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The distribution of carbapenem- and colistin-resistance in Gram-negative bacteria from the Tamil Nadu region in India

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Abstract

Purpose. The occurrence of carbapenem- and colistin-resistance among Gram-negative bacteria is increasing worldwide. The aim of this study was to understand the distribution of carbapenem- and colistin-resistance in two areas in Tamil Nadu, India.

Methodology. The clinical isolates (*n*=89) used in this study were collected from two diagnostic centres in Tamil Nadu, India. The bacterial isolates were screened for meropenem- and colistin-resistance. Further, resistance genes bla_{NDM-1}, $bla_{OXA-48-like}$, bla_{IMP}, bla_{VIM}, bla_{KPC}, *mcr-1* and *mcr-2* and integrons were studied. The synergistic effect of meropenem in combination with colistin was assessed.

Results. A total of 89 bacterial isolates were studied which included *Escherichia coli* (*n*=43), *Klebsiella pneumoniae* (*n*=18), *Pseudomonas aeruginosa* (*n*=10), *Enterobacter cloacae* (*n*=6), *Acinetobacter baumannii* (*n*=5), *Klebsiella oxytoca* (*n*=4), *Proteus mirabilis* (*n*=2) and *Salmonella paratyphi* (*n*=1). MIC testing showed that 58/89 (65 %) and 29/89 (32 %) isolates were resistant to meropenem and colistin, respectively, whereas 27/89 (30 %) isolates were resistant to both antibiotics. *Escherichia coli*, *K. pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* isolates were bla_{NDM-1}-positive (*n*=20). Some strains of *Escherichia coli*, *K. pneumoniae* and *K. oxytoca* were $bla_{OXA-181}$-positive (*n*=4). Class 1, 2 and 3 integrons were found in 24, 20 and 3 isolates, respectively. Nine NDM-1-positive *Escherichia coli* strains could transfer carbapenem resistance via plasmids to susceptible *Escherichia coli* AB1157. Meropenem and colistin showed synergy in 10/20 (50 %) isolates by 24 h time-kill studies.

Conclusion. Our results highlight the distribution of carbapenem- and colistin-resistance in Gram-negative bacteria isolated from the Tamil Nadu region in South India.

INTRODUCTION

Antibiotic resistance has become one of the increasing concerns in Gram-negative bacteria because of the lack of available treatment options. The adverse outcome of developing multidrug-resistant (MDR) bacterial infections can lead to up to a two-fold increase in severity compared to the same infections caused by susceptible strains [1]. Carbapenems are broad-spectrum antimicrobial agents that are very useful against infections caused by MDR *Enterobacteriaceae* [2]. They are drugs of choice against infections caused by extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria [3]. From the healthcare epidemiological point of view, developing resistance towards carbapenems (imipenem, meropenem, doripenem and ertapenem) is of particular importance because carbapenems are last resort antibiotics [4]. The emergence of resistant bacteria against most classes of commercially available antibiotics and the shortage in discovery of new antibiotics that have activity against

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Keywords: Integron; plasmid-bound resistance; transferability; New Delhi metallo-beta lactamases; carbapenemases.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; CR-GNB, carbapenem-resistant Gram-negative bacteria; ERIC, enterobacterial repetitive intergenic consensus; ESBL, extended-spectrum beta-lactamase; LB, Luria–Bertani; MBL, metallo-beta-lactamase; MDR, multidrug-resistant; MH, Mueller–Hinton; MHT, modified Hodge test; SND, synergy not detected.

The GenBank/EMBL/DDBJ accession numbers for the class 1 integron sequence of *Escherichia coli* are KX610373, KX660695, KX685500. The GenBank/EMBL/DDBJ accession numbers for the class 1 integrons of *Klebsiella pneumoniae* are KX660694 and KX660696.

Gram-negative bacteria had led to the use of polymyxins as a valuable therapeutic option [5]. Finding treatment options against infections caused by carbapenem-resistant Gram-negative bacteria (CR-GNB) is one of the current challenges of our time resulting from an uncontrolled and irrational use of carbapenems [6]. Gram-negative bacteria have developed various resistance mechanisms such as the production of carbapenemase enzymes including β-lactamases (NDM, IMP, VIM, OXA, DIM, SIM) [7, 8]. The therapeutic armamentarium against infections caused by CR-GNB has limited the choice of antibiotics [9]. Colistin is a member of the polymyxin group polypeptide antibiotics with a significant activity against Gram-negative bacteria and it targets lipopolysaccharide in the outer membrane, although the exact mechanism of bacterial killing is still unknown [10]. The paucity of new antibiotics for a decade has allowed clinicians to reconsider colistin as an alternative therapeutic option against infections caused by Gram-negative bacteria [11]. The unconstrained use of colistin has caused the emergence of resistance in recent times [12–15].

