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New Delhi Metallo-β-lactamase and other mechanisms of carbapenemases among Enterobacteriaceae in rural South India

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ABSTRACT

Objectives: The emergence and dissemination of carbapenem-resistant Enterobacteriaceae (CRE) is an important public health problem. This study aimed to understand the prevalence and mechanisms of carbapenem resistance in clinically important members of Enterobacteriaceae in rural South India.
Methods: Routine clinical isolates of *Escherichia coli* and *Klebsiella* spp. were tested for Ertapenem (ETP) non-susceptibility by the disk diffusion method over a 3-year period (2012–2014). The ETP non-susceptible isolates were preserved, and tested for the MIC of carbapenems and the carriage of major carbapenemase-encoding genes. Representative genes were sequenced and selective isolates were tested for the production of carbapenemase by carbapenem inactivation method.
Results: A total of 444 ETP non-susceptible isolates were identified in increasing incidence over the study period. Among them, MIC_{50} and MIC_{90} of carbapenems (excluding ETP) were 0.25–0.5 μg/mL and 8–16 μg/mL, respectively, and the prevalence of non-ETP carbapenem resistance was estimated as 3%. Among the 177 tested isolates, 65 (37%) had one or more carbapenemase-encoding genes with a predominance of New Delhi Metallo-β-lactamase (NDM; 32 of 65; 49.2%).
Conclusions: This study documented the MIC range for carbapenems, prevalence and mechanisms of carbapenem resistance among Enterobacteriaceae in rural South India. It substantiated NDM as a leading mechanism of carbapenem resistance and highlighted the importance of MIC testing in patient management.

1. Introduction

Carbapenems are often one of the last options available for the treatment of multi-drug resistant Gram-negative bacterial infections [1]. Nevertheless, in the recent past, the emergence and dissemination of carbapenem resistance have increasingly been reported across the globe [2,3], which limited the usage of carbapenems. Among the Enterobacteriaceae, carbapenem resistance in *Escherichia coli* (*E. coli*) and *Klebsiella* spp. poses the greatest risk to public health because of high prevalence, wide range of clinical infections, multi-drug resistance, and rapid spread of resistance to other bacterial strains and species. Additionally, therapeutic options for the clinical management of infections caused by these carbapenem-resistant Enterobacteriaceae (CRE) have become limited.

In India, data on the prevalence of CRE is scarce. The major study [4], which was partly reported from Chennai on the emerging mechanisms of CRE, was not aimed at measuring the prevalence, and its sample collection was focused on resistant strains. Further, there is no report on the mechanism of CRE from rural South India.

The mechanism of carbapenem resistance is primarily mediated by carbapenemases. Although non-carbapenemase-mediated carbapenem resistance has also been reported, it is not usually transferrable (via plasmids) to other bacteria. Hence, the development of carbapenem resistance without carbapenemase production is considered insignificant in terms of a public health perspective [5]. Further, among the carbapenemase encoding genes, NDM (New Delhi Metallo-β-lactamase), KPC (*Klebsiella pneumoniae* Carbapenemase), VIM (Verona Integron-encoded

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Metallo-β-lactamase), OXA (Oxacillinase), and IMP (Imipenemase) were recognised as the 'Big-Five Carbapenemases' by Public Health England, owing to their wide prevalence and public health importance [6]. Hence, this study aimed to focus on these resistance mechanisms (i.e., carbapenemase-producing Enterobacteriaceae (CPE)).

According to the World Health Organization (WHO) [7], the problem of antimicrobial resistance is burgeoning and neglected in India, and this could be due to the lack of valid surveillance data. The prevalence of antimicrobial resistance [8,9] has recently been reported, and the current study aimed to document the prevalence and mechanisms of carbapenem resistance in important members of Enterobacteriaceae. This will be helpful for designing and instigating the necessary control measures by public health authorities.

2. Methods

2.1. Study design

This cross-sectional study was designed to investigate the prevalence and mechanisms of carbapenem resistance in *E. coli* and *Klebsiella* spp. The study was conducted at the Department of Microbiology, Government Theni Medical College & Hospital (GTMC), Tamil Nadu, India. The hospital is a 900-bed tertiary care referral centre, primarily serving the rural population of Tamil Nadu and Kerala in South India.

The study was carried out among the clinical isolates recovered from the patients of our hospital during the period from January 2012 to December 2014. Representative isolates for testing purposes were randomly selected from the pool of total isolates. All of the clinical isolates identified as Ertapenem (ETP) non-susceptible (NS) were handled with Bio Safety Level 2 (BSL-2), bio-containment work precautions, throughout the study.

All experimental methods in the study protocol was carefully reviewed and approved by the Institutional Human Ethical Committee of Govt. Theni Medical College.

2.2. Bacterial isolates

Patients attending the hospital with symptoms of urinary tract infection, wound infection, respiratory tract infection, blood stream infection, diarrhoeal infection, etc., were tested for the clinically significant isolation of *E. coli*/*Klebsiella* spp. by bacterial culture of the respective clinical specimens such as urine, pus, sputum, blood, faeces and others as a routine testing practice. The organism was then identified by standard biochemical tests [10].

