

Carbapenemase and NDM-1 Production by *Escherichia coli* and *Klebsiella pneumoniae* from Patients belonging to a Rural Community in North India Hospitalized with Community-Acquired Infections

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Abstract

Carbapenem-resistant *Enterobacteriaceae*, especially New Delhi metallo- β -lactamase type-1-producing strains, exhibit multidrug resistance, thus posing a serious public health threat for treatment of infections caused by Gram-negative bacteria. However, most of the studies on the problem have been confined to hospitalized patients from urban population. The present study was carried out to detect carbapenemase production among *Escherichia coli* and *Klebsiella pneumoniae* isolated from rural population hospitalized with community acquired infections. A total of 296 non-repetitive isolates of *E. coli* and *K. pneumoniae* were subjected to Modified Hodge test for carbapenemase detection, Metallo- β -lactamase detection by MIC test strip MBL and molecular detection of bla_{NDM} gene by Polymerase Chain Reaction (PCR). Twenty three (7.8%) of the 296 isolates were detected as carbapenemase producers, of which five (21.7%) were found to harbor bla_{NDM} genes by PCR. Gene sequencing of all the five isolates revealed bla_{NDM-1} genes. The present study showed the prevalence of carbapenemase-producing *E. coli* and *K. pneumoniae* in the rural community of North India and spread of NDM-1.

Keywords: Carbapenemase-producing enterobacteriaceae, New Delhi metallo-beta-lactamase type-1, Multidrug resistance, Community-acquired infections

Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) have been increasingly reported among clinical isolates worldwide, thus posing a serious public health threat for treatment of infections caused by multidrug-resistant Gram-negative

bacteria.¹ Carbapenem resistance due to production of carbapenemases belonging to three classes of β -lactamases has been identified in *Enterobacteriaceae*, viz., the Ambler class A, B (metallo- β -lactamases) and D β -lactamases.¹ New Delhi metallo- β -lactamase type-1 (NDM-1), a plasmid mediated metallo- β -lactamase (MBL), is the more recently

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identified MBL type of carbapenemases, first reported in 2009 among *K. pneumoniae* and *E. coli* isolates from a Swedish patient of Indian origin.² Since then, pathogens harboring NDM-1 type of β -lactamase gene (*bla*_{NDM-1}) have been reported from all over the world in species belonging to *Enterobacteriaceae*, most commonly in *E. coli* and *K. pneumoniae*.¹ Plasmids carrying *bla*_{NDM-1} gene are often associated with other drug-resistance genes thus leaving very few options for the treatment of infections due to multidrug resistant NDM-1 producers.¹ Apart from its reported isolation from hospitalized patients,³ intestinal colonization in community with consequent spread in the environment has also been reported.⁴ In India, most of the reports of CRE among clinical isolates, especially harboring *bla*_{NDM-1} are confined to patients attending hospitals in metropolitan cities⁵ with hardly any report from population attending hospitals in rural area.⁶ The present study was carried out to determine the prevalence of CRE and NDM-1 among *E. coli* and *K. pneumoniae* isolated from various clinical specimens in a multispecialty hospital catering to rural community in North India.

Materials and Methods

Period of Study

The study was conducted for 12 months duration in 2015. Prior approval was obtained from institutional ethics committee constituted by SGT University comprising of external experts as members.

Study Population

The study population included patients with community-acquired infections (CAIs), admitted in various clinical departments of SGT Hospital, an 800-bedded multispecialty hospital situated in a rural belt in Northern India. CAI was defined by a positive bacterial culture obtained within 48 hours after hospital admission without any history of hospitalization or antibiotic treatment in the last 30 days.⁷

Collection of Demographic and Epidemiological Information

A pre-designed proforma was employed to collect demographic and epidemiological information on age, sex, occupation, companion livestock animals at home, history of hospitalization with more than five days duration during preceding five years based on records available with the patient and history of illness in the last 12 months suggestive of infectious nature viz., chest infections, loose motions, suppurative infections of skin, and ear infections for which treatment was sought from local practitioners.

Clinical Specimens

The clinical specimens were received by the microbiology

department of SGT Hospital under proper transport conditions and were comprised of urine, stool, pus, blood, sputum, wound swabs and body fluids. The specimens were processed for identification as per standard bacteriological techniques and only those isolates identified as *E. coli* and *K. pneumoniae* based on typical colony morphology on MacConkey agar or Cystine lactose electrolyte-deficient (CLED) medium and biochemical characteristics were selected for further processing.⁵

Antimicrobial Susceptibility Testing (AST) and Determination of Minimum Inhibitory Concentration (MIC) for Carbapenems

AST was carried out by Kirby-Bauer disc diffusion method and the results were interpreted based on zone of inhibition validated as per CLSI guidelines 2017.⁸ The antimicrobial discs were commercially procured (Himedia, Mumbai, India), which included ertapenem (10 μ g), meropenem (10 μ g) and imipenem (10 μ g).