Recently, the plasmid-mediated colistin resistant gene *mcr-1* was identified, and modification of lipid A by MCR-1 and MCR-2 was reported [16–18]. There are different mechanisms involved in the spread of antibiotic resistance, such as the acquisition of genetic elements like plasmids, integrons, resistant islands and transposons [19]. Integrons (class I integrons) are known to play a significant role in the dissemination of antibiotic resistance genes within and in between bacterial species [20–22]. For these reasons, combination therapy with different classes of antibiotics is recommended to improve efficacy and also to prevent the emergence of further resistance [23]. In this study, we investigated the mechanism of carbapenem and colistin resistance in Gram-negative bacteria isolated from a clinical setting in Tamil Nadu, India, evaluated the dissemination of integrons, and assessed whether meropenem–colistin had a synergistic effect on the clinical strains.

METHODS

Isolate collection and identification

The clinical isolates used in this study were collected from two regions, Chennai and Tiruchirappalli (separated by 300 km) in Tamil Nadu, India. A total of 89 non-duplicate, Gram-negative, clinical isolates were collected from two diagnostic laboratories during August 2014 to March 2015. The samples from which isolates were cultured included urine, blood, pus, sputum, bronical aspirate, wound swab and cerebrospinal fluid. The isolates were received in vials from the clinical diagnostic centres and further processing was carried out in the Antibiotic Resistance Laboratory at VIT University, Vellore, India. The isolates were sub-cultured onto MacConkey agar (Hi-Media) and stored at −80 °C for further analysis. Identification was done using phenotypic methods and based on the colony morphology and biochemical characteristics, and was confirmed by the VITEK identification system (bioMèrieux).

Antibiotic susceptibility test

The MIC of meropenem was determined by agar dilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, Mueller–Hinton (MH) agar was prepared and the final concentrations of meropenem ranging from 0.06 to 128 mg l^{-1} were added to MH agar (Hi-Media) in Petri plates, mixed well and allowed to solidify. Then, bacterial inoculum grown overnight was diluted in saline (10 µl in 4.99 ml) and placed on the surface of the agar (1 µl) within the marked grids placed under the plates. The inoculum was allowed to dry and was then incubated at 37 °C for 20 h. The lowest concentration of meropenem with the absence of visible bacterial growth was accepted as the MIC. The results were interpreted using the CLSI guidelines [24].

The MIC of ertapenem was determined by the Epsilometer test (E-test). Briefly, MH agar plates were prepared and bacterial inocula adjusted to 0.5 McFarland turbidity standards were swabbed on the surface of the agar. Plates were dried for 5 min and Ertapenem Ezy MIC strips (Hi-Media) were placed in the centre of the plate and incubated at 37 °C for 20 h. E-test strips were labelled from 0.002 to 32 mg l^{-1} and the MIC was determined. The results were interpreted using the CLSI guidelines.

For colistin, the MIC was determined using the micro-broth dilution method [24. Briefly, cation-adjusted MH broth (Hi-Media) was prepared and 100 µl was disbursed in a 96-well microtitre plate. Colistin was added at final concentrations ranging from 0.06 to 128 mg l^{-1} in row 1 to 12 with row 12 being used as a growth control. Then, bacterial inocula of 5×10^5 dilutions (CLSI guidelines) from overnight-grown cells were added to the respective wells and incubated at 37 °C for 20 h. The results were interpreted using the CLSI guidelines.

Identification of carbapenemase producer

The modified Hodge test (MHT) and the EDTA inhibition tests were performed for the detection of carbapenemase production as previously described [25]. The interpretation was done according to CLSI guidelines.

For the EDTA inhibition test, a liquid culture was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of an MHA plate. Two 10 µg meropenem disks were placed 15 mm apart, and 10 µl of 0.5M EDTA (Hi-Media) was added to one of the disks (final EDTA concentration was 750 µg). Plates were incubated at 37 °C for 20 h, and metallo-beta-lactamase (MBL) production was identified by any increase in zone diameter of >5 mm in the disk potentiated with EDTA. An MBL producer was defined as an isolate displaying reduced susceptibility to meropenem and tested positive in both the MHT and EDTA inhibition test.

Synergy testing

Time-kill analysis was performed for the isolates that were resistant to meropenem and colistin (27/89), and was based on the previously described method [26]. Antibiotics were used at final concentrations of 4 mg l^{-1} for colistin and 8 mg

l^{-1} for meropenem with an aim to mimic the clinical serum peak levels during standard treatments. Briefly, flasks containing MH broth with meropenem and colistin (MER-COL) were inoculated with a test organism of approximately 10^5 c.f.u. ml^{-1} and incubated at 37 °C in a shaking incubator. After the post-incubation period of 24 h, 100 µl aliquots were removed from the flask and serial dilutions were plated onto MH agar plates for determination of viable counts. After incubation for 24 h, colonies were counted to determine the synergistic effect of drugs in combination. Synergy was defined as any $\geq$2 $\log_{10}$ decrease in colony count after 24 h compared with the same drug alone and remaining as synergy not detected (SND).

