 (27.3)
Wound swab	18 (14.9)
Ear swab	6 (5)
Tracheal aspirate	6 (5)
Tissues	3 (2.4)

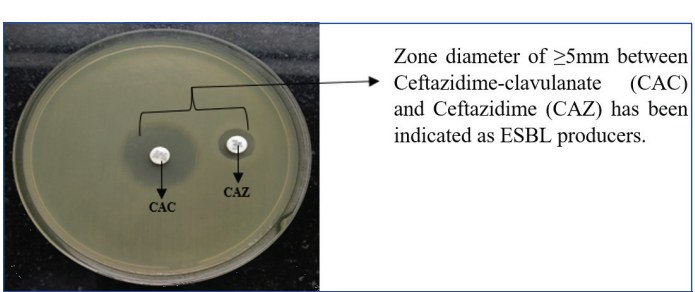
Nasal swab	1 (0.8)
Bile fluid	1 (0.8)
Blood	1 (0.8)
Species wise distribution	
Citrobacter koseri	66 (54.5)
Citrobacter freundii	55 (45.5)
Device associated *Citrobacter* spp. distribution	
Catheter-associated Urinary Tract Infection (UTI)	15 (68.2)
Ventilator-associated pneumonia	7 (31.8)

[Table/Fig-3]: Socio-demographic characteristics.

The antimicrobial sensitivity pattern of the 121 *Citrobacter* spp. isolates showed that 58 (48%) were sensitive to tigecycline, imipenem, meropenem, piperacillin-tazobactam, amikacin, ertapenem, ceftazidime-clavulanate, tetracycline, and gentamicin. As well as, 63 (52%) of the isolates were resistant to ampicillin, cefotaxime, cefazolin, cefoxitin, ampicillin-sulbactam, cefuroxime, ceftazidime-clavulanate, and amoxicillin-clavulanate. In the present study, the prevalence of ESBL producers was reported to be 49 (77.8%) among the 63 (52%) resistant *Citrobacter* isolates [Table/Fig-4]. Of the 49 ESBL producers, 29 (59.2%) tested positive in the screening test for ESBL producers, while 20 (40.8%) isolates showed positive results in the double disk synergy test (the confirmatory test for ESBL producers) [Table/Fig-5]. The comparison of ESBL producers by screening and confirmatory tests yielded no significant difference (p=0.577) [Table/Fig-4]. The species-wise distribution of *Citrobacter* spp. among the 49 (77.8%) ESBL producers. Remarkably, 39 (79.5%) of the ESBL producers were identified as *C. freundii*, while 10 (20.5%) isolates of *C. koseri* were ESBL producers [Table/Fig-4]. The ESBL gene, bla_{TEM-1}, was detected and analysed using the PCR method. Out of the 15 *C. freundii* isolates confirmed as ESBL producers through the double disk synergy test, only 10 (66.7%) were found to harbour the bla_{TEM-1} gene upon genotypic analysis [Table/Fig-5]. All 5 (100%) *C. koseri* isolates that tested positive by the phenotypic method also showed the presence of the bla_{TEM-1} gene by PCR. The expression of the bla_{TEM-1} gene in *Citrobacter koseri* samples is shown in [Table/Fig-6-8].

Isolates	ESBL producers (n=49)			p-value
	Total (n=63)	Screening test	Double disk synergy test	
Citrobacter freundii	39 (79.5%)	24 (48.9%)	15 (30.6%)	0.577
Citrobacter koseri	10 (20.5%)	5 (10.2%)	5 (10.2%)	
Total	49 (77.8%)	29 (59.2%)	20 (40.8%)	

