Invitro Antimicrobial Synergy of Carbapenem with Tigecycline and Colistin in Gram negative isolates from ICU

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Abstract

The worldwide increase in the emergence of carbapenem resistant Gram-negative (CRGN) pathogens calls for the investigation into alternative approaches for treatment. The aim of this study is to evaluate the *in vitro* effect of the colistin–carbapenem (including meropenem, doripenem, ertapenem and imipenem) combination and tigecycline-carbapenem combination against Carbapenem Resistant *Enterobacterales* (CRE) using two different techniques viz. Chequerboard and time-kill synergy method.

Methods

A total of 118 CRE isolates were included to the study. The minimum inhibitory concentrations of colistin, tigecycline and carbapenem (including meropenem, doripenem, ertapenem and imipenem) were determined with broth dilution method. In addition, PCR amplifications of the most common beta lactamases contributing to carbapenem resistance were performed. Synergistic effects of tigecycline-carbapenem and colistin-carbapenem were investigated by checkerboard technique and time kill assay.

Results

All of the isolates were resistant to carbapenems whereas none of the isolates were resistant to colistin and tigecycline. Synergistic effect for the colistin-carbapenem and tigecycline-carbapenem combination was observed using both methods. Additive effects were also detected in both combinations where the ∑FICI of carbapenem combined with colistin was 1.167 ± 0.354 and that of carbapenem with tigecycline was 1.106 ± 0.337. The combination of colistin-carbapenem showed better effects as compared to tigecycline-carbapenem ($p < 0.05$). The colistin-carbapenem and tigecycline-carbapenem combinations also showed a decrease of 2.6 and 2.8-fold, respectively. Time-kill assays additionally showed synergistic effects, and no bacterial re-growth was detected following a 24 h incubation. Synergistic effect was variable and strain-depended

against CRE isolates that have been tested.

Conclusion

Our study showed that the combination of carbapenems with colistin and tigecycline could be a promising antimicrobial strategy in treating CRE infections and holds great importance for management of patients who cannot afford expensive drugs for treatment.

Key Words: Multidrug resistance, Synergy, time kill assay, chequerboard technique

Background

Infections caused by carbapenem-resistant Gram-negative (CRGN) pathogens are increasing globally and are associated with poor patient outcomes [1]. The emergence of Carbapenem Resistant Gram-negative (CRGN) pathogens and their detection in several regions across the world makes their treatment increasingly challenging [2] .They have emerged as one of the most important nosocomial pathogens, especially in patients admitted to an intensive care unit (ICU). They can colonize multiple body sites of hospitalized patients and survive for a long time on inanimate surfaces [3]. Both these aforementioned characteristics may have contributed to the prominent role of CRGN in nosocomial infections.

A wide range of broad-spectrum antimicrobial agents have been used in the treatment of infections caused by Carbapenem Resistant Enterobacterales (CRE). Of these agents, carbapenems are often resorted to due to their low toxicity and high efficacy. Nonetheless, the overuse and misuse of carbapenems led to an increase in resistance rates against this potent class of antimicrobial agents [4].

The high carbapenem resistance rates pose serious therapeutic and infection control challenges, especially since they are associated with high mortality rates and an increase in hospital stay [5]. Moreover, the lack of effective antibiotics against CRE isolates led to the re-use of colistin [6] . Colistin, which was abandoned since the 1960s due to nephrotoxicity, gained new interest for its activity against these infections. Tigecycline also showed good in vitro bacteriostatic activity against carbapenem resistant strains that showed different susceptibilities to carbapenems. [6]

Therefore, it is important to look for combination of drugs that might be synergistic. Combination treatment with a colistin and a carbapenem has been suggested to improve effectiveness, supported by in vitro models showing synergism between the two antibiotics and this combination therapy has been adopted widely by clinicians. The aim of this study is to evaluate the in vitro effect of the colistin–carbapenem (including meropenem, doripenem, ertapenem and imipenem) combination and tigecycline-carbapenem combination against Carbapenem Resistant *Enterobacterales* (CRE) using two different techniques viz. Chequerboard and time-kill synergy method. [6]

Method

Study design and clinical isolates

The prospective study was carried out in the Department of Microbiology of JN Medical College and Hospital, AMU, Aligarh. A total of 118, non-duplicate consecutive isolates were collected from various clinical specimens from December 2020 to December 2022. Minimum inhibitory concentration (MIC) was estimated for 60 representative isolates of differing levels of drug resistance. No written consent from the patients was taken since no interventions were performed.