DNA extraction

DNA extraction was done by the boiling preparation method. Briefly, overnight-grown bacteria were centrifuged at 9 000 × g for 10 min, and to the harvested bacterial cells, 100 µl sterile distilled water was added and the cells were heated at 95 °C for 10–15 min. The mixture was centrifuged at 5000 r.p.m. for 2 min and the supernatant was extracted and used as a source of template.

Screening of antibiotic resistance gene determinants

The presence of beta-lactamase genes $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$, bla_{IMP}, bla_{VIM} and bla_{KPC} were studied by multiplex PCR using specific primers and reaction conditions as described by Doyle *et al.* [27]. The integrase genes *intI1, intI2* and *intI3* and its internal gene cassettes were amplified by multiplex PCR using specific primers and reaction conditions described by Kargar *et al.* [28]. For the amplification of class 1 integrons, two primer sets were used: IntI1-F and IntI1-R for the *intI1* gene, and 5′-CS and 3′-CS for the integron variable region as described by Kargar *et al.* [28]. For class 2 integrons, primers IntI2-F and IntI2-R were used for the *intI2* gene, and primers attI2-F and orfX-R for characterization of gene cassette arrays. Integrase gene products were sequenced for further determination of the integron gene cassette.

Primers for the *mcr-1* and *mcr-2* genes were as described earlier [14, 18]. For identification of clonality between the bacterial species enterobacterial repetitive intergenic consensus (ERIC)-PCR was performed using ERIC2 primers as described by Versalovic *et al.* [29].

Plasmid profiling and gene transferability assay

Plasmid DNA was isolated for the resistant isolates harbouring resistance genes using the HiPurA plasmid DNA miniprep purification kit (Hi-Media) following the manufacturer's protocol, and the lambda DNA/EcoRI+HindIII marker (ThermoFisher Scientific) was used to identify the size of the plasmids. A total of 24 isolates carrying resistance genes encoding NDM-1 and OXA-48 were studied. In the case of intra-genus transfer of antibiotic resistance, only NDM-1- and OXA-48-encoded *Escherichia coli* isolates (11) were chosen and the selected isolates were also found to be colistin-resistant with a MIC of $\geq$32 mg l^{-1}. A plasmid-free, streptomycin-resistant (F^-, Str^r) auxotrophic strain of *Escherichia coli* (AB1157), showing sensitivity to all the antibiotics under study, was used as a recipient, while all the NDM-1- and OXA-48-encoded *Escherichia coli* served as the donors [30]. Donor and recipient cultures were grown overnight (10^8 cells ml^{-1}) and 5 ml each bacterial culture was mixed (1 : 1) in a Luria–Bertani (LB) broth (Hi-Media) and was incubated without shaking for 16 h at 37 °C. The transconjugants were selected on LB agar plates supplemented with streptomycin (100 µg ml^{-1}) in addition to either meropenem (10 µg ml^{-1}) or colistin (10 µg ml^{-1}). In order to study the transfer of multiple resistance markers, combinations containing streptomycin with both meropenem and colistin were used, so a total of 10 *Escherichia coli* isolates resistant to both meropenem and colistin were studied. The transformants grown on the antibiotic plates were screened for resistance genes encoding NDM-1 and OXA-48 by PCR.

RESULTS

Identification of bacterial isolates

A total of 89 bacterial isolates identified as Gram-negative were included in this study. The identified clinical isolates included the following: 48 % (*n*=43) *Escherichia coli*, 20 % (*n*=18) *Klebsiella pneumoniae*, 11 % (*n*=10) *Pseudomonas aeruginosa*, 7 % (*n*=6) *Enterobacter cloacae*, 7 % (*n*=5) *Acinetobacter baumannii*, 4 % (*n*=4) *Klebsiella oxytoca*, 2 % (*n*=2) *Proteus mirabilis* and 1 % (*n*=1) *Salmonella paratyphi*. Of the 89 isolates, 81 % were found to be *Enterobacteriaceae* (Fig. 1).

Susceptibility to antibiotics

All the isolates received from clinical laboratories were found to be multiple-drug-resistant by the disk diffusion method at the clinical centres. Throughout this study, results were interpreted using the CLSI guidelines. Agar dilution MIC results showed that 58/89 (65 %) isolates were meropenem-resistant. The MIC_{50} and MIC_{90}values for meropenem agar dilution were 16 and 32 µg ml^{-1}, respectively. Among the different isolates, the percentage of *Escherichia coli* that were meropenem-resistant was 65 % (28/43), *K. pneumoniae* 67 % (12/18), *Pseudomonas aeruginosa* 60 % (6/10) and *K. oxytoca* 100 % (4/4). Meropenem resistance was also observed in isolates such as *Enterobacter cloacae* (3/6), *A. baumannii* (3/5), *Proteus mirabilis* (1/2) and *S. paratyphi* (1/1).

