# Phenotypic detection and molecular characterization of beta-lactamase genes among *Citrobacter* species in a tertiary care hospital

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## ABSTRACT

**Objective:** To examine the distribution, emergence, and spread of genes encoding beta-lactamase resistance in *Citrobacter* species isolated from hospitalized patients in a tertiary care hospital. **Methods:** A prospective study was conducted in a 1000-bed tertiary care center in Pune, India from October 2010 to October 2013. A total of 221 *Citrobacter* spp. isolates were recovered from clinical specimens from different patients (one isolate per patient) admitted to the surgical ward, medical ward and medical and surgical Intensive Care Units. Polymerase chain reaction (PCR) assays and sequencing were used to determine the presence of beta-lactamase encoding genes. Conjugation experiments were performed to determine their transferability. Isolate relatedness were determined by repetitive element based-PCR, enterobacterial repetitive intergenic consensus-PCR and randomly amplified polymorphic DNA. **Results:** Among 221 tested isolates of *Citrobacter* spp. recovered from various clinical specimens, 179 (80.9%) isolates showed minimum inhibitory concentration (MIC) >4 µg/ml against meropenem and imipenem. One hundred and forty-five isolates with increased MICs value against carbapenems were further processed for molecular characterization of beta-lactamase genes. Susceptibility profiling of the isolates indicated that 100% retained susceptibility to colistin. Conjugation experiments indicated that $bla_{NDM\text{-}1}$ was transferable via a plasmid. **Conclusion:** The ease of NDM-1 plasmid transmissibility may help their dissemination among the *Citrobacter* species as well as to others in *Enterobacteriaceae*. Early detection, antimicrobial stewardship and adequate infection control measures will help in limiting the spread of these organisms.

**Key words:** $bla_{NDM\text{-}1}$, $bla_{VIM\text{-}2}$, combined-disc synergy test, *Citrobacter freundii, Citrobacter koseri*, double-disc synergy tests, metallo-beta-lactamase, modified Hodge test

## INTRODUCTION

*Citrobacter* species are an important cause of nosocomial infections, particularly involving the urinary and respiratory tracts of hospitalized patients and are inhabitants of the human gastrointestinal tract, often found in human feces and hospital environment.[1,2] In recent years, *Citrobacter* species have been commonly isolated from various clinical specimens such as urine, pus, and blood. A significant increase in nosocomial infections caused by *Citrobacter* species has been reported, especially in Neonatal Intensive Care Units (NICUs).[3-5] It has been reported to cause neonatal sepsis, brain abscess, urinary tract infections (UTIs), bloodstream infections, skin and surgical site infections, burns infections, intra-abdominal sepsis, meningitis, and pneumonia.[3-5] Fatality in *Citrobacter* septicemia ranges

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from 33% to 48%[6] Infant survivors may have significant damage to the central nervous system, including profound mental retardation, seizures, and hemiparesis.[7] There is very little data dealing with *Citrobacter* isolates in India: Neither its antibiotic sensitivity pattern nor the molecular characterization of its resistance genes. This study focused on determining the antibiotic resistance pattern and prevalence of metallo-beta-lactamase (MBL) genes in carbapenem-resistant *Citrobacter* spp. isolated in a tertiary care center.

## MATERIALS AND METHODS

### The bacterial isolates

A prospective study was conducted in a 1000-bed tertiary care center in Pune, India from October 2010 to October 2013. A total of 221 *Citrobacter* spp. isolates were recovered from clinical specimens of hospitalized patients admitted to the medical and surgical ICUs. Samples were collected from patients, using strict aseptic precautions and in accordance with standard protocols[8] and immediately processed without delay. The isolates were obtained from various clinical specimens such as urine, blood, pus, respiratory secretions (sputum, endotracheal secretions, broncho-alveolar lavage (BAL), and bronchial wash), and other sterile body fluids. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques.[8] The organism was identified up to the species level using VITEK-GNI cards (bioMérieux, Marcy l'Etoile, France).

### Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed by the Kirby-Bauer disc diffusion technique on Mueller-Hinton agar, as per Clinical Laboratory Standard Institute (CLSI) guidelines.[9] The antibiotics tested were as follows (potency in µg/disc): Ampicillin (10), cefuroxime (30), cefpodoxime (CPD) (30), ceftazidime (30), cefepime (30), cefotaxime (30), piperacillin (100), ticarcillin (75), piperacillin-tazobactam (100/10), ticarcillin-clavulanic acid (75/10), aztreonam (30), imipenem (IP) (10), meropenem (10), ertapenem (10), colistin (10), gentamicin (10), tobramycin (10), amikacin (30), netilmicin (30), ciprofloxacin (5), levofloxacin (5), lomefloxacin (10), and ofloxacin (5) (Hi-Media Laboratories Pvt., Ltd., Mumbai, India). *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains.

### Minimum inhibitory concentration determination

Minimum inhibitory concentrations (MICs) of antibiotics were determined by VITEK-2 AST-GN25 and AST-GN280 susceptibility cards in accordance with the CLSI recommendations and manufacturer's instructions, except that the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for tigecycline and colistin.[9,10] MICs were further determined by the E-test (bioMérieux, Marcy l'Etoile, France).

### Phenotypic screening for carbapenemase production

Isolates with reduced susceptibility to meropenem and IP (diameter of zones of inhibition ≤13 mm) by disc diffusion method and showed higher MICs as determined by the E-test were further screened for the production of carbapenemase. The phenotypic detection of the carbapenemase production was performed by the modified Hodge test (MHT) using ertapenem and meropenem discs (10 µg) for each isolate as per CLSI guidelines.[9] For MHT *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were used as positive and negative controls, respectively. MBL production detected by double-disc synergy tests (DDST) with both IP and meropenem discs (10 ug) plus ethelenediaminetetraacetic acid (EDTA) (750 ug) for all the carbapenem resistant isolates, as described earlier by Lee *et al*. and combined-disc synergy test (CDST) as described previously by Franklin *et al*. using IP and meropenem discs (10 µg) and 0.1 M EDTA (292 µg).[11,12] *K. pneumoniae* ATCC BAA-2146 and *P. aeruginosa* ATCC 27853 were used as positive and negative controls, respectively. MBL (IP/IP-inhibitor [IPI]) E-test was carried out to detect MBL as per manufacturer's instructions.