2.3. Susceptibility testing

Antimicrobial susceptibility test (AST) was performed on Mueller-Hinton agar (MHA) (HiMedia, India) using Kirby-Bauer disk diffusion method by following Clinical and Laboratory Standards Institute (CLSI) guidelines with ATCC 25922 *E. coli* as a quality control [11]. According to the CLSI [12], ETP was used as a screening agent for the selection of CPE. All freshly recovered pure clinical isolates of *E. coli* or *Klebsiella* spp. were subjected to ETP susceptibility test by Kirby-Bauer disk diffusion method using in-house prepared 10 μg ETP (Merck) disk. Isolates showing zone of inhibition (ZOI) < 22 mm to ETP were recognised as NS; further, ZOIs of 19–21 mm were categorised as intermediate resistant, and ZOIs ≤ 18 mm were identified as resistant [12]. Randomly selected non-susceptible (NS) isolates were preserved in 15% glycerol broth and stored at −80 °C.

The MIC of carbapenems was determined by agar dilution AST method. Thirteen doubling dilutions ranging from 0.016–64 μg/mL of carbapenems, including ETP (Merck), imipenem (IPM) (Sigma-Aldrich), meropenem (MEM) (Sigma-Aldrich), and doripenem (DOR) (Sigma-Aldrich) were tested for MIC against preserved ETP NS isolates on MHA by following CLSI guidelines [13]. The susceptibility cut-off point was defined as ≤0.5 μg/mL for ETP and ≤1 μg/mL for other carbapenems such as IPM, MEM, and DOR; similarly, the resistance cut-off point was defined as ≥2 μg/mL for ETP and ≥4 μg/mL for other carbapenems. The test isolates were interpreted as susceptible (S), intermediate (I) or resistant (R) as per CLSI recommendations [14].

2.4. Multiplex polymerase chain reaction

Total bacterial DNA was extracted by boiling lysis method from overnight colonies grown on nutrient agar (HiMedia, India). Total bacterial DNA extracted from the preserved isolates (ETP NS by disk diffusion method) was subjected to multiplex polymerase chain reaction (PCR) for the amplification of one or more of the carbapenemase encoding genes (bla_{NDM},bla_{KPC}, bla_{OXA-48}, bla_{VIM} and bla_{IMP}) using previously published primers (Supplementary Table S1) [15] with positive and negative controls. The positive controls were bla_{NDM} (*Klebsiella pneumoniae*, *K. pneumoniae*), bla_{KPC} (ATCC BAA 1705 *K. pneumoniae*), bla_{IMP} (*Acinetobacter baumannii*), bla_{VIM} (*Acinetobacter calcoaceticus*), and bla_{OXA-48} (*K. pneumoniae*); the negative control was master mix without DNA template. In a 25 μL reaction mixture, final components included DNA template 4 μL, *Taq* DNA polymerase 1U, betaine 1 M, primer mix (0.2 μM for KPC, OXA-48, and VIM; 0.3 μM for NDM; 1 μM for IMP), dNTP 0.2 mM, $MgCl_2$ 1 mM, 1 × PCR buffer and water. The cycling conditions were initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 56 °C for 45 s, and 72 °C for 60 s; final extension at 72 °C for 5 min. PCR amplicons were visualised on a 3% agarose gel containing ethidium bromide.

2.5. Sequencing PCR and gene sequencing

Randomly selected PCR positive DNA samples were subjected for sequencing PCR using previously published primers (Supplementary Table S1). The reaction mixture was set at a final volume of 50 μL with 1 × PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers (forward and reverse – each 0.2 μM), 0.5 U Hi-fidelity *Taq* DNA polymerase and 5 μL DNA template. The reactions were carried out as 2-min initial denaturation at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s at annealing temperature (62 °C for NDM, 60 °C for KPC, OXA-48 and VIM), and 120 s (60 s for VIM) extension at 72 °C. Finally, a 10-min extension was carried out at 72 °C. PCR amplicons were visualised on a 2% agarose gel containing ethidium bromide, and purified by ammonium acetate ethanol precipitation technique [16]. Nucleotide sequencing was performed by Macrogen Inc., (Seoul, Korea) and results were analysed and aligned with previously published sequences using BLAST program at National Centre for Biotechnology Information.

2.6. Phenotypic testing for carbapenemase production

Selective isolates were tested for the phenotypic production of carbapenemase by carbapenem inactivation method (CIM) [17]. A loopful of test bacteria grown on MHA was suspended in 400 μL of sterile water and an in-house prepared 10 μg MEM disk (Sigma-Aldrich) was placed in a flip cap vial; the suspension was then incubated at 35 °C for 2 h. Then the MEM disk was taken out and tested for retention of significant amounts of antimicrobial activity against ATCC 25922 *E. coli* by disk diffusion method [11]. Test results were measured as carbapenemase positive when there was no ZOI after 18 h of incubation; while, a ZOI of ≥15 mm was

measured as no carbapenemase production by the test isolate. ATCC BAA 1705 *K. pneumoniae*, and ATCC 25922 *E. coli* were used as positive and negative controls for carbapenemase production, respectively [17,18].