In the case of colistin, MIC results showed that 29 of 89 (33 %) isolates were resistant with MIC_{50} of 1 µg ml^{-1} and MIC_{90} of 16 µg ml^{-1}. Colistin resistance was observed in isolates of *Escherichia coli* (61 %, 11/43), *K. pneumoniae* (33 %, 6/18) and *Pseudomonas aeruginosa* (30 %, 3/10). We also observed colistin resistance in *Enterobacter cloacae* (3/6), *A. baumannii* (2/5), *K. oxytoca* (2/4), *Proteus mirabilis* (1/2) and *S. paratyphi* (1/1), but since these numbers are small they do not reflect the real problem of resistance among these organisms. From the MIC results, it was found that 30 % (27/89) of the isolates used in this study were resistant to both meropenem and colistin (Fig. 1).

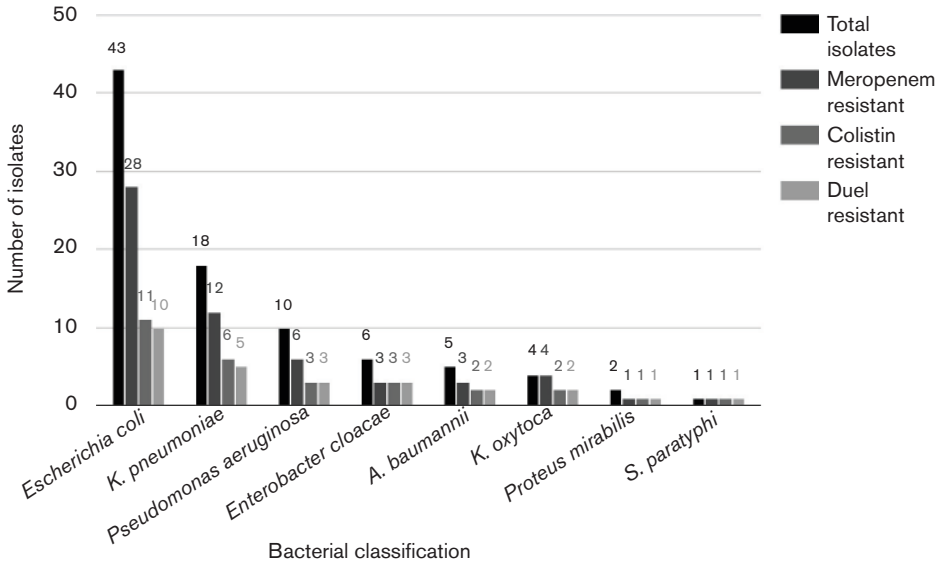

Fig. 1. Classification of bacterial isolates used for the study and comparison of MIC resistance pattern of bacterial isolates among antibiotics meropenem and colistin. Dual resistant, resistant to both meropenem and colistin.

Identification of carbapenemase producer

Using phenotypic tests, carbapenemase activity was detected in 52/89 (58.4 %) isolates by the MHT method and 50/89 (56.1 %) isolates by the EDTA inhibition test. A comparison of, disk diffusion and MIC results for meropenem showed 100 % and 65 % resistance, respectively (Table 1). Overall, 50 (56 %) of the 89 tested clinical isolates were positive for the production of MBLs including carbapenemases.

Screening of antibiotic resistance genes

The presence of resistance genes $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$, bla_{IMP}, bla_{VIM}, bla_{KPC} and *mcr-1* and *mcr-2* was tested for all 89 isolates. PCR results showed that $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48-like}}$ were the most predominant genes in carbapenem-resistant Gram-negative bacteria in our study. In total, 24/89 isolates carried resistant genes $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48-like}}$. Genes bla_{IMP}, bla_{VIM} and bla_{KPC} were absent in all the isolates tested. Among $bla_{\text{NDM-1}}$ (20/89) carriers were *Escherichia coli* (*n*=9), *K. pneumoniae* (*n*=4), *Pseudomonas aeruginosa* (*n*=3), *K. oxytoca* (*n*=3) and *Enterobacter cloacae* (*n*=1). Four $bla_{\text{OXA-48}}$ producers were *Escherichia coli* (*n*=2), *K. pneumoniae* (*n*=1) and *K. oxytoca* (*n*=1). Interestingly, four of the *Escherichia coli* isolates (EC8, EC12, EC15 and EC33) were positive for $bla_{\text{NDM-1}}$ and two *Escherichia coli* isolates (EC14 and EC22) carried $bla_{\text{OXA-48-like}}$ and were identified in plasmid DNA and studied further to determine if these genes were transferable. ERIC-PCR results showed that two of the carbapenem-resistant *Escherichia coli* (EC8 and EC15) carrying $bla_{\text{NDM-1}}$ and two *Escherichia coli* (EC14 and EC22) carrying $bla_{\text{OXA-48-like}}$ had the same ERIC profile. Two of the $bla_{\text{NDM-1}}$*Escherichia coli* could not be grouped. None of the meropenem-susceptible isolates were positive for the tested carbapenem resistance genes and also none of the isolates carried more than one carbapenem resistance gene. Sequencing results showed that isolates positive for the bla_{NDM} gene also had $bla_{\text{NDM-1}}$, and those positive for the $bla_{\text{OXA-48-like}}$ gene also had $bla_{\text{OXA-181}}$. In the case of colistin resistance, neither *mcr-1* and *mcr-2* were amplified in any of the isolates. Of the $bla_{\text{NDM-1}}$ carriers, two *Escherichia coli* isolates (EC8 and EC10) and one $bla_{\text{OXA-181}}$-positive *K. pneumoniae* isolate (KP4) were found to be resistant to both meropenem and colistin.