### DNA extraction and molecular detection

DNA was extracted from the bacterial isolates using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions. Polymerase chain reaction (PCR)-based detection of beta-lactamase (extended-spectrum beta-lactamase [ESBL]) genes (*bla*$_{CTXM}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$), Ambler class B MBLs (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$, *bla*$_{GIM}$, *bla*$_{SIM}$ and *bla*$_{NDM-1}$), Ambler class D (*bla*$_{OXA-23}$, *bla*$_{OXA-24}$ and *bla*$_{OXA48}$) and serine carbapenemases (*bla*$_{KPC}$, *bla*$_{GES}$ and *bla*$_{NMC}$) were carried out on the isolates using Gene Amp 9700 PCR System (Applied Biosystems, Singapore).[13-16] PCR products were run on 1.5% agarose gel, stained with ethidium bromide visualized under ultraviolet light and photographed. The amplicons were purified using QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany).

### DNA sequencing and sequence analysis

Automated sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (http://blast.ncbi.nlm.nih.gov).

### Conjugation experiments

Transfer of resistance genes by conjugation was assayed by mating experiments in Luria-Bertani broth using the clinical *Citrobacter* isolates (parental strains) as donors and an azide-resistant *E. coli* J53 as the recipient strain using 1:10 ratio. The transconjugants were selected on Luria-Bertani agar with selection based on growth on agar in the presence of ceftazidime (30 µg/ml) and sodium azide (100 µg/ml).[16] Plasmids were separated and compared by co-electrophoresis with plasmid of known sizes from *E. coli* (V517 and 39R861) on a horizontal 0.5% agarose gel at 50 volts for 3 h. Bands were visualized with UV transilluminator after staining with 0.05% ethidium bromide.

### Strain molecular typing

Repetitive element based-PCR (REP-PCR), enterobacterial repetitive intergenic consensus (ERIC-PCR) and randomly amplified polymorphic DNA (RAPD) assays were performed to characterize *Citrobacter* spp. recovered from patients.[17,18]

### Plasmid analysis

Plasmids from each parental strain and its transconjugants were extracted by using Qiagen plasmid mini kit (GmbH, Hilden, Germany) as per manufacturer's Instructions. Extracted plasmid DNA were subjected to plasmid-based replicon incompatibility (Inc.) typing by using eighteen pairs of primers to perform five multiplex and three single PCRs which recognized F, FIA, FIB, FIC, B/O, X, Y, N, P, W, T, A/C, HI1, HI2, I1-Ic, L/M, K, and FII replicons as described previously.[19] Plasmid replicons were determined for the ESBL and carbapenemase-producing clinical isolates.

## RESULTS

A total of 221 *Citrobacter* spp. isolates were recovered from clinical specimens from different patients (one isolate per patient) admitted to the surgical ward, medicinal ward and medical and surgical ICUs of a tertiary care center. Distribution of *Citrobacter* spp. isolates from various samples is shown in Figure 1 and Table 1.

The largest proportion of specimens were from UTI (98 or 44%), followed by 19% (43) in skin and soft tissue infections (SSTIs), 13% (29) in blood stream infections (BSIs), 14% (30) in Intra-abdominal infections (IAIs) and miscellaneous and 10% (21) in Respiratory tract infections (RTIs), respectively. Among 221 tested isolates, 179 (80.9%) isolates showed MIC >4 µg/ml against IP and meropenem. The majority of Carbapenem-resistant *Citrobacter* spp. were from urine 48% (87), followed by 21% (37) in wound swabs and pus, 12% (21) in IAIs and miscellaneous, 11% (20) in blood and endo-tracheal aspirate (09), BAL (05) both together constitute 08% (14), respectively [Table 1].

One hundred and ninety-eight out of 221 isolates, showed resistance to penicillins and third generation cephalosporins by the disc diffusion method, among them 179 (80.99%) were found to exhibit reduced susceptibility to IP and meropenem (diameter of zones of inhibition ≤15 mm) and 145 were found to have MIC values for IP, meropenem and ertapenem ranging from 8 to 32 µg/ml as per CLSI breakpoints. All the 221 isolates were found to be susceptible to colistin while (167/221) 75.56% were susceptible to tigecycline *in vitro* as per EUCAST MIC breakpoints. Of 221 isolates, 179 were found carbapenem-resistant as MICs was >4 µg/ml against IP and meropenem as determined by the E-test and VITEK-2, MHT for carbapenemase production was positive for 34.84% (77), DDST in 51.58% (114), CDST in 50.67% (112) isolates and MBL (IP/IPI) E-test was positive for 58.37% (129) isolates. Results of different phenotypic tests of *Citrobacter* spp. recovered from various clinical specimens are shown in Tables 2 and 3.

In these phenotypic tests from different infection sites among 130 *Citrobacter freundii* tested, carbapenem resistance was detected in 82.30% (107) isolates. MBL E-test was found positive for 78.64% (81), followed by CDST in 54.6% (71), DDST in 53.8% (70), and MHT in 39.2% (51) Table 2. Among 91 *Citrobacter koseri* tested, carbapenem resistance was detected in 79.1% (72) isolates MBL E-test found positive for 52.74% (48) isolates, followed by CDST in 47.3% (43), DDST in 46.15% (42) and MHT in 28.57% (26) [Table 3].