2.7. Data analysis

All patient and isolate details and susceptibility data were documented and analysed with WHONET software ver. 5.6 (www.whonet.org). Second and subsequent isolates of the same species from the same patient within 30 days were considered as duplicate and excluded from the analysis, irrespective of susceptibility profile and specimen type. Scatterplot analysis was performed for the MIC of carbapenems among ETP NS isolates. Statistical analysis was performed with χ^2 test and the significance level set at $P < 0.05$.

3. Results

During the study period, 2384 clinical isolates of *E. coli* (n = 1407) and *Klebsiella* spp. (n = 977) were isolated. Among them, 449 isolates were identified as ETP NS by disk diffusion method, and the exclusion of repetitive isolates from the same patients revealed 444 (129 were intermediate resistant, and 315 were resistant); this included 207 *E. coli* (15.5%) and 237 (24.8%) *Klebsiella* spp. [212 *K. pneumoniae*, plus 25 *K. oxytoca*] (Fig. 1). The prevalence of ETP non-susceptibility was presumptively observed as 19.4% and its annual incidence significantly increased during the

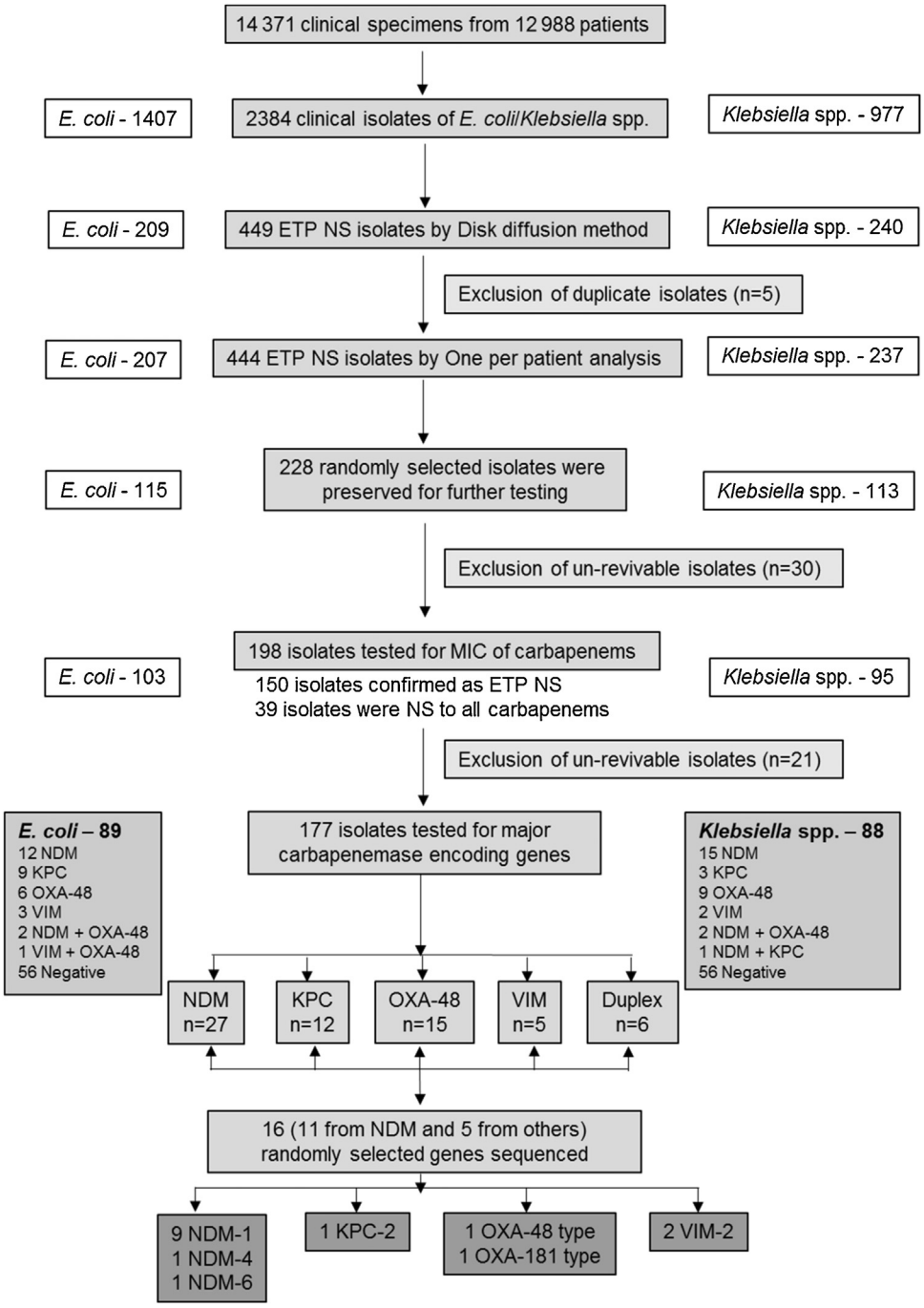

Fig. 1. Overview of the study.

study period ($P_{trend} < 0.01$) (Fig. 2). These strains were primarily isolated from urine and pus specimens collected from the inpatients of the 50–60 age group admitted to surgical wards (Supplementary Table S2).

