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

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**Abstract**

**Background**: 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 regions in Tamil Nadu, India.

**Materials and Methods**: 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, 2*and integrons were studied. 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), *Salmonella* *paratyphi* (n=1). MIC testing showed that 58/89 (65%) isolates and 29/89 (32%) isolates were resistant to meropenem and colistin respectively whereas 27/89 (30%) were resistant to both antibiotics. *E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. aeruginosa*, and *E. cloacae* isolates were *bla*NDM-1 positive (n=20). Some strains of *E. coli*, *K. pneumoniae*, *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 *E. coli* strains could transfer carbapenem resistance via plasmids to susceptible *E. 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 Tamil Nadu region in South India.

**Keywords:** Integron, Plasmid-bound resistance, Transferability, New Delhi metallo-beta lactamases, Carbapenemases.

**Introduction**

Antibiotic resistance has become one of the increasing concerns in Gram-negative bacteria (GNB) because of the lack of available treatment options. The adverse outcome of developing multi-drug resistant (MDR) bacterial infections can lead to up to 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-lactamases (ESBLs) 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 the last resort antibiotics [4]. The emergence of resistant bacteria against the most classes of commercially available antibiotics and shortage in discovery of new antibiotics that has activity against Gram-negative bacteria had led to the use of polymyxins as a valuable therapeutic option [5]. Treatment options against infections caused by carbapenem-resistant Gram-negative bacteria (CR-GNB) are the current challenges of our time due to 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 polymyxin group of polypeptide antibiotic with a significant activity against Gram-negative bacteria and they target lipopolysaccharide (LPS) 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 the recent times [12-15].

Recently, 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 be playing a significant role in the dissemination of antibiotic resistance genes within and in between the bacterial species [20-22]. For these reasons, combination therapy with different classes of antibiotics is recommended to improve the 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 and evaluated the dissemination of integrons, and assessed whether meropenem-colistin had a synergistic effect on the clinical strains.

**Materials and 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- 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 the vials from clinical diagnostic centers and further processing was carried out in Antibiotic Resistance Laboratory at VIT University, Vellore, India. The isolates were sub-cultured onto MacConkey agar (Hi-Media, India) and stored at -80°C for further analysis. Identification was done using phenotypic methods and based on the colony morphology and biochemical characteristics and confirmed by the VITEK identification system (bioMèrieux Inc., USA).

**Antibiotic susceptibility test**

Minimal Inhibitory Concentration (MIC) of meropenem was performed by agar dilution method following the CLSI guidelines. Briefly, Mueller-Hinton (MH) agar was prepared and the final concentrations of meropenem ranging from 0.06 mg/L to 128 mg/L were added in MH agar (Hi-Media, India) in petri plates, mixed well and allowed to solidify. Then, overnight grown bacterial inocula 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. Inoculum was allowed to dry, and was incubated at 37ºC for 20 h. Lowest concentration with the absence of visible bacterial growth was accepted as the MIC. The results were interpreted using the CLSI guidelines [24].

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

For colistin, MIC was performed using micro-broth dilution method (CLSI guidelines, 2015). Briefly, cation-adjusted MH broth (Hi-Media, India) was prepared and 100 µL of broth was disbursed in 96 wells microtiter plate. Colistin was added at the final concentrations ranging from 0.06 mg/L to 128 mg/L in row A1 to 12 with row 12 being used as a growth control. Then, bacterial inocula of 5x105 dilutions (CLSI guidelines) from overnight grown cells were added to respective wells and were incubated at 37ºC for 20 h. The results were interpreted using the CLSI guidelines.

**Identification of carbapenemase producer**

The Modified Hodge Test (MHT) and the EDTA (Ethylene Diamine Tetra Acetic acid) inhibition tests were performed for the detection of carbapenemase production as described earlier [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 separated by 15 mm apart, and 10 μl of 0.5M EDTA (Hi-Media, India) was added to one of the disks (final concentration EDTA 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 the EDTA. An MBL producer was defined as an isolate displaying reduced susceptibility to meropenem and tested positive for MHT and positive for EDTA inhibition test.

**Synergy testing**

Time-kill analysis was performed for the isolates that were resistant to meropenem and colistin (27/89) and based on the method described earlier [26]. Antibiotics were used at the final concentrations of 4 mg/L for colistin and 8 mg/L 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 105 CFU/mL and incubated at 37°C in 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 24 h of incubation, colonies were counted to determine the synergistic effect of drugs in combination. Synergy was defined as any ≥2-log10 decrease in colony count after 24 h comparing with the same drug alone and remaining as SND (synergy not detected).