Of 221 isolates, 179 (80.99%) were found to exhibit reduced susceptibility to IP and meropenem and were ESBL

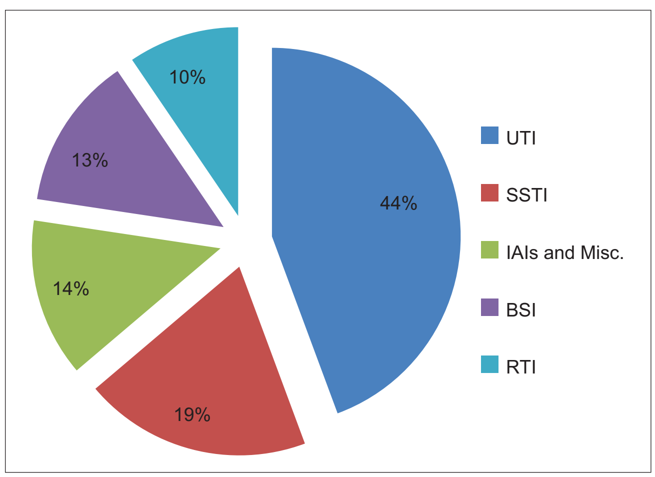


**Figure 1:** Distribution of *Citrobacter* spp. from various sites of infection

**Table 1: The distribution of carbapenem resistant *Citrobacter* spp. from total isolated**

| Specimen | Wound and Pus *n* (%) | Blood *n* (%) | Endo tracheal aspirate *n* (%) | BAL *n* (%) | Urine *n* (%) | Drain tip, other fluids *n* (%) | Miscellaneous *n* (%) | Total |
|---|---|---|---|---|---|---|---|---|
| Total isolated *Citrobacter* spp. | 43 (19.45) | 29 (13.12) | 13 (5.88) | 8 (3.6) | 98 (44.34) | 21 (9.5) | 9 (4) | 221 |
| Resistant *Citrobacter* spp. | 37 (20.7) | 20 (11.2) | 9 (5) | 5 (2.8) | 87 (48.60) | 16 (8.9) | 5 (2.8) | 179 |
| *C. frundii* | 23 (21.5) | 12 (11.2) | 6 (5.7) | 4 (3.7) | 49 (45.8) | 11 (10.3) | 2 (1.8) | 107 |
| *C. koseri* | 14 (19.4) | 8 (11.1) | 3 (4.2) | 1 (1.4) | 38 (52.8) | 5 (6.9) | 3 (4.2) | 72 |

*C. freundii*: *Citrobacter freundii*, *C. koseri*: *Citrobacter koseri*, BAL: Broncho-alveolar lavage

**Table 2: Percentage and result of different phenotypic tests of *C. freundii recovered* from various infection sites**

| Infection site | Total | Carbapenem resistant by MIC[a] | MBL E-Test[b] | MHT[c] | CDST[d] | DDST[e] |
|---|---|---|---|---|---|---|
| SSTI | 25 | 23 | 19 | 15 | 18 | 18 |
| BSI | 17 | 12 | 8 | 5 | 7 | 7 |
| UTI | 58 | 49 | 36 | 20 | 28 | 28 |
| IAIs and others | 18 | 13 | 10 | 7 | 10 | 10 |
| RTI | 12 | 10 | 8 | 4 | 7 | 8 |
| Total (%) | 130 | 107 (82.30) | 81 (78.64) | 51 (39.2) | 70 (53.8) | 71 (54.6) |

[a]MIC values for imipenem, meropenem, and ertapenem ≥4 μg/ml, [b]MBL (IP/IPI) E-test, [c]MHT: Modified Hodge test, [d]CDST: Combined-disc synergy test, [e]DDST: Double-disc synergy tests. *C. freundii*: *Citrobacter freundii*, MBL: Metallo-beta-lactamase, SSTI: Skin and soft tissue infection, BSI: Blood stream infection, UTI: Urinary tract infection, IAIs: Intra-abdominal infections, RTI: Respiratory tract infection, MIC: Minimum inhibitory concentration, IP: Imipenem, IPI: Imipenem inhibitor

**Table 3: Percentage and result of different phenotypic tests of *C. koseri* recovered from various infection sites**

| Infection site | Total | Carbapenem resistant by MIC[a] | MBL[b] E-test | MHT[c] | CDST[d] | DDST[e] |
|---|---|---|---|---|---|---|
| SSTI | 18 | 14 | 12 | 8 | 11 | 11 |
| BSI | 12 | 8 | 3 | 2 | 2 | 2 |
| UTI | 40 | 38 | 24 | 12 | 22 | 22 |
| IAIs and others | 12 | 8 | 6 | 2 | 5 | 6 |
| RTI | 9 | 4 | 3 | 2 | 2 | 2 |
| Total (%) | 91 | 72 (79.12) | 48 (52.74) | 26 (28.57) | 42 (46.15) | 43 (47.25) |

*C. koseri*: *Citrobacter koseri*, SSTI: Skin and soft tissue infection, BSI: Blood stream infection, UTI: Urinary tract infection, IAIs: Intra-abdominal infections, RTI: Respiratory tract infection, MIC: Minimum inhibitory concentration, MBL: Metallo-beta-lactamase, MHT: Modified Hodge test, CDST: Combined-disc synergy test, DDST: Double-disc synergy tests, a- MIC values for imipenem, meropenem, and ertapenem ≥4 μg/ml, b- MBL (IP/IPI) E-test, c- MHT: Modifi ed Hodge test, d- CDST: Combined-disc synergy test, e- DDST: Double-disc synergy tests

producers and among them 145 were found to have MIC values for IP, meropenem, and ertapenem ranging from 8 to 32 μg/ml as per CLSI breakpoints. The presence of $bla_{NDM-1}$ was detected in 55.30% (99/179) while $bla_{VIM}$ was present in 17.87% (32/179) of carbapenem-resistant strains. Based on Automated sequencing the genes were characterized and known sequences were compared using the BLAST facility (http://blast.ncbi.nlm.nih.gov). The sequences of *bla*$_{NDM-1}$ from *C. freundii and C. koseri* determined in this study have been assigned GenBank accession no. KR816561 and KR816562.