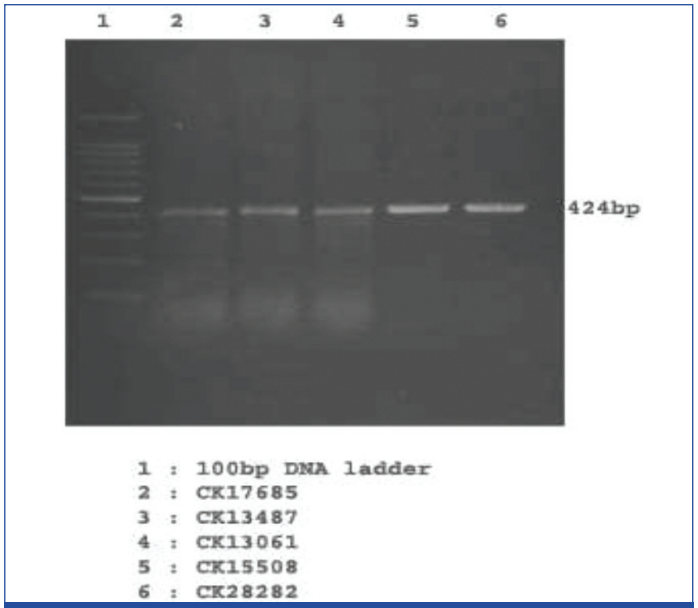
[Table/Fig-4]: Comparison of ESBL producers by screening and confirmatory test. A p-value with two tails of 0.05 or less was considered as statistically significant by Chi-square test



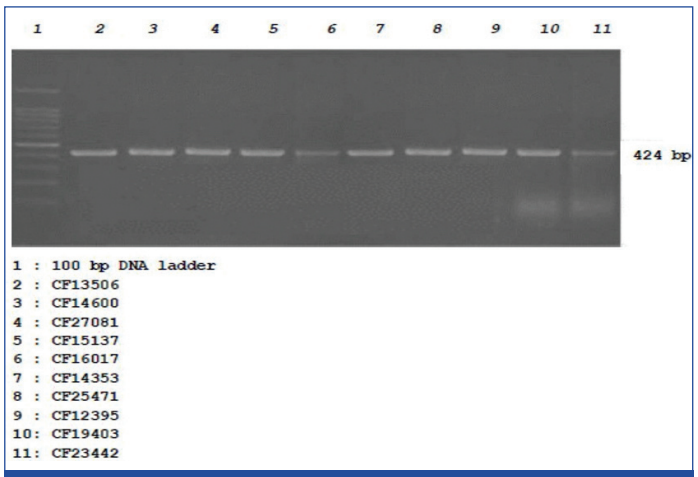
[Table/Fig-5]: Combination disk diffusion method for ESBL producers.

Isolates	Combined disk diffusion test	bla_{TEM-1}
Citrobacter freundii	15	10 (66.7%)
Citrobacter koseri	5	5 (100%)

[Table/Fig-6]: Genotypic detection of bla_{TEM-1} gene by PCR technique.



[Table/Fig-7]: Expression of bla_{TEM-1} gene in *Citrobacter koseri* samples.



[Table/Fig-8]: Expression of bla_{TEM-1} gene in *Citrobacter freundii* samples.

DISCUSSION

Citrobacter spp. are common inhabitants of the gastrointestinal system and are included in the coliform family. However, under specific situations, such as those involving weakened host defenses or favourable conditions in other tissues, they have the potential to cause serious infections [13]. Patients with underlying risk factors, such as diabetes, hepatic or renal conditions, Chronic Obstructive Pulmonary Disease (COPD), prior medical procedures, and previous antibiotic use, are at a higher risk of developing *Citrobacter* infections in hospital environments [14].

The findings of this study indicate that a higher number of *Citrobacter* spp. were isolated from male patients (63.8%) than from female patients (37.2%). Similar results were reported in the study by Poonam AR et al., which showed that *Citrobacter* spp. were more commonly isolated from male patients (70%) rather than female patients (30%) [15]. In the current study, the highest prevalence of *Citrobacter* spp. (31.4%) was found among individuals aged between 46 and 60 years. These results were not in concordance with the findings of Metri BC et al., which showed a higher incidence of *Citrobacter* spp. (35.5%) among individuals aged ≥60 years [1].