Identification and antimicrobial susceptibility testing

The clinical samples received in the laboratory were inoculated on 5% sheep blood agar (BA), MacConkey agar (MCA), nutrient broth and brain heart infusion (BHI) broth. These were incubated at 35°C for 18-24 hours. Isolates obtained were further subjected to various biochemical reactions. The organisms were identified on the basis of morphology, cultural characteristics and biochemical tests. Susceptibility to different classes of antimicrobial agents was determined by the disc diffusion method [7]. Antimicrobial susceptibility of organisms was also performed by automated method using VITEK-2 (Biomeuriux).

In addition, Minimum Inhibitory Concentrations (MICs) of colistin, tigecycline, meropenem, imipenem, doripenem and ertapenem were performed by broth dilution methods [7]. Cutoff values were ≤ 2 µg/ ml≥ 4 µg/ ml for colistin and ≤ 1µg/ ml≥ 4 µg/ ml for meropenem, imipenem, doripenem and ≤ 1µg/ ml≥ 2 µg/ ml for ertapenem [7]. The FDA tigecycline breakpoints for *Enterobacterales* were applied to due to lack of breakpoint criteria in the CLSI guidelines [8] .

Polymerase chain reactions

DNA extraction was performed for all the isolates as described by [9]. The DNA extracts were preserved at −20°C until used. bla_{VIM}, bla_{NDM} and bla_{OXA} were tested for by PCR using the primers listed in Table 1 [10,11,12,13]. Positive and negative controls for the tested genes were provided from previous studies performed in the laboratory [10,14].

Table 1: Primers used for PCR amplification with their different amplicon size.

Beta-lactamases	bla gene	Primer direction	Sequence (5'–3')	Size (bp)
CLASS B				
	VIM	VIM F	ATTCCGGTCGG(A=G) GAGGTCCG	601
		VIM R	TGTGCTKGAGCAAKTCYAGACCG	
	NDM	NDM F	GGGCCGTATGAGTGATTGC	825
		NDM R	GAAGCTGAGCACCGCATTAG	
CLASS D				
	OXA-48	OXA48 F	GCTTGATCGCCCTCGATT	281
		OXA48 R	GATTTGCTCCGTGGCCGAAA	

R, reverse primer; F, forward primer.

The checkerboard technique

The checkerboard technique was performed in using the combinations of colistin-carbapenem (including meropenem, imipenem, doripenem and ertapenem) and tigecycline-carbapenem combination. Concentration ranges of 16xMIC to 1/16xMIC for colistin and tigecycline and 256xMIC to 1/256xMIC for carbapenems were prepared. The bacterial inoculum was adjusted to 5×10^5cfu/ml and distributed in all the tubes. Two wells were reserved for positive and negative controls. After incubation at 37°C for 24 h, the Fractional Inhibitory Concentration Index (FICI) was calculated using the formula “FICA + FICB = FICI” where “FICA” is the MIC of the drug A

in combination/ MIC of the drug A alone; and "FICB" is the MIC of the drug B in combination/ MIC of the drug B alone (Daoud et al., 2013). The sum of FICI was then interpreted as follows: synergy if ∑FICI ≤ 0.5, additive effect if 0.5 < ∑FIC ≤ 2, indifference if 2 < ∑FIC ≤ 4, and antagonism if ∑FIC > 4 (Pillaii et al., 2005).