From UTIs, a single NDM-1 gene was present in 26 *C. freundii* isolates. NDM-1, TEM-1 and CTXM-15 altogether were found in 13 isolates while SHV, CTXM-15, and NDM-1 gene were present in 15 isolates. SHV, CTXM-15 and VIM-2 gene were present in 12 isolates whereas VIM-2, TEM-1, and CTXM-15 were found in 10 isolates.

In *C. koseri*, a single NDM-1 gene was present in 21 isolates, NDM-1, TEM-1, SHV, and CTXM-15 together were found in 18 isolates while CTXM-15 and NDM-1 gene were present in 18 isolates. VIM-2, CTXM-15, and TEM-1 altogether were present in 03 isolates [Figure 2].

From BSIs, NDM-1, SHV, TEM-1, and CTXM-15 were found in 5 *C. freundii* isolates while VIM-2, TEM-1, SHV, and CTXM-15 were altogether detected in 3 isolates whereas In *C. koseri* NDM-1 along with TEM-1, CTXM-15, and SHV genes was present in 03 isolates [Figure 3].

From RTIs, NDM-1, CTXM-15, SHV, and TEM-1, genes altogether were present in 06 *C. freundii* isolates while one isolate had the co-presence of VIM-2, TEM-1, CTXM-15, and SHV-12 gene. In *C. koseri* co-presence of NDM-1, TEM-1, CTXM-15, and SHV genes was detected in 03 isolates [Figure 4].

From SSTIs, *C. freundii* NDM-1, CTXM-15, TEM-1, and SHV genes altogether were present in 11 isolates, while copresence of VIM-2, CTXM-15, TEM-1, and SHV gene were detected in 5 isolates, 08 isolates, 05 isolates with