Conjugation

Plasmid analysis revealed that the resistant isolates carried one or more plasmids ranging from 10 to 100 kb (Table 2). Conjugation studies were used to elucidate the intra-genus gene transfer of plasmid-borne resistance. The resistance was transferred to *Escherichia coli* AB1157. Out of nine *Escherichia coli* isolates carrying NDM, only four (EC8, EC12, EC15 and EC33) were found to transfer resistance plasmids to susceptible *Escherichia coli* AB1157. However, both *Escherichia coli* EC14 and EC22 -OXA carriers were able to transfer their plasmid-borne gene to susceptible *Escherichia coli* AB1157. The resistance was transferred at a frequency of $4–6\times10^{-5}$ for both the transformants. No transformants were observed in the case of colistin. Additionally, multiple resistance transferability was studied using meropenem and colistin. In this case, 1/10 *Escherichia coli* isolate (with $bla_{\text{NDM-1}}$) showed the simultaneous transfer of both markers at a low frequency of 3×10^{-6}.

Distribution of integrons in carbapenem- and colistin-resistant isolates

Of the 89 clinical isolates studied, 24 (26.9 %) were identified as positive for class 1 integrons, 20 (22.4 %) for class 2 integrons and three (3.3 %) for class 3 integrons (Table 3).

Table 1. Carbapenem resistance in Gram-negative isolates according to different phenotypic tests

Species	Number of carbapenem-resistant isolates from phenotypic tests			
	Disk-diffusion*	MIC†	MHT‡	EDTA inhibition test§
Escherichia coli	43	28	20	21
K. pneumoniae	18	12	12	10
Pseudomonas aeruginosa	10	6	7	6
Enterobacter cloacae	6	3	2	5
A. baumannii	5	3	4	4
K. oxytoca	4	4	4	4
Proteus mirabilis	2	1	2	0
S. paratyphi	1	1	1	0

*Disk-diffusion represents Kirby–Bauer test results obtained for meropenem.

†MIC results of agar dilution method for meropenem.

‡MHT (modified Hodge test) data obtained using meropenem disk.

§EDTA inhibition test results obtained from meropenem-EDTA (M-EDTA).

Sequencing results showed that the variable regions in class 1 integrons carried antibiotic-resistant genes and showed that one isolate had $bla_{\text{NDM-1}}$ in its gene cassette (Fig. 2a, b). Interestingly, 2/6 of the donor *Escherichia coli* (EC8 and EC10) involved in conjugation carried $bla_{\text{NDM-1}}$ along with class 1 integrons, and the same was also amplified in transformants by PCR using specific primers, indicating the development of competence and uptake of DNA, leading to recombination and thus, transformation. Interestingly, in the *Escherichia coli* EC8 isolate, $bla_{\text{NDM-1}}$ was found inside the class 1 integron (variable region) arrangement along with $bla_{\text{OXA-30}}$, which correlates with our earlier PCR studies, but the gene encoding OXA-30 was not amplified with specific primers in this study. However, dual resistance was found in *Escherichia coli* isolates producing plasmid-bound $bla_{\text{NDM-1}}$ and also involved in co-transformation of dual resistance in the presence of class 1 integrons.

Synergy testing

For synergy testing, isolates were chosen based on the DNA fingerprinting results obtained from ERIC-PCR. On observing ERIC results, five patterns of *Escherichia coli*, five patterns of *K. pneumoniae*, three patterns of *Pseudomonas aeruginosa*, two patterns of *A. baumannii*, two patterns of *K. oxytoca*, and one pattern each for *Enterobacter cloacae*, *Proteus mirabilis* and *S. paratyphi* were observed. A total of 20 isolates were included for the synergy testing; differences in colony count of ≥ 2 $\log_{10}$ after 24 h exposure to the drugs in combination and as single agents was considered as synergy. The time-kill analysis showed synergy for 10/20 (50 %) isolates after 24 h (Table 4). Among the 10 synergetic isolates, 4/5 were *Escherichia coli*, 2/5 *K. pneumoniae*, and one each of *Pseudomonas aeruginosa, K. oxytoca, Enterobacter cloacae* and *S. paratyphi*. It was also noted that 4/10 synergetic isolates (two *Escherichia coli*, one *K. pneumoniae* and one *Pseudomonas aeruginosa*) were NDM-1 producers and 1/10 (*K. pneumoniae*) was an OXA-181 producer.

DISCUSSION

Our present study demonstrated that 65 % and 33 % of the Gram-negative bacteria under study were resistant to meropenem and colistin, respectively. The study simultaneously analysed the potential role of the plasmid-borne resistance in transferability and the type of integrons involved in the resistance of clinical Gram-negative isolates from Tamil Nadu, India. The molecular characteristics of integrons and plasmids in carbapenem- (NDM- and OXA-producing) and colistin-resistant Gram-negative clinical isolates were described to emphasize the rapid spread of resistance within clinical bacteria.