Time-kill curve assay

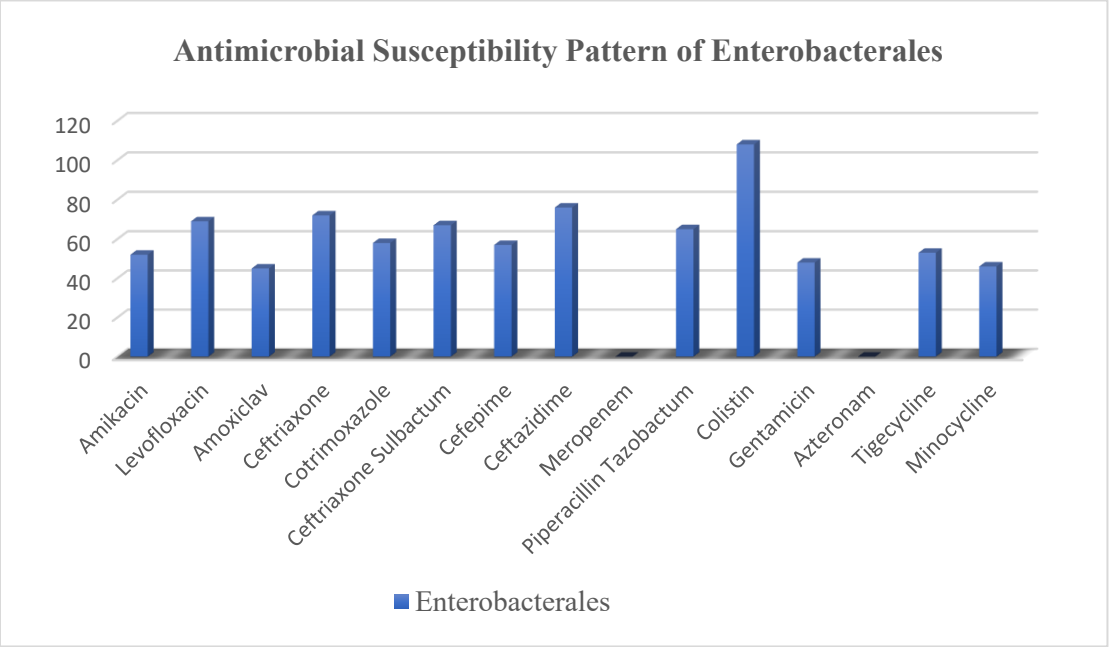
Briefly, concentration ranges of 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125xMIC were prepared in Mueller Hinton Broth for colistin and tigecycline and carbapenems (including imipenem, meropenem, ertapenem and doripenem alone, and in combination (colistin-carbapenem and tigecycline-carbapenem). A 5 x × 10^5cfu/ml inoculum of the tested organism was also prepared. The suspensions were then incubated at 37°C for 4 h. An antibiotic-free growth control was also included. At predetermined time points (4 h and 24 h), subcultures were done from each tube next day onto Mueller- Hinton agar plates. Time kill curves were then constructed as a function of time and the results were represented as a difference in log10 between the cfu/mL at 4 h and 24 h.

Synergistic effects were determined by a decrease of 3 log10 in colony count at 24 h by the combination compared to most active single agent. Additivity/indifference were interpreted as <3 log10 increase or decrease in colony count at 24 h by the combination compared with that by the most active drug alone. Antagonism was interpreted as 3 log10 increase in colony count at 24 h by the combination with that by the most active drug alone.

Results

Bacterial isolates and susceptibility testing

In this study, majority of isolates constituted *Escherichia coli* (n=57;52.7%), *Klebsiella pneumoniae* (n=22; 20.3%), *Klebsiella oxytoca* (n=10;9.2%) followed by *Enterobacter cloacae* (n=10; 9.2%) and *Citrobacter freundii* (n=9; 8.3%). The antibiotic resistance profile of *Enterobacterales* to different antibiotics is depicted in Figure 1. Of the various groups tested, *Enterobacterales* showed maximum sensitivity to colistin: 100%, Ceftazidime: 70.3%, followed by Ceftriaxone 66.6%. *Enterobacterales* showed a moderate degree of sensitivity to Ceftriaxone sulbactum: 62%, Piperacillin tazobactum: 60.1% and Cefepime: 52.7%. The prevalence of antimicrobial resistance was considerably high in *Enterobacterales.*



Carbapenem resistance genes

PCR amplification of the common carbapenemase genes detected three bla_{OXA} (n=3;10%) genes to be positive out of 29 carbapenemase producing isolates. The rest of the genes tested for were not detected in any of the isolates.

In vitro combination effects

Synergistic effects were detected while combining carbapenem with colistin (10 isolates) and tigecycline (10 isolates) using the checkerboard and time kill assay. Additive effects were also detected in both combinations where the ∑FICI of carbapenem combined with colistin was 1.167 ± 0.354 and that of carbapenem with tigecycline was 1.106 ± 0.337. The combination of colistin with carbapenem showed a better additive effect than the tigecycline with carbapenem combination ($p < 0.05$). The combinations of carbapenem-colistin and carbapenem-tigecycline resulted in a decrease of 2.8- and 2.6-folds in the MIC of carbapenems, respectively.

Time-kill curves showed that there was bactericidal activity detected for all three antibiotics (colistin, tigecycline and carbapenem), where all the isolates showed a decrease in colony counts from 4 hours to 24 hours. A significant bactericidal effect of colistin-imipenem when compared to colistin-meropenem ($p < 0.05$) was determined at 0.5XMIC. Moreover, no bacterial re-growth was detected at the different concentrations of colistin and colistin-carbapenem combinations. It is important to note that, due to limitations that were faced during the experiment, only 10 out of the 60 isolates were tested for at 16XMIC, which could have resulted in obtaining rather high values at this concentration as compared to the other concentrations.