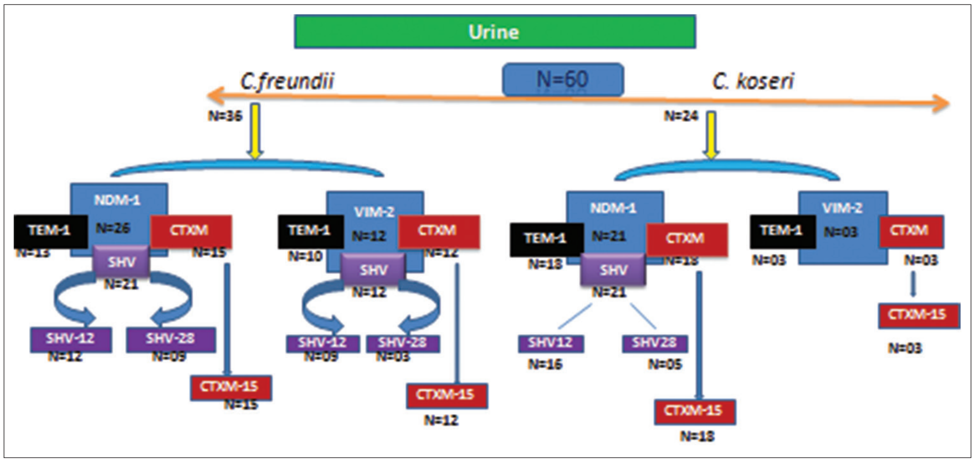


**Figure 2:** Distribution of beta-lactamase genes in *Citrobacter* spp. isolated from urine

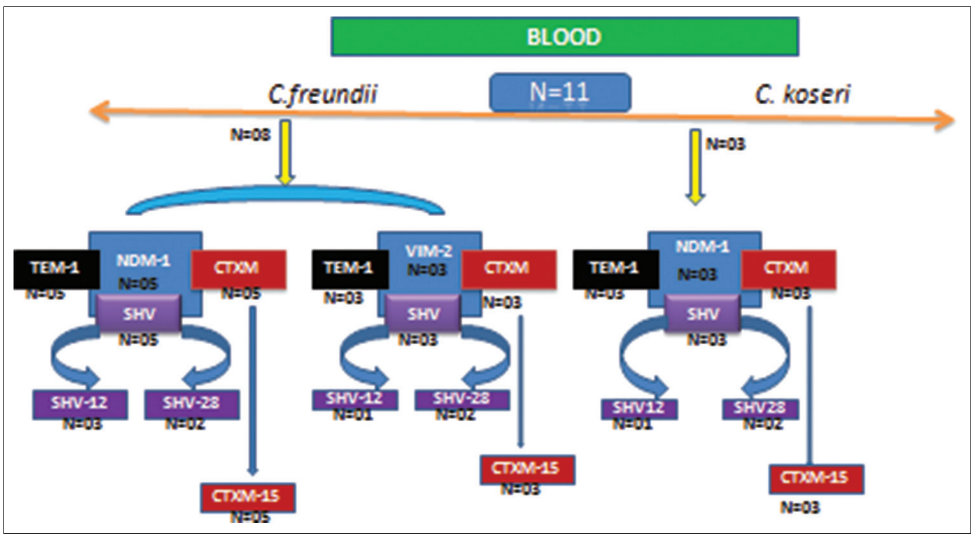


**Figure 3:** Distribution of beta-lactamase genes in *Citrobacter* spp. isolated from blood stream infections

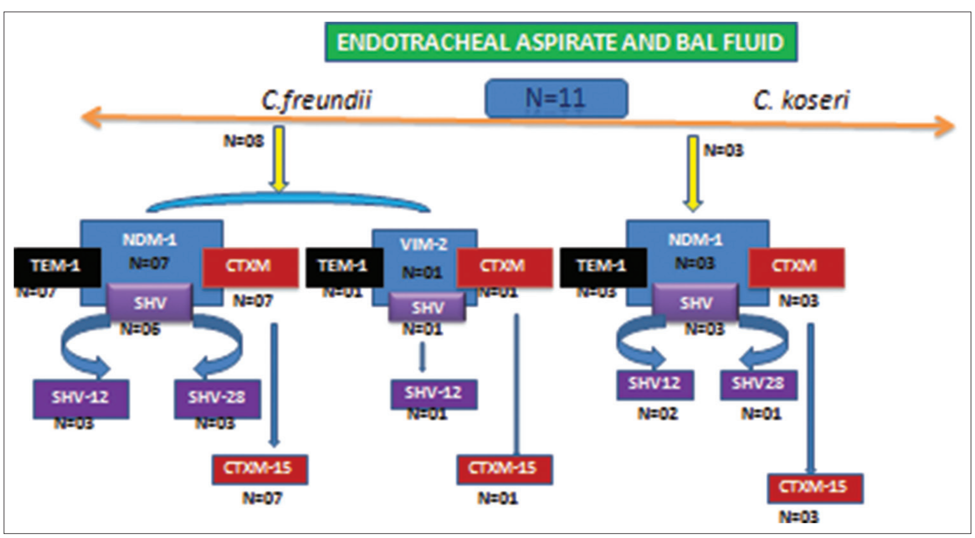


**Figure 4:** Distribution of beta-lactamase genes in *Citrobacter* spp. isolated from respiratory tract infections

VIM-2 also had and 05 isolates with also had CTXM-15 whereas in *C. koseri* NDM-1, SHV, TEM-1, and CTXM-15 genes were present in 7 isolates while copresence of VIM-2, CTXM-15, and TEM-1 was detected in 3 isolates [Figure 5].

From IAIs and miscellaneous in *C. freundii* NDM-1, CTXM-15, TEM-1, and SHV altogether were present in 8 isolates while VIM-2, CTXM-15. Moreover, TEM-1 were detected in 02 isolates whereas in *C. koseri* 6 isolates had co presence of NDM-1, SHV, CTXM-15, and TEM-1 genes [Figure 6].

## Strain molecular typing

Genotypic analysis by molecular typing of 81 strains of *C. freundii* (MBL producers) using RAPD PCR produced an average of 14–18 fragments per *C. freundii* strains. There were all together 10 RAPD pattern assigned as CF-A to CF-J [Figure 7].

As per ERIC PCR and REP PCR banding pattern, the isolates showed a genotypic diversity with 08 clonal clusters exhibited by 81 isolates. Genotypic analysis using REP PCR produced an average of 6–8 fragments per *C. freundii* strains [Figure 8].

Genotypic analysis by molecular typing of 48 strains of *C. koseri* using RAPD PCR produced an average of 10–12 fragments per *C. koseri* strains. There were all together 6 RAPD pattern assigned as CK-A to CK-F [Figure 9].

As per ERIC PCR and REP PCR banding pattern, 06 clonal clusters were exhibited by 48 isolates (MBL producers). Genotypic analysis using ERIC PCR produced an average of 12–18 fragments per *C. koseri* strains [Figure 10].

RAPD PCR distinguishes the various clones from one another better than REP PCR and ERIC PCR [Figures 7-10]. In molecular strain typing RAPD types distributed between various REP and ERIC types.

## Plasmid replicon typing, transferability and conjugation studies

Conjugation experiments revealed that $bla_{NDM\text{-}1}$ was transferable via a plasmid along with other beta-lactamase genes carried on other plasmids. Plasmid profiling of the

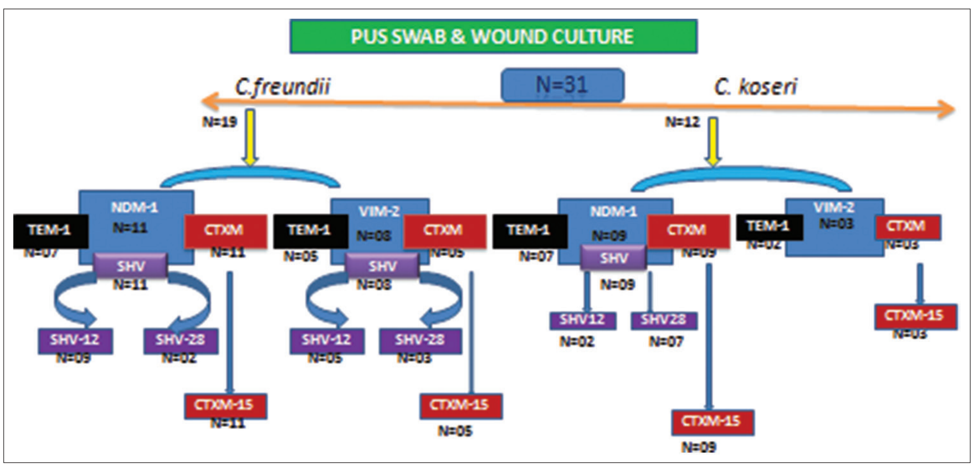


Figure 5: Distribution of beta-lactamase genes in *Citrobacter* spp. isolated from skin and soft tissue infections