Many studies have demonstrated the wide spread of NDM-1- and OXA-48-like-producing carbapenem-resistant Gram-negative bacteria from India [31–33]. In addition, our previous studies also reported the distribution of

Table 2. Distribution of plasmids in Gram-negative bacteria used in this study

Plasmid size (kb)*	Frequency of plasmid distribution according to organism† (%)					
	Escherichia coli, *n*=43	*K. pneumoniae*, *n*=18	*Pseudomonas aeruginosa*, *n*=11	*Enterobacter cloacae*, *n*=6	*A. baumannii*, *n*=5	*K. oxytoca*, *n*=4
1.0	3 (6.9)	0	1 (9.0)	0	0	0
3.0	1 (2.3)	1 (5.5)	0	0	1 (20.0)	0
5.0	0	0	0	1 (16.6)	0	0
10.0	9 (21)	3 (16.6)	1 (9.0)	0	0	0
20.0	2 (4.6)	1 (5.5)	2 (18.1)	0	0	0
50.0	3 (6.9)	0	0	0	1 (20.0)	1 (25.0)
100.0	5 (12)	2 (11.1)	1 (9.0)	1 (16.6)	0	1 (25.0)

*Plasmid sizes were identified using lambda DNA/EcoRI+HindIII marker.

†Plasmids were absent in *S. paratyphi* and *Proteus mirabilis*.

Table 3. List of clinical isolates harbouring integrons and resistance genes

+, Present; −, absent.

Sample no.	Organism	Class 1 integron	Class 2 integron	Class 3 integron	Integron gene cassette region
	Escherichia coli				
1	EC4	+	−	−	−
2	EC7	+	−	−	+
3	EC8-NDM*†	+	−	−	+
4	EC10-NDM*†	+	−	−	+
5	EC16†*	+	−	−	
6	EC17*	+	−	−	+
7	EC19†	+	−	−	+
8	EC22-OXA*	+	+	−	−
9	EC23*	+	+	−	−
10	EC25*	+	+	−	+
11	EC29	+	+	−	−
12	EC31	+	+	−	−
13	EC32*	−	+	−	+
14	EC35*	−	+	−	−
15	EC37*	−	+	−	+
16	EC38	−	+	−	+
17	EC41†	−	+	−	+
18	EC42	−	−	+	−
	K. pneumoniae				
19	KP1†	+	−	−	+
20	KP5†	+	−	−	−
21	KP6†	+	−	−	−
22	KP7-NDM*	+	−	−	+
23	KP8*	+	−	−	+
24	KP9*	+	+	−	−
25	KP11	+	+	−	−
26	KP13	+	+	−	−
27	KP14†	−	+	−	+
28	KP15*	−	+	−	+
29	KP17*	−	+	−	−
30	KP18	−	+	−	−
	K. oxytoca				
31	KO1*†	+	−	−	+
32	KO2	+	−	−	−
33	KO3*†	−	+	−	+
34	KO4*	−	−	+	−
	Enterobacter cloacae				
35	EL1-NDM*	+	−	−	+
36	EL3	+	−	−	−
37	EL4*	−	+	−	+
38	EL5	−	+	−	+
39	EL6*	−	−	+	−

*Carbapenem-resistant.

†Colistin-resistant.

$bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$ and bla_{IMP} among carbapenem-resistant clinical isolates and also the distribution of colistin resistance among clinical isolates in Tamil Nadu [15, 34]. The rapid spread of carbapenem and colistin resistance among Gram-negative bacteria has become a major threat for the treatment of infectious diseases, not only in India, but also in other parts of the world. This study also showed the discrepancies among the results obtained by disk-