Table 2: Log 10 values of the cfu/ml of the *Enterobacterales* isolates obtained by the time kill curve assays after incubation, as compared to the initial inoculum.

Antibiotics	Bactericidal effect of colistin and carbapenems alone and in combination (time kill curve)							
	16xMIC	8xMIC	4xMIC	2xMIC	1xMIC	0.5xMIC	0.25xMIC	0.125xMIC
	ΔLog10							
Col	0.429	0.155	0.214	0.441	1.18	1.368	1.621	1.826
Imp	0.325	0.139	0.137	0.121	0.218	1.12	1.914	2.174
Mer	0.413	0.131	0.116	0.282	1.12	1.922	2.054	2.194
Erta	0.403	0.178	0.098	0.195	0.106	0.815	1.06	1.802
Dori	0.325	0.139	0.137	0.121	0.218	1.12	1.914	2.174
Col-Imp	0.437	0.196	0.115	0.066	0.096	0.349	0.915	1.828
Col-Mer	0.403	0.178	0.098	0.195	0.106	0.815	1.06	1.802
Col-Erta	0.001	0.031	−0.11	−0.404	−1.077	−1.059	−0.732	−0.024
Col-Dori	−0.006	0.05	−0.119	−0.274	−0.937	−0.553	−0.554	−0.014
p-value	0.528	0.698	0.679	0.103	0.831	0.018	0.112	0.731

Col, colistin; Imp, Imipenem; Mer, Meropenem; Erta, Ertapenem; Dori, Doripenem; Col-Imp, colistin Imipenem combination, Col-Mer, colistin meropenem Col-Erta, colistin ertapenem, Col-Dori, colistin doripenem combination

Table 3: Log 10 values of the cfu/ml of the *Enterobacterales* isolates obtained by the time kill curve assays after incubation, as compared to the initial inoculum.

Antibiotics	Bactericidal effect of tigecycline and carbapenems alone and in combination (time kill curve)							
	16xMIC	8xMIC	4xMIC	2xMIC	1xMIC	0.5xMIC	0.25xMIC	0.125xMIC
	ΔLog10							
Tgc	0.413	0.131	0.116	0.282	1.12	1.922	2.054	2.194
Imp	0.403	0.178	0.098	0.195	0.106	0.815	1.06	1.802
Mer	0.325	0.139	0.137	0.121	0.218	1.12	1.914	2.174
Erta	0.001	0.031	−0.11	−0.404	−1.077	−1.059	−0.732	−0.024
Dori	−0.006	0.05	−0.119	−0.274	−0.937	−0.553	−0.554	−0.014
Tgc-Imp	0.437	0.196	0.115	0.066	0.096	0.349	0.915	1.828
Tgc-Mer	0.403	0.178	0.098	0.195	0.106	0.815	1.06	1.802
Tgc-Erta	0.001	0.031	−0.11	−0.404	−1.077	−1.059	−0.732	−0.024
Tgc-Dori	−0.006	0.05	−0.119	−0.274	−0.937	−0.553	−0.554	−0.014
p-value	0.528	0.698	0.679	0.103	0.831	0.018	0.112	0.731

Tgc, Tigecycline; Imp, Imipenem; Mer, Meropenem; Erta, Ertapenem; Dori, Doripenem; Tgc-Imp, Tigecycline Imipenem combination, Tgc-Mer, Tigecycline meropenem Tgc-Erta, Tigecycline ertapenem, Tgc-Dori, Tigecycline doripenem combination

Discussion

The possibility and the probability of acquiring infections in ICU has increased in the past two decades. Such infections constitute a significant problem for the patients with substantial morbidity and mortality [15]. These infections represent a leading cause of death and represent important health care cost [16]. Treatment of ICU infections is increasingly hampered by the emergence of antibiotic resistance. Rapid and accurate diagnosis of ICU infections is an important aspects of intensive care medicine [17].

MIC values of six drugs namely Colistin, Tigecycline, Meropenem, Ertapenem, Doripenem and Imipenem were determined by the broth dilution method. For Colistin the MIC range was 0.5-1μg/ml. Most of the isolates showed the MIC value of less than 0.5 μg/ml. The MIC range of

Tigecycline came out to be 1-4 µg/ml. Most of the isolates showed the MIC value of 2 µg/ml. For carbapenems the MIC range was 32-512 µg/ml. Most of the isolates showed MIC values of 32 µg/ml. The prevalence of antimicrobial resistance was considerably high in Enterobacterales.