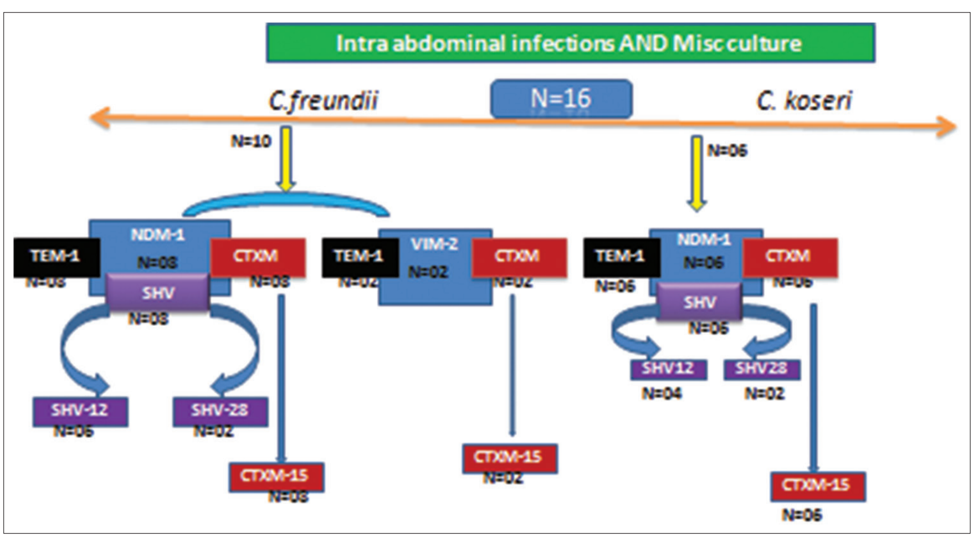


Figure 6: Distribution of beta-lactamase genes in *Citrobacter* spp. isolated from intra-abdominal infections and miscellaneous culture

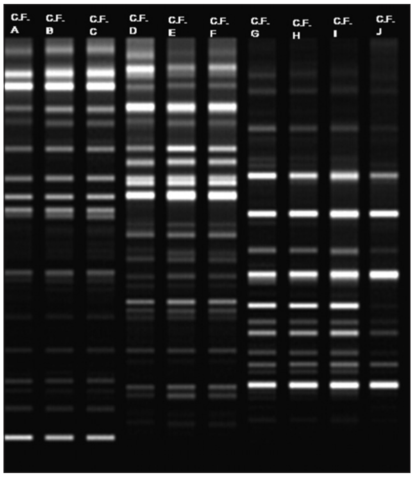


**Figure 7:** Randomly amplified polymorphic DNA polymerase chain reaction banding pattern among 10 clonal clusters of *C. freundii*

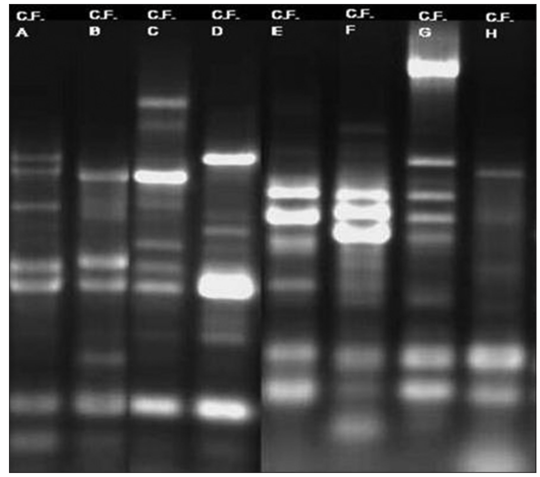


**Figure 8:** Repetitive element based-polymerase chain reaction banding pattern among 8 clonal cluster of *Citrobacter freundii*

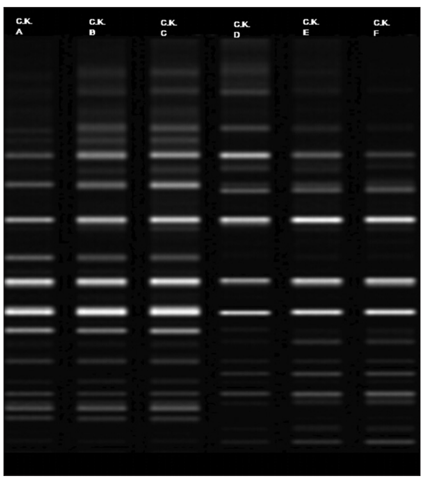


**Figure 9:** Randomly amplified polymorphic DNA polymerase chain reaction banding pattern among 6 clonal clusters of *Citrobacter koseri*

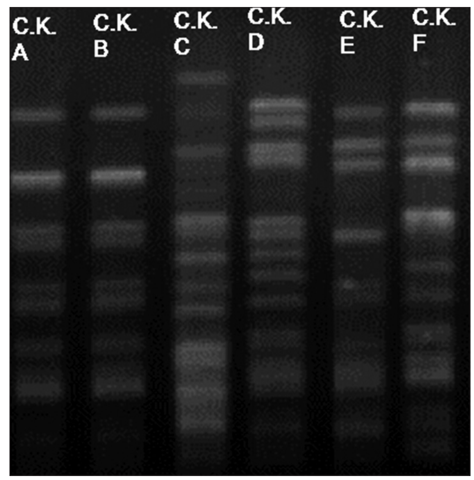


**Figure 10:** Enterobacterial repetitive intergenic consensus-polymerase chain reaction banding pattern among 6 clonal cluster of *Citrobacter koseri*

isolates showed that $bla_{\text{NDM-1}}$ was carried on plasmids ranging in sizes from 35 to 130 kb and $bla_{\text{VIM}}$ was carried on 50 to 200 kb size plasmids. All of the plasmid types were transferable. From UTI 50% ($n$ = 20), SSTIs, BSIs, RTIs and IAIs and others 50% ($N$ = 23) of multidrug resistant *C. freundii* were randomly selected as a donor *Citrobacter* spp. strains for conjugation studies and plasmid typing [Table 4].

From SSTIs, BSIs, UTIs, RTIs, and IAIs and others 50% ($N$ = 24) of multidrug resistant *C. koseri* were randomly selected as a donor *Citrobacter* spp. strains for conjugation studies and plasmid typing [Table 5].