The location of specimen collection from areas below the waist may have boosted the likelihood of isolating *Citrobacter* spp. This was due to the possibility of colonisation, even in otherwise healthy people, as a result of these locations being close to the perianal region. The increased correlation of *Citrobacter* spp. with UTIs may possibly be explained by this proximity [16]. In the present research, it was observed that the greatest proportion of

Citrobacter spp. was isolated from urine samples (43%), followed by pus swabs (27.3%) and wound swabs (14.9%). These findings align with the study reports of Poonam AR et al., which showed that maximum incidence from urine samples (44.5%), followed by pus samples (21.6%) [15]. Also, the study results of Avinash G et al., demonstrated similar findings, with the highest frequency of *Citrobacter* spp. isolated from urine samples (23.9%), followed by pus samples (7.17%) [17].

In contrast, the research findings of Metri BC et al., and Mohan S et al., reported that *Citrobacter* spp. were isolated more frequently from pus samples (10.52% and 41.1%, respectively) [18,19]. The study by Nayar R et al., noted that the lowest number of *Citrobacter* spp. isolates were found in samples such as pleural fluid, tracheal swabs, and endotracheal tubes [20].

In this study, *C. koseri* (54.5%) was the most predominant species among the *Citrobacter* spp. isolated, followed by *C. freundii* (45.5%). No other species of *Citrobacter* were isolated. The study results from Sami H et al., and Kumari R et al., also yielded similar outcomes, showed *C. koseri* (53.2% and 75.4%) to have a higher incidence than *C. freundii* (18.2% and 24.6%) [21,22]. In contrast, investigations carried out by Mohan S et al., documented that *C. freundii* (49%) was the most common species isolated, followed by *C. koseri* (28%) [19].

The present study demonstrates that the prevalence of ESBL producers was 77.8%, among which only 10 (66.6%) isolates of *C. freundii* were detected with the bla_{TEM-1} gene, while all 5 (100%) isolates of *C. koseri* were detected with the bla_{TEM-1} gene by PCR. Lavigne JP et al., reported an ESBL prevalence of 17.8% among *Citrobacter* isolates [23]. Similarly, an ESBL prevalence of 19.3% was reported in a study held by Kanamori H et al., in 2011 [24]. In contrast to these analyses, a study conducted by Mohan S et al., reported that 54% of *Citrobacter* isolates were positive for ESBL production in 2014 [19].

Comprehending the frequency and attributes of ESBLs in *Citrobacter* isolates is essential for shaping suitable treatment approaches. This knowledge contributes to crafting efficient infection control measures and encourages the careful utilisation of antibiotics to reduce the spread of resistant strains. Moreover, insights into the ESBL profile in *Citrobacter* isolates add to the comprehensive monitoring of antibiotic resistance patterns in the area [25]. Such monitoring aids in recognising emerging trends and evaluating the efficacy of existing therapeutic interventions. Ongoing investigations in this domain holds promise for pioneering novel treatment strategies, such as discovering new antimicrobial agents or combining existing ones, to proficiently address *Citrobacter* strains that exhibit ESBL production [26]. These advancements have the potential to enhance patient outcomes and minimise complications linked to antibiotic resistance. This understanding is crucial for optimising patient wellbeing and thwarting the exacerbation of antibiotic resistance, ultimately promoting public health in the North East Coast of Tamil Nadu, India.

Limitation(s)

The present study involved a limited number of samples, as it might limit the generalisability of the findings to a broader population. A more extensive and diverse sample set would afford a more comprehensive insight into the prevalence and characteristics of ESBLs in *Citrobacter* isolates.

CONCLUSION(S)

It is noteworthy that the results of this study provide insight into the changing patterns of antibiotic resistance in *Citrobacter* spp., with a particular emphasis on ESBL profiles. It is evidently necessary to continuously evaluate and modify healthcare procedures as ESBLs become more diverse and complicated in order to stop their spread. Thus, it is imperative to implement intervention techniques to reduce the ongoing selection and spread of these more resistant bacteria, as well as efficient infection-control measures to manage epidemics. These results advance the comprehension of antibiotic resistance patterns and guide initiatives to combat the increasing danger of antibiotic resistance.

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