Multidrug-resistant Enterobacterales and Nil fermenters with combined decreased susceptibility to Imipenem, Meropenem, Ertapenem and Doripenem is increasingly being found as a cause of nosocomial infections. It is important to look for combination of drugs that might be synergistic. The putative benefits are to increase efficacy by achieving synergistic killing and preventing the emergence of antibiotic resistance, but data are sparse [18]. Combinations of carbapenems/colistin and carbapenems/tigecycline have synergistic effects against gram negative isolates. Combinations of carbapenems and colistin and combination of carbapenems with tigecycline have found to be synergistic. This supported an in vivo synergistic or additive effect of the carbapenem plus colistin combination.

Resistance is becoming increasingly common among gram negative bacteria. Therefore, making empirical therapy decisions more difficult. Infections caused by carbapenem-resistant Gram-negative (CRGN) pathogens are increasing globally and are associated with poor patient outcomes. The most serious resistance patterns now emerging among Gram-negative organisms include resistance to extended-spectrum cephalosporins and penicillins [19]. This resistance is commonly mediated by ESBLs in Escherichia coli and Klebsiella species, or by the hyper production of chromosomally mediated cephalosporinases (Bush group I AmpC enzymes) in Serratia and Citrobacter species [20]. The ESBL genes generally result from point mutations in the genes of broad-spectrum ß-lactamase Ambler class A enzymes, such as CTX-M, TEM or SHV. They are usually located in conjugative megaplasmids, which often carry genes responsible for resistance to other antibacterial drugs, making it extremely difficult to treat infections caused by bacteria that produce these enzymes [20].

The role of antibiotic combinations in the treatment of CRE infections is a matter of long-standing debate. The potential advantages of combination treatment are improved effectiveness due to synergism and prevention of resistance development. The potential disadvantages are increased side effects and increased selection pressure (because of increased antibiotic use), which favours the spread of antibiotic-resistant organisms. While some observational studies reported greater survival in patients treated with colistin combination regimens. The rationale behind synergy

testing as a basis for choosing combination treatment is attractive: it could improve clinical outcomes in patients for whom the combination is synergistic and reduce antibiotic use compared with giving combination treatment to everyone.[20]

However, there is a paucity of clinical data regarding the effectiveness of synergy-guided treatment for CRGN infections, and most data come from case reports. With synergy-guided treatment, it is important to select the synergy testing method that best correlates with clinical outcome. Various in vitro testing methods have been used, including checkerboard, E-test and time-kill assays. The checkerboard assay has the advantage of testing a wide range of drug concentration combinations simultaneously. Time-kill assay provides data on the rapidity of synergistic killing compared to each drug alone, but it is impractical when testing a large number of isolates or in the routine clinical microbiology laboratory, as it is complicated and time consuming. Multidrug-resistant Enterobacterales and Nil fermenters with combined decreased susceptibility to Imipenem, Meropenem, Ertapenem and Doripenem is increasingly being found as a cause of nosocomial infections.

It is important to look for combination of drugs that might be synergistic. The putative benefits are to increase efficacy by achieving synergistic killing and preventing the emergence of antibiotic resistance, but data are sparse [Vladimir Chachanidze et al]. Combinations of carbapenems/colistin and carbapenems/tigecycline have synergistic effects against gram negative isolates. Combinations of carbapenems and colistin and combination of carbapenems with tigecycline have found to be synergistic. This supported an in vivo synergistic or additive effect of the carbapenem plus colistin combination

Conclusion

Nowadays, the rate of carbapenem resistance among nosocomial isolates is high, particularly in ICUs. Increasing rates of carbapenem resistance have led to widespread use of combination treatment for the treatment of diverse infectious disease. Although the isolates tested were resistant to one or both antibiotics, synergy was observed which suggests that treatment with combination of antibiotics is a good option in multidrug resistant isolates and should be tried. This finding holds great importance for management of patients infected with multidrug resistant gram-negative organisms and in poor patients who cannot afford expensive drugs for treatment.

These results suggest that even if Enterobacterales isolates are resistant to carbapenems drugs by disc diffusion method or even if they have high MIC values, they can be used with combination of colistin and tigecycline. This protocol can help in overcoming increasing resistance in carbapenems.

We suggest that synergy testing should be performed at various centres to assess which combination of antibiotics is most synergistic

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