Among the ETP NS isolates, 198 (*E. coli* 103 and *Klebsiella* spp. 95) were tested for MIC of carbapenems (Table 1). Notably, 48 (24.2%) isolates were found to be susceptible to ETP by MIC, which was identified as NS by ZOI. Among them, 30 were *E. coli* and 18 were *Klebsiella* spp. Based on the MIC results observed from the sample data, the prevalence of carbapenem resistance was extrapolated to the original population and the prevalence was estimated as 3% for non-ETP carbapenems (Table 2).

Among the ETP NS isolates, the MIC_{50} of ETP was 4 μg/mL and 16 μg/mL for *E. coli* and *Klebsiella* spp., respectively; similarly, the MIC_{50} of other carbapenems were ca. 0.25 μg/mL and 1 μg/mL, respectively, which is within the susceptible range of CLSI breakpoints. Thus, more than half (59.3%; 89/150) of the ETP NS isolates were susceptible to other carbapenems. Scatterplot analysis of MIC results on non-ETP carbapenems was performed and strong concordance (>80%) among all the three carbapenems (Fig. 3) was observed; although the DOR has lower value for MIC_{50}, especially with *E. coli*, its overall susceptibility rate is similar to IPM (Table 1).

When compared with *E. coli,* the *Klebsiella* spp. had approximately two-fold higher values for MIC_{50} and MIC_{90} for all carbapenems; further, the prevalence of resistance was always higher among *Klebsiella* spp., and the difference was statistically significant with IPM (13.7% vs. 27.3%; $P = 0.04$) and DOR (15.1% vs. 28.6%; $P = 0.046$).

About 177 preserved isolates (131 ETP NS plus 46 ETP S by MIC) were tested for carbapenemase encoding gene by multiplex PCR. Among them, 65 were found positive for at least any one of the five carbapenemase-encoding genes tested, with NDM (32/65; 49.2%) being the most common mechanism of carbapenemase (Fig. 4). The impact of different types of carbapenemase encoding gene on the MIC of carbapenems was analysed, which revealed NDM as the leading mechanism with highest MIC values among all carbapenemases (Table 3).

Among the 150 ETP NS isolates confirmed by elevated MIC, 131 isolates were tested by PCR and 37.4% (n = 49) of them were found to be positive for carbapenemase encoding gene(s); thus, 62.6% (n = 82) of the isolates had elevated MIC for ETP by other unknown mechanisms. Further, among the 48 ETP S isolates (however, NS by initial screening test i.e. disk diffusion method), 46 were tested by PCR and about 37% (n = 17) of them were found positive for carbapenemase encoding gene, predominantly OXA-48 (47%; n = 8).

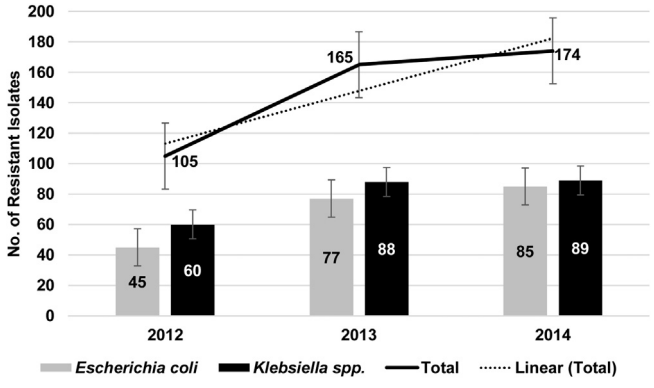

Fig. 2. Screening of ETP-NS isolates - annual incidence. Whiskers indicate standard error.

Sixteen randomly selected samples (selected from each group to cover all gene types; 11 from NDM, two each from OXA and VIM, and one from KPC) were subjected to sequencing PCR. Among them, nine were identified as NDM-1, one each as NDM-4 and NDM-6; one isolate was KPC-2 and two isolates were VIM-2 and one each as OXA-48 and OXA-181.

Sixty-eight isolates, including the isolates NS to all carbapenems (n = 39), isolates susceptible to ETP but PCR positive (n = 14), isolates NS to ETP but susceptible to MEM (n = 12) and isolates susceptible to ETP and PCR negative (n = 3) were subjected to CIM, and 43 of them were found positive and 25 of them were negative for carbapenemase. The study observed strong concordance between MEM MIC of ≥2 μg/mL and carbapenem inactivation by CIM (Table 4).