**DNA extraction**

DNA extraction was done by the boiling preparation method. Shortly, overnight grown bacteria were centrifuged at 10,000 rpm for 10 min, and to the harvested bacterial cells, 100 µL of sterile distilled water was added and the cells were heated at 95°C for 10-15 min. The content was centrifuged at 5000 rpm for 2 min and the supernatant was extracted and used as a source of a template.

**Screening of antibiotic resistance gene determinants**

The presence of beta-lactamase genes *bla*NDM-1, *bla*OXA-48-like, *bla*IMP, *bla*VIM and *bla*KPC were studied by multiplex polymerase chain reaction (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*. For the amplification of class 1 integrons, two primer sets were used: IntI1-F and IntI1-R for *intI1* gene and 5’-CS and 3’-CS for the integron variable region as described earlier by Kargar *et al*., [28]. For class 2 integrons, primers IntI2-F and IntI2-R for *intI2* gene and attI2-F and orfX-R for characterization of gene cassette arrays. Integrase gene products were sequenced for further determination of integron gene cassette.

Primers for the *mcr-1*, *mcr-2* gene 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 HiPurA plasmid DNA miniprep purification kit (Hi-Media, India) following manufacture’s protocol and lambda DNA/EcoRI+HindIII marker (ThermoFisher Scientific, USA) was used to identify the size of the plasmids. A total of 24 isolates carrying resistance genes NDM-1 and OXA-48 were studied. In the case of intra-genus transfer of antibiotic resistance, only NDM-1 and OXA-48 encoded *E. coli* (11) isolates were chosen and the selected isolates were also found to be colistin resistant with MIC ≥32. A plasmid-free, streptomycin-resistant (F-, Strr) auxotrophic strain of Escherichia coli (E. coli AB 1157), showing sensitivity to all the antibiotics under study, was used as a recipient, while all the NDM-1 and OXA-48 encoded E. coli served as the donors [30]. Donor and recipient cultures were grown overnight (108 cells/mL) and 5 mL of each bacterial culture was mixed (1:1) in a Luria-Bertani broth (Hi-Media, India) and was incubated without shaking for 16 h at 37°C. The transconjugants were selected on the LB agar plates supplemented with streptomycin (100 µg/mL) in addition with either meropenem (10 µg/mL) or colistin (10 µg/mL). 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 *E. coli* isolates resistant to both meropenem and colistin were studied. The transformants grown on the antibiotic plates were screened for resistant genes 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 our present study. The identified clinical isolates included; 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*, 1% (n=1) *Salmonella* *paratyphi*. In a total of 89 isolates, 81% were found to be *Enterobacteriaceae* (Figure 1).

**Susceptibility to antibiotics**

All the isolates received from clinical laboratories were found to be multiple drug resistant by 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. MIC50 and MIC90 for meropenem agar dilution were 16 µg/mL and 32 µg/mL respectively. Among meropenem resistant isolates percentage of *E. coli* was 65% (28/43), *K. pneumoniae* 67% (12/18), *P. aeruginosa* 60% (6/10), *K. oxytoca* 100% (4/4). Meropenem resistance was also observed in isolates such as *E. cloacae* (3/6), *A. baumannii* (3/5), *P. mirabilis* (1/2) and *S. paratyphi* (1/1).

In the case of colistin, MIC results showed that 29 of 89 (33%) isolates were resistant showing MIC50= 1 µg/mL and MIC90= 16 µg/mL. Colistin-resistance was observed in *E. coli* (61%, 11/43), *K. pneumoniae* (33%, 6/18), *P. aeruginosa* (30%, 3/10). We also observed colistin resistance in *E. cloacae* (3/6), *A. baumannii* (2/5), *K. oxytoca* (2/4), *P. 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 (Figure 1).

**Identification of carbapenemase producer**

Phenotypic studies for carbapenemase activity was detected in 52/89 (58.4%) by MHT method and 50/89 (56.1%) by the EDTA inhibition test. Comparing, 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 metallo beta-lactamases including carbapenemases.