MIC values for IP, meropenem and ertapenem among transconjugants are ranging from 8 to 32 µg/ml as per CLSI breakpoints. Both *bla*TEM-1 and *bla*SHV were associated with Inc. FIA, Inc. FIB, Inc. FIC multiple replicons. The $bla_{\text{NDM-1}}$ gene was located on Inc. A/C, Inc. FII and Inc. N plasmids. $Bla_{\text{VIM-2}}$ was carried on plasmids belonging to Inc. FII replicons, Inc. B/O replicons and Inc. nreplicons. Majority of *bla*CTX-M-15 was associated with multiple replicons either (Inc. FIA, Inc. FIB) OR (Inc. FIIB, Inc. FIB) type [Tables 4 and 5].

## DISCUSSION

*Citrobacter* is an opportunistic pathogen causing outbreaks where there are local or systemic breaches to host defenses. Common infections caused by *Citrobacter* spp. are UTI, bacteremia, meningitis, pneumonia, osteomyelitis, peritonitis, and endocarditis.[3,6,7,20-25] It has been a cause of neonatal sepsis,[4-7] and IAI.[26] *Citrobacter* bacteremia is associated with a high mortality rate between 33% and 48%.[6,7,27] *C. freundii* and *C. koseri* are the two most common pathogens and infections can be acquired from exogenous as well as endogenous

**Table 4: Transferability of MBL and ESBL gene present along with plasmid typing of *C. freundii* isolates**

| Isolate | MBL gene | Plasmid type | Transferability | Other ESBL gene present | | | Plasmid type | | | Transferability |
|---|---|---|---|---|---|---|---|---|---|---|
| UC324 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIA | FIA, FIB | Transferable |
| UC384 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIC | FIA | FIA, FIB | Transferable |
| UC641 | NDM-1 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| UC729 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIC | FIB | FIA, FIB | Transferable |
| UC899 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIA | FIA, FIB | Transferable |
| UC1013 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| UC1117 | NDM-1 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| UC1209 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIC | FIA | FIA, FIB | Transferable |
| UC1303 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| UC1495 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIC | FIB | FIA, FIB | Transferable |
| UC1532 | VIM-2 | N | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIA | FII, FIB | Transferable |
| UC1583 | VIM-2 | B/O | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIA | FII, FIB | Transferable |
| UC1681 | VIM-2 | B/O | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIC | FII, FIB | Transferable |
| UC1805 | VIM-2 | FII | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIC | FIA | FII, FIB | Transferable |
| UC2620 | VIM-2 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIC | FII, FIB | Transferable |
| UC3030 | VIM-2 | B/O | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIC | FIB | FII, FIB | Transferable |
| UC3786 | VIM-2 | B/O | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIA | FII, FIB | Transferable |
| UC4423 | VIM-2 | B/O | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FII, FIB | Transferable |
| UC4503 | VIM-2 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FII, FIB | Transferable |
| UC4522 | VIM-2 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIC | FII, FIB | Transferable |
| ETB 127 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIA | FIC | FIA, FIB | Transferable |
| ETB 273 | VIM-2 | N | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| ETB 487 | NDM-1 | N | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| ETB561 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| BACT49 | VIM-2 | N | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| BACT301 | VIM-2 | N | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIA | FIC | FIA, FIB | Transferable |
| BACT437 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| BACT78 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| PC39 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| PC46 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIC | FIC | FIA, FIB | Transferable |
| PC71 | NDM-1 | A/C | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIB | FIC | FIA, FIB | Transferable |
| PC89 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| PC103 | NDM-1 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIA | FIA, FIB | Transferable |
| PC148 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIC | FIB | FIA, FIB | Transferable |
| PC195 | NDM-1 | N | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIA | FIA, FIB | Transferable |
| PC201 | VIM-2 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FII, FIB | Transferable |
| PC242 | VIM-2 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIA | FII, FIB | Transferable |
| PC312 | VIM-2 | FII | Transferable | TEM-1 | SHV-28 | CTXM-15 | FIA | FIC | FII, FIB | Transferable |
| DTP27 | NDM-1 | FII | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIB | FIA, FIB | Transferable |
| DTP41 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIA | FIC | FIA, FIB | Transferable |
| DTP69 | NDM-1 | A/C | Transferable | TEM-1 | SHV-12 | CTXM-15 | FIB | FIB | FIA, FIB | Transferable |
| DTP75 | VIM-2 | N | Transferable | TEM-1 | ND | CTXM-15 | FIA | ND | FIA, FIB | Transferable |
| DTP93 | VIM-2 | N | Transferable | TEM-1 | ND | CTXM-15 | FIA | ND | FIA, FIB | Transferable |

*C. freundii*: *Citrobacter freundii*

sources, being ubiquitous in nature as a saprophyte in soil and sewage and as a commensal in human gastrointestinal tract.

In our study, carbapenem-resistant *C. freundii* was the most prominent species isolated 59.78% (107/179) followed by *C. koseri* 40.22% (72/179) and our finding [Table 1] were similar to others as reported earlier.[28,29] These isolates showed a high level of resistance to the beta-lactam antibiotics as well as to the beta-lactam/beta-lactamase inhibitor combination which were tested in the study. Sixty-five percentage (145/221) isolates were found to be multi drug resistant, the resistance being to penicillins, cephalosporins, fluoroquinolones, and aminoglycosides using disc diffusion method. The majority of specimens were from urine 44%, followed by SSTI 19%, Drain tip, tissue, other body fluids, and miscellaneous culture constitute 14%, blood 13% and respiratory secretions 10%, respectively.[25,30] CPD resistance can be used as a phenotypic marker for ESBL detection in cases of UTI. The worldwide prevalence of ESBLs available at PubMed in *Citrobacter* spp. was reported to be 0.5–36%.[31,32] In our study, 80.9% (179/221) of *Citrobacter* isolates were ESBL producers and this study correlates well with another study by Khanna *et al.* from India.[25] $Bla_{\text{CTX-M-15}}$ was the only CTX-M reported in our study while others have reported $bla_{\text{CTX-M-35}}$, $bla_{\text{CTX-M-30}}$, $bla_{\text{CTX-M-14}}$ $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-3}}$ from USA,[33] Canada,[34] China,[35] UK,[36] France,[37] Poland,[38] Korea,[39] and Spain.[40] There are very few studies in Medical literature, regarding MBL detection among *Citrobacter* spp. in India and abroad as compared to other members of family *Enterobacteriaceae*. In our study,