Additional discs of ampicillin (10 μ g), amoxicillin-clavulanic acid (20/10 μ g), piperacillin/tazobactam (100/10 μ g), amikacin (30 μ g), gentamicin (10 μ g), netilmicin (30 μ g), cefoxitin (30 μ g), cefotaxime (30 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), cefepime (30 μ g), aztreonam (30 μ g), ciprofloxacin (5 μ g), ofloxacin (5 μ g) and co-trimoxazole (25 μ g) were also put up as a part of routine AST. Tigecycline (15 μ g) and colistin (10 μ g) were further employed for AST to study co-resistance pattern of the strains showing resistance to any of the carbapenems.

MIC for ertapenem, imipenem and meropenem was determined by MIC test strip with gradient of antimicrobial concentrations of ertapenem, imipenem and meropenem from 0.002 μ g/mL to 32 μ g/mL (Liofilchem, Italy; www.liofilchem.net). MIC was determined for ertapenem based on CLSI guidelines considering MIC (μ g/mL) ≤ 1 as susceptible, >1 to <2 as intermediate and ≥ 2 as resistant while for meropenem and imipenem, MIC (μ g/mL) ≤ 1 as susceptible, >1 to <4 as intermediate and ≥ 4 as resistant.^{5,8}

Phenotypic Test for Carbapenemases Detection

Modified-Hodge Test (MHT)

This was carried out as per CLSI guidelines 2017.⁸ Isolates showing resistance to ertapenem or imipenem or meropenem were subjected to detection of carbapenemase production. Inoculum of *E. coli* ATCC 25922 in Mueller-Hinton broth matched with 0.5 McFarland standard was used for lawn culture on to Mueller-Hinton agar plate. Ertapenem (10 μ g) disc was placed in the center of the plate and test strain was streaked in a straight line from the edge of the disc towards the edge of the plate. The inoculated plate was incubated aerobically overnight at 35°C. The isolate showing a clover leaf-like indentation

in the zone of inhibition of *E. coli* ATCC 25922 around the disc along the streak of test strain was considered positive and absence of indentation in the zone was considered negative. *K. pneumoniae* ATCC BAA-1705 (MHT positive) and *K. pneumoniae* ATCC BAA-1706 (MHT negative) were used for quality control.

Test for MBL Detection

Screening Test

Commercially available MIC test strip MBL (Liofilchem, www.liofilchem.net) was used to carry out presumptive screening of MBL-producing strains.⁵ The MBL strip had imipenem (IMI) gradient at one end (4–256 µg/mL) and gradient of imipenem (1–64 µg/mL) plus a constant level of EDTA (4 µg/mL) at other end (IMD). The MBL production was considered positive when a strain showed MIC ratio of (IMI/IMD) as ≥ 8 .⁵

Detection of *bla*_{NDM} Gene

Polymerase chain reaction (PCR) was carried out for strains positive for NDM production in MIC test strip MBL using the pre-published sequences, 5'-ACCGCTGGACCGATGACCA-3' and reverse 5'-GCCAAAGTTGGGCGCGGTTG-3' which amplified 264 bp fragment of the *bla*_{NDM} gene⁹ (Fig. 1). Sequencing was done in PCR products of all the positive strains with amplicons purified by PCR purification kit (QIAGEN, Hidden, Germany), followed by sequencing on ABI PRISM 3130XL sequencer using Big Dye Terminator cycle sequencing kit (Perkin Elmer). The accuracy of the base calling with the chromatogram peaks for the sequences obtained were checked using BIOEDIT software followed by blast at the National Center for Biotechnology Information website search (<https://blast.ncbi.nlm.nih.gov/Blast>).

cgi). The derived sequences were aligned with reference sequences from the database of GenBank and accession numbers were obtained after submission to the GenBank.

Statistical Analysis

Student's T-test was used for continuous variable, viz., age while Chi-square test was done for discrete variables, viz., carbapenemase positivity and epidemiological risk factors. Odds ratio was used to find out the association of carbapenemase positivity with male/female.

Results

A total of 296 non-repetitive *E. coli* and *K. pneumoniae* isolated in various clinical specimens from patients with CAI during 2015 were included in the study. The age group of the patients ranged from 13 to 75 years (mean 34.6±18.2 year) with male:female ratio as 1:2.1. Out of the total 296 isolates of *E. coli* and *K. pneumoniae*, 26 (8.8%) isolates were found to be carbapenemase producers by disc diffusion method out of which 23 (7.8%) isolates were confirmed phenotypically by MIC determination and MHT comprising of *E. coli* 19 (82.6%) and *K. pneumoniae* 4 (17.4%). *E. coli* (n=19) were mainly isolated from urine (n=12), followed by pus (n=5), blood (n=1) and sputum (n=1) while *K. pneumoniae* (n=4) were isolated from urine (n=2), pus (n=1) and sputum (n=1). Majority of the patients were farmers and had companion livestock animals within the same residential premises. However, associated history of hospitalization and treatment from a local practitioner for ailments suspected to be infectious in nature were significantly higher in carbapenemase-producing group compared to the non-carbapenemase-producing group (Table 1).