4. Discussion

Enterobacteriaceae infections have been globally increasing due to the wide spectrum of resistance to common antimicrobial agents. Further, the recent emergence of carbapenem resistance in these organisms worsens the clinical management [19]. It is believed that this is the first report to document the mechanisms of carbapenem resistance in rural India. Although a few reports have been published, they were not designed to measure the prevalence, have a high sampling bias towards the selection of resistant strains, and all of them were from the urban regions or metropolitan cities [4,20–23]. Further, only one study has used the most sensitive ETP for CRE screening [20]. Hence, the present study could help to predict the prevalence of CRE in rural parts of India.

The study observed higher prevalence of carbapenem resistance when compared with the report of Xu et al. [24] in India (2.6%) as well as the average of Asian countries (0.9%). However, the prevalence is lower than most of the earlier reports from India [25].

Interestingly, the susceptibility pattern did not significantly differ between the carbapenems, except ETP, which has a four-fold higher resistance rate. Hence, the susceptibility result cannot be generalised to other carbapenems when the isolate is resistant to ETP. A similar observation was recently reported from Taiwan [26]. This information is clinically significant because ETP is an approved antibiotic for the treatment of complicated UTI, and skin and soft tissue infections [27], which may pose a risk to treatment failure owing to its higher resistance rate compared with other carbapenems.

Antimicrobial susceptibility test by disk diffusion method with 10 μg ETP is highly sensitive in detecting CRE [28]. However, the information on the specificity (78.2%) is very limited [29]. The present study observed that 24.2% (48 of 198) of the susceptible (by MIC) test results were misleadingly identified as non-susceptible (i.e., resistance (n = 27) or intermediate (n = 21)) by disk diffusion method. More specifically, 13.6% (27 of 198) of the susceptible isolates were incorrectly identified as resistant by disk diffusion method. This is explained by the fact that the ETP disk test was intended for screening of potential CPE; that is, most of the OXA-48 producers have low MIC to carbapenems (often susceptible to IPM, MEM, DOR, and intermediate or susceptible to ETP) [30]. In that way, the disk diffusion method is more sensitive than 'true' MIC to suspect the production of OXA-48-type carbapenemases. Further, it is well known that the zone diameters are not extremely well correlated with the MIC of ETP. Thus, it is better to take the value of zone diameter instead of true MIC for the screening of CPE. Hence, ETP has a slightly reduced susceptibility breakpoints (19–21; the isolate is identified as non-susceptible if the ZOI is ≤21 mm) when compared with other carbapenems (breakpoints 20–22) as per CLSI. Further, EUCAST have proposed a reduced screening cut-off for ETP, which is different from clinical breakpoints [31]. Thus, some of the ETP NS isolates screened with the disk test may be false positives owing to its limited specificity.

Table 1
MIC of carbapenems against ETP NS isolates (MIC > 0.5 μg/mL).

Organism	Antibiotic name	R (%)	S (%)	%R 95% CI	MIC (μg/mL)			
					MIC_{50}	MIC_{90}	Mean	MIC range
Escherichia coli (n = 73)	Doripenem	11 (15.1)	59 (80.8)	8.1–25.8	0.064	4	0.217	0.032 to 64
	Ertapenem	50 (68.5)	0	56.4–78.6	4	>64	6.616	1 to >64
	Imipenem	10 (13.7)	54 (74)	7.1–24.2	0.25	4	0.539	0.125 to >64
	Meropenem	13 (17.8)	55 (75.3)	10.2–28.9	0.125	8	0.27	0.016 to 32
Klebsiella spp. (n = 77)	Doripenem	22 (28.6)	44 (57.1)	19.2–40.2	1	16	0.893	0.032 to >64
	Ertapenem	67 (87)	0	76.9–93.3	16	>64	14.105	1 to >64
	Imipenem	21 (27.3)	48 (62.3)	18.1–38.8	1	16	1.095	0.064 > 64
	Meropenem	20 (26)	40 (51.9)	17.0–37.5	1	16	0.986	0.032 to >64
Total (n = 150)	Doripenem	33 (22)	103 (68.7)	15.8–29.6	0.25	8	0.449	0.032 to >64
	Ertapenem	117 (78)	0	70.4–84.2	8	>64	9.759	1 to >64
	Imipenem	31 (20.7)	102 (68)	14.7–28.2	0.5	8	0.776	0.064 to >64
	Meropenem	33 (22)	95 (63.3)	15.8–29.6	0.5	16	0.525	0.016 to >64

Table 2
Estimated prevalence of carbapenem resistance.