**Table 5: Transferability of MBL and ESBL gene present along with plasmid typing of *C. koseri* isolates**

| Isolate | MBL gene | Plasmid type | Transferability | Other ESBL gene present | | | Plasmid type | | | Transferability |
|---|---|---|---|---|---|---|---|---|---|---|
| UC69 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIA | FIA, FIB | FIB | Transferable |
| UC145 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIB | FIA, FIB | FIC | Transferable |
| UC218 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIA | FIA, FIB | FIB | Transferable |
| UC356 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIB | FIA, FIB | FIC | Transferable |
| UC378 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIC | FIA, FIB | FIB | Transferable |
| UC615 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIB | FIA, FIB | FIB | Transferable |
| UC719 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIC | FIA, FIB | FIB | Transferable |
| UC861 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIC | FIA, FIB | FIB | Transferable |
| UC937 | A/C | A/C | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIA | FIA, FIB | FIC | Transferable |
| UC1148 | VIM-2 | FII | Transferable | TEM-1 | CTXM-15 | ND | FIC | FII, FIB | ND | Transferable |
| UC1361 | VIM-2 | FII | Transferable | TEM-1 | CTXM-15 | ND | FIB | FII, FIB | ND | Transferable |
| UC1417 | VIM-2 | FII | Transferable | TEM-1 | CTXM-15 | ND | FIA | FII, FIB | ND | Transferable |
| DTP43 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIB | FIA, FIB | FIC | Transferable |
| DTP81 | NDM-1 | FII | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIA | FIA, FIB | FIC | Transferable |
| DTP97 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIB | FIA, FIB | FIB | Transferable |
| PC21 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIA | FIA, FIB | FIB | Transferable |
| PC52 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIC | FIA, FIB | FIC | Transferable |
| PC98 | NDM-1 | FII | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIB | FIA, FIB | FIB | Transferable |
| PC112 | NDM-1 | N | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIA | FIA, FIB | FIC | Transferable |
| PC131 | VIM-2 | N | Transferable | TEM-1 | CTXM-15 | ND | FIB | FII, FIB | ND | Transferable |
| PC157 | VIM-2 | N | Transferable | TEM-1 | CTXM-15 | ND | FIA | FII, FIB | ND | Transferable |
| BACT64 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIB | FIA, FIB | FIB | Transferable |
| BACT58 | NDM-1 | N | Transferable | TEM-1 | CTXM-15 | SHV-28 | FIB | FIA, FIB | FIC | Transferable |
| ETB 375 | NDM-1 | A/C | Transferable | TEM-1 | CTXM-15 | SHV-12 | FIA | FIA, FIB | FIB | Transferable |

ESBL: Extended-spectrum beta-lactamase, MBL: Metallo-beta-lactamase, *C. koseri*: *Citrobacter koseri*

58.37% (129/221) of *Citrobacter,* were producing MBL genes. A study from Kolkata, India[41] have reported 41.67% of MBL production among *Citrobacter* spp. [Tables 2 and 3]. Their lower frequency might be due to the sample size and geographical region or to timing of the studies as the prevalence of these resistance genes in increasing with time.[30,41] Emergence of $bla_{\text{NDM-1}}$ producing *Citrobacter* isolates reported from Bangladesh,[42] Turkey,[43] Thailand,[44] France,[45] South Africa,[46] United Arab Emirates,[47] Canada,[48,49] and India.[50] We detected presence of $bla_{\text{NDM-1}}$ in 55.30% (99/179) while $bla_{\text{VIM}}$ was present in 17.87% (32/179) of carbapenem resistant strains. The presence of $bla_{\text{IMP}}$[51,52] and $bla_{\text{GIM}}$[53] has been reported in *Citrobacter* isolates in other countries, but we did not find any of these MBL in our study. Likewise, we found no $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$ as has been reported in *Citrobacter* spp. by Deshpande *et al.*[54] and Mavroidi *et al.*[55] PBRT of purified plasmids from the clinical isolates of *Citrobacter* spp. revealed Inc. N, Inc. A/C and Inc. FII type plasmids associated with NDM-1 carriage which correlates well with previous studies.[47,48,50] Carriage of NDM-1 has also been reported on plasmid Inc. HII, Inc. X-type and Inc. L/M.[47,48,50-52] Inc. FII, Inc. B/O and Inc. N replicon type plasmids were associated with $bla_{\text{VIM}}$ carriage suggesting that MBL genes are carried on multiple plasmids. RAPD PCR was better as compared to REP PCR and ERIC PCR [Figures 7-10]. This study has shown that the MBL genes are transmissible by conjugation, which suggests that the presence of plasmid-borne MBL genes among the organisms making up the gut flora may facilitate transmission of resistance genes from one organism to another.

## CONCLUSION

A high prevalence of carbapenem resistance was reported among *Citrobacter* isolates investigated in this study. This indicates spread of NDM-1 producing *Citrobacter* in central India. Early detection is important as the simultaneous presence of other resistance genes makes the organisms refractory to most of the common antibiotics used in clinical practice. Furthermore, the presence of these genes on plasmids that are transmissible to other species. Thus, the detection of genes for carbapenem resistance should be a major focus of infection control to prevent transmission of MBL genes to other patients and to other bacterial species within the same patient.

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### Conflicts of interest

There are no conflicts of interest.

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