Table 1. Demographic and Epidemiological Profile of Patients with Community Acquired Infections by Carbapenemase-producing *E. coli* and *K. pneumoniae*

Characteristics		<i>E. coli</i> and <i>K. pneumoniae</i> Isolates from Patients with CAI (n=296)		Statistical Analysis (p-value)
		Non-carbapenemase producers (n=273)	Carbapenemase producers (n=23)	
Age (Mean±SD)		34.6±18.2	44.1±19	p <.05
Male:Female		1:2.1	1:1.5	0.7*
Occupation as farmers		227 (83.2)	20 (86.9)	NS (0.66)
Livestock as companions		185 (68)	16 (69.6)	NS (0.86)
History of hospitalization (>5 days duration) in last 5 years)		38 (13.9)	12 (52.2)	p <.05
History of ailments suggestive of infectious nature requiring treatment from a local practitioner in last 12 months	No. with positive history	54 (19.8)	21 (91.3)	p <.05

CAI: Community-acquired infections

Figures within parenthesis indicate percentage

*Odds ratio

Antibiotic-resistance profile for *E. coli* and *K. pneumoniae* isolates with evidence for carbapenemase production against various antimicrobials evaluated through routine AST showed the following co-resistance profiles: ampicillin (100%), amoxicillin-clavulanic acid (100%), piperacillin/tazobactam (60.9%), amikacin (52.2%), gentamicin (69.6%), netilmicin (65.2%), cefoxitin (69.6%), cefotaxime (100%), ceftazidime (91.3%), ceftriaxone (100%), cefepime (69.6%), aztreonam (100%), ciprofloxacin (87%), ofloxacin (82.6%) and co-trimoxazole (73.9%). All the isolates were susceptible to tigecycline and colistin.

MBL Detection

Out of the 23 carbapenemase producers, five *E. coli* isolates were found to be MBL producers by MIC test strip MBL. All these *E. coli* strains were found to harbor *bla_{NDM}* by PCR (Table 2).

Table 2. Characteristics of *bla_{NDM-1}* Producing *E. coli* Isolates from Patients with Community-Acquired Infections

Strain ID No.	Specimens	PCR for <i>bla_{NDM}</i>	Sequencing Results	GenBank Accession No.	Epidemiological Characteristics		
					Companion Livestock Animals	H/O Hospitalization	H/O Treatment from Local Practitioner
<i>E. coli</i> NSU_4	Urine	+	<i>bla_{NDM-1}</i>	KU158104	-	+	-
<i>E. coli</i> NSU_5	Pus	+	<i>bla_{NDM-1}</i>	KU158105	-	-	+
<i>E. coli</i> NSU_6	Urine	+	<i>bla_{NDM-1}</i>	KU158106	-	+	+
<i>E. coli</i> NSU_7	Urine	+	<i>bla_{NDM-1}</i>	KU158107	-	-	+
<i>E. coli</i> NSU_8	Urine	+	<i>bla_{NDM-1}</i>	KU158108	-	-	+

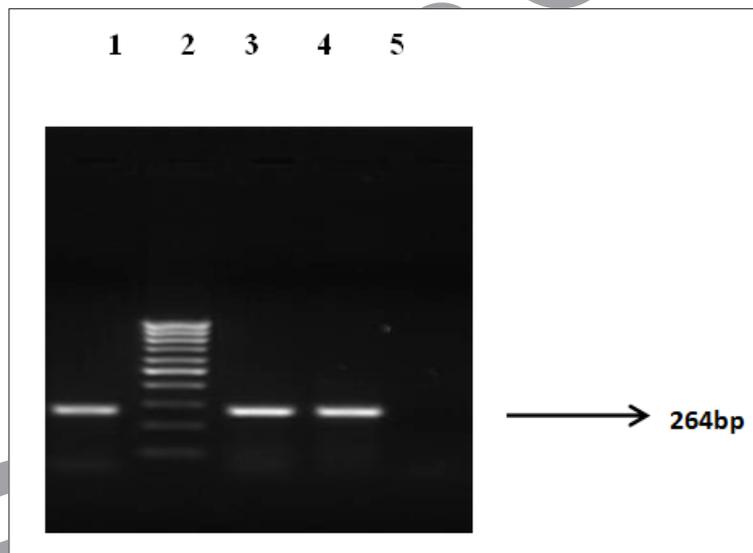


Figure 1: PCR Assay for Detection of *bla_{NDM}* Gene from Phenotypically MBL-Positive *E. coli* Isolates. Lanes: 1-Positive for NDM-1, 2-Marker 100bp, 3-Positive for NDM-1, 4-Positive for NDM-1 and 5-Negative-control

Molecular Characterisation of *bla_{NDM}* Gene

The partial nucleotide sequence of isolates revealed identity with the sequence of reference strain of *bla_{NDM-1}* (Fig. 1). The sequences were deposited in GenBank and the accession numbers were obtained as KU158104 to KU158108.