Organism	Antibiotic name	Estimated* No. of R isolates	95% CI for the estimated number of R isolates	Prevalence (%R) [a]
Escherichia coli (n = 1338)	Doripenem	22	12.6–37.5	1.64%
	Ertapenem	100	80.1–120.2	7.47%
	Imipenem	20	11.1–35.1	1.49%
	Meropenem	26	15.6–42.2	1.94%
Klebsiella spp. (n = 954)	Doripenem	55	37.5–77.2	5.77%
	Ertapenem	167	143.8–186.6	17.51%
	Imipenem	52	35.4–74.5	5.45%
	Meropenem	50	31.1–71.8	5.24%
Total (n = 2292)	Doripenem	77	61.8–95.8	3.36%
	Ertapenem	267	238.5–298.6	11.65%
	Imipenem	72	57.3–90.2	3.14%
	Meropenem	76	60.9–94.7	3.32%

[a] The prevalence was estimated from sample data (n = 103 for *E. coli* and n = 95 for *Klebsiella* spp. randomly selected isolates of 444 ETP NS isolates detected by disk diffusion method during the study period); Population size: *E. coli* 1338 (n = 207 are ETP NS) and *Klebsiella* spp. 954 (n = 237 are ETP NS). Example: 11 out of 103 isolates were resistant to DOR in *E. coli*; thus, (11 × 207)/103 = 22 and (22/1338) × 100 = 1.64%.
* Refer to reference [9].

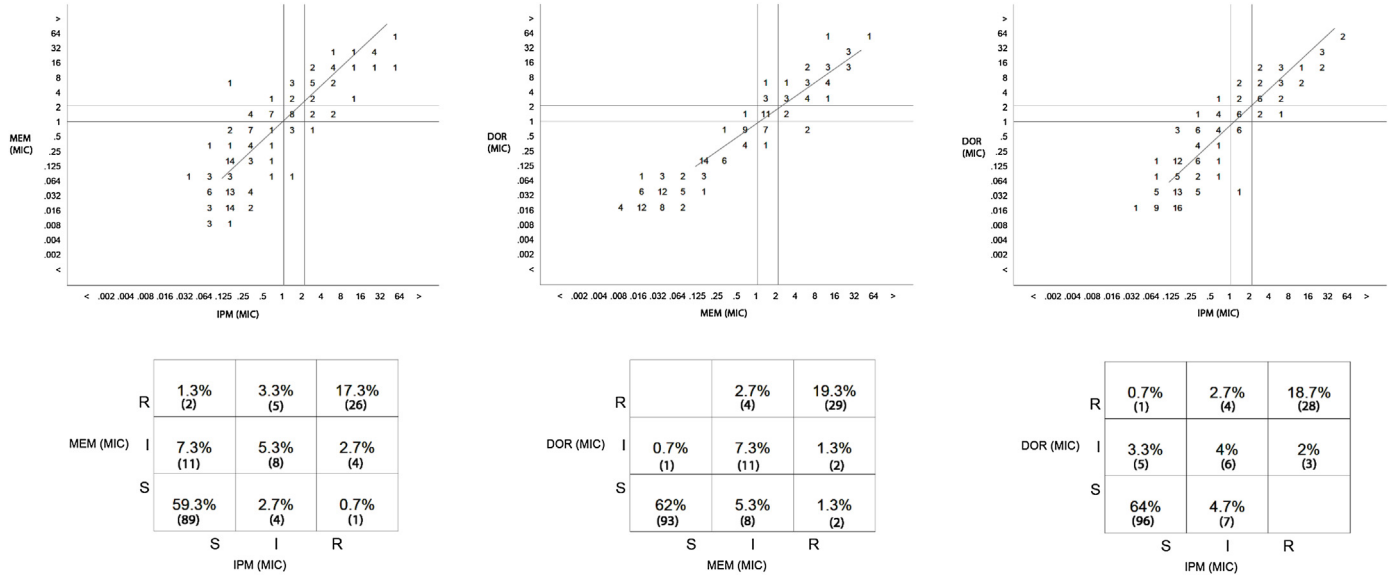

Fig. 3. Scatterplot comparison of MIC results of carbapenems on ETP NS (MIC > 0.5 μg/mL) isolates.
Numbers in the graph represents number of isolates (n = 150). Regression line value are $\log_2$ (MIC) = –0.45 + 1.24 $\log_2$ (MIC) [r^2 = 0.72; n = 148], $\log_2$ (MIC) = –0.3 + 0.93 $\log_2$ (MIC) [r^2 = 0.92; n = 149], and $\log_2$ (MIC) = –0.68 + 1.24 $\log_2$ (MIC) [r^2 = 0.79; n = 148] respectively for MEM vs. IPM, DOR vs. MEM, and DOR vs. IPM.

Detecting CPE in a clinical laboratory is a challenging process as it is not detected or differentiated by the routine susceptibility tests [32,33]. Hence, both CLSI and EUCAST have proposed not testing for resistance mechanisms in routine clinical isolates, justifying that the MIC testing with lower cut-off values would be adequate for treatment purposes [14,34].