**Screening of antibiotic resistance genes**

The presence of resistance genes *bla*NDM-1, *bla*OXA-48-like, *bla*IMP, *bla*VIM, *bla*KPC and *mcr-1, mcr-2* were tested for all the 89 isolates. PCR results showed that *bla*NDM-1 and *bla*OXA-48-like were the most predominant genes in carbapenem-resistant Gram-negative bacteria in our study. In total, 24/89 carried resistant genes *bla*NDM-1, and *bla*OXA-48-like. Genes *bla*IMP, *bla*VIM, and *bla*KPC were absent in all the isolates tested. Among *bla*NDM-1 (20/89)carriers were *E. coli* (n=9), *K.* *pneumoniae* (n=4), *P. aeruginosa* (n=3), *K. oxytoca* (n=3), *E. cloacae* (n=1). Four *bla*OXA-48 producers were *E. coli* (n=2), *K*. *pneumoniae* (n=1) and *K. oxytoca* (n=1). Interestingly, four of *E. coli* isolates (EC8, EC12, EC15, EC33) were positive for *bla*NDM-1 and two *E. coli* isolates (EC14, EC22) carried *bla*OXA-48-like and were identified in plasmid DNA and studied further to determine if these genes were transferrable. ERIC-PCR results showed that two of the carbapenem resistant *E. coli* (EC8, EC15) carrying *bla*NDM-1 and two *E. coli* (EC14, EC22) carrying *bla*OXA-48-like had the same ERIC profile. Two of the *bla*NDM-1 *E. 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 genes. Sequencing results showed that isolates positive for *bla*NDM gene had *bla*NDM-1 and *bla*OXA-48-like gene had *bla*OXA-181. In the case of colistin resistance, both *mcr-1* and *mcr-2* genes were not amplified in any of the isolates. Of the *bla*NDM-1 carriers two *E. coli* (EC8, EC10) and one *bla*OXA-181 positive *K. pneumoniae* 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 100kb (Table 2). Conjugation studies were used to elucidate the intra-genus gene transfer of plasmid-borne resistance. The resistance was transferred to *E. coli* AB1157. The results showed that out of 9 *E. coli* isolates carrying NDM, only 4 (EC8, EC12, EC15, EC33) were found to transfer resistance plasmids to susceptible *E. coli* AB1157. But, both the *E. coli* EC14 & EC22 -OXA carriers were able to transfer its plasmid-borne gene to susceptible *E. coli* AB1157. The resistance was transferred at a frequency of 4-6 x 10-5 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 *E. coli* isolate (with *bla*NDM-1) showed the simultaneous transfer of both the markers at a low frequency of 3 x 10-6.

**Distribution of integrons in carbapenem and colistin resistant isolates**

Of the 89 clinical isolates studied, 24 (26.9%) isolates were identified as positive for class 1 integrons, 20 (22.4%) for class 2 integrons and 3 (3.3%) for class 3 integrons (Table 3). Sequencing results showed that the variable regions in class 1 integrons carried antibiotic resistant genes and showed that one isolate had *bla*NDM-1 in its gene cassette (Figure 2A and 2B). Interestingly, 2/6 of the donor *E. coli* (EC8, EC10) involved in conjugation carried NDM-1 along with class 1 integrons and the same was also amplified in transformants by PCR using specific primers, indicating the development of the competence and uptake of DNA, leading to recombination and thus, transformation. Interestingly, one of the *E. coli* EC8 isolate carrying *bla*NDM-1 was found inside the class 1 integron (variable region) arrangement along with *bla*OXA-30 which correlates with our earlier PCR studies but OXA-30 was not amplified with specific primers in this study. But dual resistance was found in *E. coli* isolates producing plasmid-bound *bla*NDM-1 and also involved in co-transformation of dual resistant in 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, 5 patterns of *E. coli*, 5 patterns of *K. pneumoniae*, 3 patterns of *P. aeruginosa*, 2 patterns of *A. baumannii*, 2 patterns of *K. oxytoca*, and 1 pattern each for *E. cloacae*, *P. mirabilis*, and *S. paratyphi* were observed. A total of 20 isolates were included for the synergy testing and differences in colony count of ≥2 log10 after 24 h of exposure to the drugs in combination and as a single agent was considered as synergy. The time-kill analysis results showed synergy for 10/20 (50%) isolates after 24h (Table 4). Our results showed that among 10 synergetic isolates included, 4/5 were *E. coli*, 2/5 *K. pneumoniae*, and one belonged to *P. aeruginosa, K. oxytoca, E. cloacae* and *S. paratyphi*. It was also noted that 4/10 synergetic isolates were NDM-1 (2 *E. coli*, 1 *K. pneumoniae* and 1 *P. aeruginosa*) producer 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 analyzed 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 widespread of NDM-1 andOXA-48-likeproducing carbapenem-resistant Gram-negative bacteria from India [31-33]. In addition, our previous studies also reported the distribution of *bla*NDM-1, *bla*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 these 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 discrepancy among the results obtained by disk-diffusion, MIC, MHT and EDTA inhibition test for the detection of carbapenem resistance. So the survey studies should not depend on the single identification strategy for reporting carbapenem resistance until adequate measures are established.