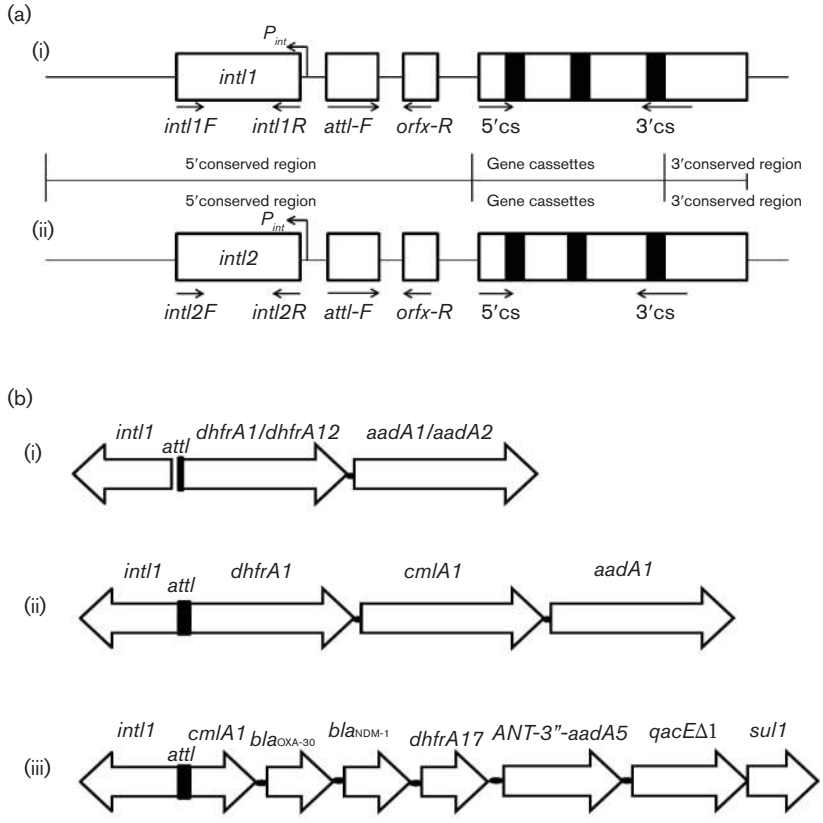

Fig. 2. (a) Schematic representation for PCR detection of class 1 and class 2 integron structures. [a(i)] Primers intI1F and intI2R were used to detect IntI1 integrase and 5′CS/3′CS (conserved region) were used to amplify variable regions of class 1 integrons. [a(ii)] Primers intI2F and intI2R were used to detect intI2 integrase and attI-F and orfX-R were used to characterize class 2 integron gene cassette arrays. (b) Variable regions (5′CS-3′CS) of class 1 integrons in isolates. [b(i)] *Escherichia coli* EC16 and *K. pneumoniae* KP1, [b(ii)] *Escherichia coli* EC7 and [b(iii)] *Escherichia coli* EC8.

diffusion, MIC, MHT and EDTA inhibition test for the detection of carbapenem resistance. Therefore the survey studies should not depend on a single identification strategy for reporting carbapenem resistance until adequate measures are established.

In recent years, there have been increasing reports of carbapenem and colistin resistance in *Enterobacteriaceae* harbouring both carbapenem-resistance and colistin-resistance genes in their plasmids [35]. Acquired resistance to colistin is extremely worrying considering that colistin is used as a last-resort antibiotic against carbapenem-resistant Gram-negative bacteria, especially *Enterobacteriaceae* [35]. Recently, plasmid-mediated carbapenem- and colistin-resistance in a clinical *Escherichia coli* isolate was reported in Switzerland [35, 36]. *Enterobacter cloacae* isolated from clinical samples were found to be resistant to both carbapenem and colistin in Colorado [37]. Colistin resistance in *K. pneumoniae* causing bacteraemia was reported from Tamil Nadu, India [38], and the presence of the colistin-resistance *mcr-1* gene was identified in *Escherichia coli* from the Indian subcontinent [39]. Recently, identification of a plasmid-mediated *mcr-1* gene conferring resistance to colistin in carbapenemase-producing *Escherichia coli* and *K. pneumoniae* from animals and patients in China and other parts of the world has been reported [40]. A recent study also found that the carbapenem- and colistin-resististant to both carbaant *Escherichia coli* producing plasmid-borne NDM-9 and MCR-1 was recovered from chicken meat samples in China [41]. Our present study showed the dissemination of carbapenem and colistin resistance (30 %) among Gram-negative clinical isolates including 78 % (21/27) in *Enterobacteriaceae*. The earlier studies also showed the co-transformation of plasmid-bound carbapenem and colistin resistance genes encoding NDM-9 and MCR-1 through conjugation [41]. In this study, we found that plasmid-borne carbapenem resistance genes bla_{NDM-1} and $bla_{OXA-181}$ were transferred through intra-genus gene transfer. The role of class 1 integrons in disseminating antibiotic resistance genes is well studied. A recent study characterized the carbapenem resistance gene bla_{VIM-2} inside the class 1 integron arrangement and reported the new integrons In1054 (*intI1-aacA56-qacEΔ1-sul1*) and In1160 (*intI1-aacA4-aacC1d*-IS*Kpn4-gcuE-qacEΔ1-sul1*) in *Pseudomonas aeruginosa* [42]. Accordingly, our study characterized

Table 4. Characteristics of bacterial isolates investigated for synergy studies, including resistance determinants, MICs of meropenem and colistin and MER-COL results from time-kill analysis

Bacterial isolate	Meropenem MIC (μg ml^{-1})	Colistin MIC (μg ml^{-1})	Time-kill analysis results‡	Synergistic concentration (MER +COL) (μg ml^{-1})§
Escherichia coli				
EC8*	64	32	S	4+4
EC16	32	8	S	8+2
EC17	16	16	S	4+2
EC21	32	8	SND	
EC24	8	8	S	1+0.5
Klebsiella pneumoniae				
KP4†	32	16	S	4+2
KP7*	64	>128	SND	
KP2*	64	16	SND	
KP3	>128	32	SND	
KP12	16	8	S	4+0.5
Pseudomonas aeruginosa PA3*	>128	16	SND	
PA5	64	16	SND	
PA8	16	32	S	2+4
Klebsiella oxytoca				
KO1	16	8	S	2+1
KO3	32	16	SND	
Acinetobacter baumannii				
AB1	>128	32	SND	
AB5	>128	32	SND	
Enterobacter cloacae EL4	32	16	S	8+2
Proteus mirabilis PM1	16	>128	SND	
Salmonella paratyphi SPT1	8	16	S	1+4

*NDM-1 producer.