Interestingly, a significant proportion of ETP-susceptible isolates were found positive for carbapenemase encoding gene, suggesting

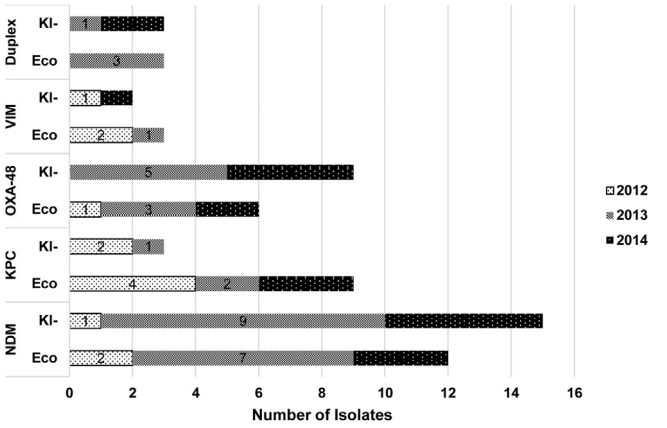

Fig. 4. Distribution of carbapenemase encoding gene(s).
Duplex in *Escherichia coli* = 2 NDM + OXA-48 and 1 VIM + OXA-48.
Duplex in *Klebsiella* spp. = 1 NDM + KPC in 2013 and 2 NDM + OXA-48 in 2014.
Total of 65 isolates were found positive for carbapenemase encoding gene, including 27 NDM, 12 KPC, 15 OXA-48, 5 VIM, 4 NDM + OXA-48 and one each of VIM + OXA-48 and NDM + KPC. Eighty-nine isolates of *E. coli* and 88 isolates of *Klebsiella* spp. were negative for carbapenemase encoding gene by multiplex PCR (n = 177; 27 in 2012, 76 in 2013, and 74 in 2014).

that the mere presence of carbapenemase encoding gene could not confer clinical resistance to any of the carbapenems [35] and it needs to be optimally expressed to attain resistance. Further, the MIC_{50} of non-ETP carbapenems were observed to be within the susceptible range (i.e., <1 μg/mL) for all types of carbapenemases except NDM. Additionally, infections with CPE with MEM MIC up to 16 μg/mL were successfully treated with the high-dose carbapenem infused for longer duration, which highlights the importance of MIC testing [36]. In the current study, >80% of the CRE isolates had an MIC of ≤16 μg/mL for non-ETP carbapenems; hence, carbapenem (other than ETP) could be considered even for an isolate known to produce carbapenemase, unless its MIC exceeds this cut-off value. This would be clinically useful as the beta lactams were an effective bactericidal agent than any other antimicrobial class. Therefore, MIC testing could be valuable for patient management; while the detection of resistance mechanism could help in predicting possible MIC. Further, the presence of carbapenemase encoding gene does not eliminate the clinical utility of carbapenem unless it has adequately expressed to neutralise the antibiotic. However, it has also been demonstrated that treatment of CPE is better with combination therapy than that of therapy with high-dose carbapenem alone [37]. Thus, combination therapy has to be considered.

Interestingly, CIM identified carbapenemases in 37 of 39 MEM NS isolates. However, 23 of them were positive for carbapenemase encoding gene by PCR, suggesting the possibility of other carbapenemases that were not tested by PCR. Notably, about 64% (14 of 22) of the isolates recovered after late 2013 were negative by PCR but positive for carbapenemase by CIM (Supplementary Table S3), which requires further investigation. However, this could be explained by the fact that the PCR is limited by its primer binding capacity; thus, the rare carbapenemase encoding genes that were not tested, new variants of the existing genes with mutations at primer binding site, or the emergence of novel carbapenemases might be the reason for the negative results with PCR. Further, isolates producing weak carbapenemases (MEM MIC < 2 μg/mL), especially OXA-48, were often not detected by CIM, but detected by PCR. Interestingly, two isolates with elevated carbapenem MIC were negative for carbapenemase by both PCR and CIM, suggesting non-carbapenemase-mediated carbapenem resistance possibly by efflux pump/porin loss coupled with ESBL/AmpC enzymes [5].

Interestingly, the MIC_{90} of all non-ETP carbapenems was observed (0.25 μg/mL) to be within the susceptible cut-off point for OXA-48 producers, highlighting the weakness of this carbapenemase enzyme (Table 3). Further, the $MIC_{50} = MIC_{90}$ for IPM among OXA-48 producers could be due to the elevated baseline MIC of IPM, as it has been used since 1981. Although, the OXA-48 type carbapenemases were known for this limitation [38], the current study documented that any type of carbapenemase may be inadequate to confer clinical resistance to carbapenem, including NDM/KPC, suggesting very-low/non-expression of carbapenemase encoding genes in those isolates.

In agreement with earlier reports from India [20,23], the present study documented NDM as the predominant mechanism of carbapenemase in the region; and all tested carbapenemases except IMP were detected, which is common among non-fermenters [39]. Further, mixed gene combinations were observed in 9% (6 of 65) of the positive isolates, in which NDM + KPC had significantly higher MIC values than NDM + OXA-48. A recent report from Morocco documented a combination of NDM + VIM + OXA-48 in a *K. pneumoniae* isolate [40]. These observations suggest that the carbapenemase encoding genes were rapidly evolving and highly diversified, and require stringent infection control measures to contain their dissemination [41]. This study established the mechanism of non-ETP carbapenem resistance in 95% of the tested isolates; however, the mechanism of ETP resistance was not established for the major proportion (63%) of isolates. This reduced susceptibility to ETP might be attributed to the combination of porin loss with ESBL production [5].