Discussion

In the present study, the prevalence of carbapenemase production among *E. coli* and *K. pneumoniae* isolated

from patients with CAI was found to be 7.8% indicating the existence of CRE in the rural community of north India. Studies in tertiary care hospitals from developing countries, viz., Nigeria and Uganda, reported prevalence of CRE as 15.2% and 22.4% respectively.^{10,11} Studies reported from India before 2006 failed to show any evidence of resistance for *E. coli* and *K. pneumoniae* to carbapenems.⁵ However, subsequently CRE were reported from many parts of India with a prevalence ranging between 5.2% and 51%.^{5,12} There are few reports on prevalence of CRE in rural population

from India. One study from rural southern India reported 22.1% of CRE in isolates from hospitalized children with blood stream infections,¹³ while another study from the same rural population on hospitalized patients reported a prevalence of carbapenemase-producing *E. coli* and *K. pneumoniae* among all categories of infections, i.e., 19.4%.⁶ However, in the later study, resistance to ertapenem was detected by disc diffusion method only in contrast to our study where detection rate of CRE was based on positivity by both MHT and MIC. Nevertheless, these reports indicate substantial evidence of CRE in rural community. The present study revealed 67.9% of the CAI patients having companion livestock animals in their residential premises which is pointing out that close association with companion livestock animals could be a risk factor for acquisition of CRE producers in the rural community due to contamination of water and food with animal faeces.¹⁴ However, CRE positivity in our study was more significantly associated with history of prior hospitalization in last 5 years and treatment in last 12 months for ailments suggestive of infections.

In the present study, all the MBL-positive isolates identified by using MIC test strip MBL were found to harbor *bla*_{NDM} and their sequence analysis showed all to be positive for *bla*_{NDM-1}. While studies conducted in the city of Delhi, India, reported other variants of *bla*_{NDM} among *E. coli* isolates, i.e., *bla*_{NDM-4}, *bla*_{NDM-5}, *bla*_{NDM-6}, *bla*_{NDM-7} and *bla*_{NDM-8},^{5,15} our study demonstrated only NDM-1 variety, which is in accordance with a study from northeast India.¹⁶ The prevalence of NDM-1 in the present study was found to be 1.7% among the *E. coli* isolates from patients with CAI, majority of the strains (four out of five) being isolated from urine while there was no evidence of NDM-1 production among *K. pneumoniae* isolates. This finding on prevalence of NDM-1 among *E. coli* and *K. pneumoniae* isolates is comparable with other studies from Vietnam (1.1%).¹⁷ On the other hand multiple studies conducted in different places of India reported the prevalence of *bla*_{NDM-1} as 5.2–8.1% among enterobacteriaceae isolated from clinical specimens, which is higher as compared to our findings,^{12,16,18} although these reports of NDM-1 positive isolates have mostly been limited to ICU and non-ICU patients rather than with CAI.^{12,17}

In the present study, CRE isolates exhibited a high degree of multidrug resistance to antimicrobials other than tigecycline and colistin with resistance rate ranging from 52.2% to 100%, which is comparable to a study from northeast India where all the CREs were susceptible to tigecycline and colistin and resistance rate ranging from 85.7% to 100% for other antimicrobials.¹⁶ Among the NDM-1-positive isolates in the present study, the AST profile revealed resistance to all the antimicrobials tested except for its 100% susceptibility to tigecycline and colistin. Other studies from different parts of India reported NDM-1-producing *E. coli* exhibiting sensitivity to tigecycline.^{16,18} However, a study from western India reported 3% and 6% resistance to

tigecycline and colistin, respectively among CRE isolates¹⁹ while a study from Vietnam reported colistin resistance (2.1%) among NDM-1-producing enterobacteriaceae.¹⁷ Although India serves as the major reservoir of NDM-1 producers, studies are mostly confined to urban hospital settings.²⁰ The present study raised its spread in rural settings, thus posing as a public health concern.

Conclusion

The present pilot study highlights the need for larger studies to find out the possible reservoirs and burden of NDM-1-producing *E. coli* and *K. pneumoniae* in rural community.

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Conflict of Interest: None

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