†OXA-48 producer.

‡Time-kill analysis was performed for 20 isolates and the results represent the synergy after 24 h. S, synergy; SND, synergy not detected.

§Minimum inhibitory concentration at which synergy was obtained (meropenem+colistin).

the $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-30}}$ beta-lactamase genes inside the class 1 integron variable region (*intI1-cmlA5-*$bla_{\text{OXA-30}}$*-*$bla_{\text{NDM-1}}$*-dhfrA17*-ANT-3′ the best of our knowle-*aadA5-qacEΔ1-sul1*) and has been described in *Escherichia coli* for the first time, to the best of our knowledge. GenBank accession numbers of the class 1 integron sequences are: KX610373, KX660694, KX660695, KX660696 and KX685500.

As resistance plasmids are the major sources of antibiotic resistance transmission, these transferable elements conferring resistance to multiple antibiotics yield MDR bacteria. It is also possible that other mechanisms like integrons can co-transfer various plasmid-mediated resistance factors, accounting for the phenomenon of co-resistance as observed in our study. Plasmid-mediated transformation of resistance is of great concern and contributes to the spread of antibiotic resistance throughout bacterial species. Our study confirmed the plasmid-mediated transfer of carbapenem and colistin resistance and also contributed to the finding of $bla_{\text{NDM-1}}$ inside class 1 integrons in plasmids. There are not many previous studies reporting the association of carbapenem resistance genes and class 1 integrons in plasmids. Our finding is a relatively low occurrence of class 1 integrons within carbapenem- and colistin-resistant clinical isolates, but demonstrating the transfer of this plasmid-mediated resistance is the important outcome of this study.

Combination therapy is preferred to overcome the multiple-drug-resistant Gram-negative (MDR-GN) clinical pathogens. For carbapenem- and colistin-resistant isolates, combination therapy was used to improve the microbiological cure in critically-ill patients [43]. In earlier studies, broth-based methods were found to be accurate for assessing synergistic effects [44]. Colistin in combination with carbapenem is found to have synergistic effect against *K. pneumoniae*, *Pseudomonas aeruginosa* and *A. baumannii* [45–47]. Our *in vitro* combination therapy (meropenem +colistin) showed synergy in 50 % of the tested isolates which include $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$ producers. Though there are no accepted standard procedures to perform synergy testing in routine microbiological laboratories, the

recent clinical data show the positive effect of combination therapy to combat developing MDR pathogens.

Our study highlights the distribution of carbapenem- and colistin-resistant Gram-negative clinical pathogens harbouring plasmid-mediated resistance genes in Tamil Nadu, India. Additionally, we also identified the class 1 integron gene cassette regions harbouring antibiotic resistance genes. Our study also shows that plasmid-associated carbapenem resistance was transferable in some *Escherichia coli* strains harbouring the $bla_{\text{NDM-1}}$ gene determinant and was associated with class 1 integrons. Our study demonstrated that carbapenem-resistant genes $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$coded for the most predominant enzymes in Gram-negative bacteria isolated from the Tamil Nadu region in India.

CONCLUSION

The distribution of antibiotic-resistant bacteria in clinical settings is worrying. Clinical pathogens, especially bacteria developing resistance to last-resort antibiotics such as carbapenem and colistin, are rapidly increasing because of horizontal gene transfer. Necessary steps are to be taken immediately to combat this serious healthcare problem in the hospital setting (nosocomial infections), and available alternative treatment options should be employed to overcome the problem. This study identifies that plasmids and integrons do play an important role in mobilization of resistance gene determinants. Plasmid-borne carbapenem resistance genes, NDM-1 and OXA-181 in *Escherichia coli* were found to be involved in horizontal gene transfer; hence proper surveillance and detection of resistance genes are essential to prevent their spread and diversification. We know that even if the core genome is stable, the accessory genome is more fluid and carbapenem resistance among Gram-negative pathogens has become common, usually by the acquisition of carbapenemase genes. Our study shows that the combination of meropenem and colistin can exert a synergistic effect against antibiotic-resistant Gram-negative bacteria, and with further clinical studies we can observe its improved therapeutic outcomes. Combination therapy has proven to be effective in the treatment of many cancers, viral infections and tuberculosis. To combat multiple-drug-resistant Gram-negative bacterial infections, alternative approaches are urgently needed as the 'one drug-one target' model has limited viability, so combination therapy is indeed a novel way of tackling highly resistant bugs. In addition , combination therapy also has the potential to decrease the likelihood of resistance development.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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