The current study presents important information on the prevalence and mechanisms of carbapenem resistance among clinically important members of Enterobacteriaceae. Further, it highlights that carbapenemase production may be inadequate in certain situations to neutralise carbapenem and, hence, MIC testing is more important than genotypic tests for carbapenem resistance in terms of patient management. However, being a single-centre study, the information presented here may not reflect the susceptibility profile of the entire country or South India and may not be suitable for generalisation across rural parts of India. In addition, the study lost 51 of the initial screening isolates for molecular investigations, which might have influenced the original prevalence of carbapenemase encoding genes. Other limitations include continual reduction in the number of isolates tested at each level of study progression, and a very limited number of genes were sequenced. Nevertheless, the

Table 3
Relationship between MIC and carbapenemase encoding gene.

Criteria	DOR				ETP				IPM				MEM			
	NDM	KPC	OXA-48	VIM	NDM	KPC	OXA-48	VIM	NDM	KPC	OXA-48	VIM	NDM	KPC	OXA-48	VIM
Mean MIC	4.5	11.5	0.6	3.5	51.5	23.6	11.9	28.1	4.4	11.4	0.3	6.8	6.9	11.6	0.6	6.7
MIC_{50}	2	0.125	0.064	0.25	32	1	0.5	4	2	0.25	0.25	0.5	2	0.125	0.064	0.5
MIC_{90}	16	8	0.25	16	128	128	16	128	16	4	0.25	32	32	8	0.25	32
Min. MIC	0.016	0.032	0.032	0.125	0.125	0.25	0.064	0.25	0.125	0.125	0.125	0.25	0.016	0.032	0.016	0.25
Max. MIC	32	128	8	16	128	128	128	128	32	128	2	32	32	128	8	32

Note: Mean MIC is calculated as an average of all MIC values observed in specified category (e.g., average MIC of DOR in NDM positive isolates); MIC of >64 were calculated as 128; n = 27, 12, 15, and 5 for NDM, KPC, OXA-48 and VIM positive isolates, respectively (total = 59).

Table 4
Results of CIM and PCR with MIC of carbapenems.

Organism	PCR	MIC Range (μg/mL)		CIM
		ETP	MEM	
Escherichia coli (n = 28)				
5	Negative	0.064–4	0.016–0.064	4 Negative; 1 Positive[b]
6	Negative	32 – >64	2–16	5 Positive[a]; 1 Negative
2	KPC	0.25–1	0.032–0.125	1 Positive; 1 Negative
3	NDM	0.125–16	0.016–0.5	2 Positive; 1 Negative
6	NDM	64 – >64	2–32	Positive
1	NDM + OXA-48	>64	8	Positive
1	VIM	0.25	0.25	Negative
1	VIM + OXA-48	0.5	0.064	Negative
2	OXA-48	0.5	0.032	Negative
1	OXA-48	>64	8	Positive
Klebsiella pneumoniae (n = 34)				
5	Negative	0.032–2	0.016–0.25	4 Negative; 1 Positive[b]
8	Negative	16 – >64	2–16	7 Positive[a]; 1 Negative
2	KPC	>64	8 – >64	Positive
2	NDM	0.25–4	0.032–0.064	Negative
7	NDM	16 – >64	2–32	Positive
1	NDM + OXA-48	>64	16	Positive
1	NDM + KPC	>64	32	Positive
7	OXA-48	0.064–16	0.016–0.25	6 Negative; 1 Positive[b]
1	VIM	>64	32	Positive
Klebsiella oxytoca (n = 6)				
3	Negative	32 – >64	2–32	Positive[a]
1	NDM	0.25	0.032	Negative
2	NDM	>64	16	Positive

2 isolates with MEM MIC ≥ 2 μg/mL were CIM and PCR negative.
[a] 15 isolates with MEM MIC ≥ 2 μg/mL were CIM positive but PCR negative.
[b] 3 isolates with MEM MIC ≤ 1 μg/mL were CIM positive but PCR negative.

results could be used as a predictive factor for the prevalence and possible mechanisms of carbapenem resistance in *E. coli* and *Klebsiella* spp. isolated from similar clinical settings in rural India. This would be helpful for choosing empirical therapy in life-threatening infections.

5. Conclusion

This study documented a 3% prevalence of carbapenem resistance (excluding ETP, which had a four-fold higher resistance rate) with NDM as the major mode of carbapenemase production in Enterobacteriaceae from rural South India. Although the study assured the efficacy of carbapenems for empirical therapy, an increasing trend of incidence and potentially emerging resistance mechanisms were observed. Further, the study highlights the importance of MIC testing for patient management and should be used more than carbapenemase/genotypic tests.

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Competing interests

None.

Ethical approval

The study was reviewed and approved by the Institutional Human Ethical Committee of Govt. Theni Medical College (GTMC/Ref. No. 2443/P&D/2012 dated 13-Dec-2012).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.05.028.

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