In recent years, there are increasing reports of carbapenem and colistin resistance in *Enterobacteriaceae* harbouring both carbapenem-resistant and colistin-resistant 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 *E. 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 bacteremia was reported from Tamil Nadu, India [38] and the presence of colistin-resistant *mcr-1* gene was identified in *E. coli* from Indian sub-continent [39]. Recently, identification of plasmid-mediated *mcr-1* gene conferring resistance to colistin in carbapenemase-producing *E. coli* and *K. pneumoniae* from animals and patients in China and other parts of the world is an additional data [40]. The recent study also found that the carbapenem and colistin-resistant *E. coli* producing plasmid-borne NDM-9 and MCR-1 that were 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 encoding NDM-9 and MCR-1 through conjugation [41]. In this study, we found that plasmid-borne carbapenem-resistant genes *bla*NDM-1 and *bla*OXA-181 were transferred through intra-genus gene transfer. The role of class 1 integrons in disseminating antibiotic resistant gene is well studied. A study recently characterized the carbapenem resistant gene *bla*VIM-2 inside the class 1 integron arrangement and reported the new integrons In1054 (*intI1-aacA56-qacE*Δ*1-sul1*) and In1160 (*intI1*-*aacA4*-*aacC1d*-IS*Kpn4*-*gcuE-qacE*Δ*1-sul1*) in *P. aeruginosa* [42]. Accordingly, our study characterized the *bla*NDM-1 and *bla*OXA-30 beta-lactamase genes inside the class 1 integron variable region (*intI1*-*cmlA5*-*bla*OXA-30-*bla*NDM-1-*dhfrA17*-ANT-3”-*aadA5*-*qacEΔ1-sul1*) and has been described in *E. coli* for the first time in general, to the best of our knowledge. Accession numbers (NCBI) of the class 1 integron sequences: KX610373, KX660694, KX660694, KX660696, KX685500

As resistance plasmids are the major sources of antibiotic resistance transmission, these transferable elements conferring resistant to multiple antibiotics yielding MDR bacteria. It is also possible that the 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-resistant throughout the bacterial species. Our study confirmed the plasmid-mediated transfer of carbapenem and colistin resistance and also contributed to the finding of *bla*NDM-1 inside class 1 integrons in plasmids. There are no many previous studies reporting the association of carbapenem-resistant genes and class 1 integrons in plasmids. However, our finding is relatively a low occurrence of class 1 integrons within carbapenem and colistin resistant clinical isolates. But 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 the synergistic effect against *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* [45-47]. Our *in vitro* combination therapy (meropenem +colistin) showed synergy in 50% of the tested isolates which includes *bla*NDM-1, and *bla*OXA-181 producers. Though there are no accepted standard procedures to perform synergy testing in routine microbiological laboratories, the recent clinical data shows the positive effect of combination therapy to combat the developing MDR pathogens.

Our study highlights the distribution of carbapenem and colistin resistant Gram-negative clinical pathogens harbouring plasmid-mediated resistant genes in Tamil Nadu, India. Additionally, we also identified the class 1 integron gene cassette regions harbouring antibiotic resistant genes. Our study also shows that plasmid-associated carbapenem resistance was transferable in some *E. coli* strains harbouring *bla*NDM-1 gene determinant and was associated with class 1 integrons. Our study demonstrated that carbapenem-resistant genes *bla*NDM-1 and *bla*OXA-181 were the most predominant enzymes in Gram-negative bacteria isolated from 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 of antibiotics like carbapenem and colistin are rapidly increasing because of horizontal gene transfer. Necessary steps are to be taken immediately to combat this serious health-care problem in the hospital setting (nosocomial infections) and available alternative treatment options should be undertaken to overcome the problem. This study identifies that plasmids and integrons do play an important role in mobilisation of resistance gene determinants. Plasmid-borne carbapenem resistant genes, NDM-1 and OXA-181 in *E. coli* were found to be involved in horizontal gene transfer hence proper surveillance and detection of resistant genes is essential in order 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 treatment of many cancers, viral infection and tuberculosis. To combat multiple drug resistant Gram negative bacterial infections, alternative approaches are urgently needed as ‘one drug-one target’ model has limited viability so combination therapy is indeed a novel way of tackling highly resistant bugs in addition to that it also has the potential to decrease the likelihood of resistance development.

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**Conflict of interest**

There is no conflict of interest.

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**Figure 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.**

**Figure 2: (A) Schematic representation for PCR detection of class 1 and class 2 integron structures.**

**2A-1) 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.**

**2A-2) 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.**

**Figure 2: (B) Variable regions (5’CS-3’CS) of class 1 integrons in isolates**

**(2B-1) *E. coli* EC16 and *K. pneumoniae* KP1**

**(2B-2) *E. coli* EC7 and (2B-3) *E. coli* EC8.**