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In vitro evaluation of antibiotic synergy for carbapenem-resistant *Klebsiella pneumoniae* clinical isolates

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***Background & objectives*: The prevalence of severe infections due to carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains has increased worldwide. With rising resistance to polymyxins, the treatment has become challenging. Given the paucity of novel agents and limited data on combination therapy for CRKP, the present study was performed to test antibiotic combinations, for synergy against clinical isolates of CRKP.**

***Methods*: A total of 50 clinical isolates of CRKP were included. Modified carbapenem inactivation method was performed for the detection of carbapenemases. *In vitro* synergy testing was done for the following combinations: meropenem+colistin, imipenem+tigecycline and polymyxin B+levofloxacin. It was performed with epsilometric test and microdilution checkerboard method. The time kill assay (TKA) was used to confirm the results. The fractional inhibitory concentration was also calculated.**

***Results*: All CRKP isolates (100%) were ESBL producers and were completely resistant to amoxicillin-clavulanic acid, cefepime, cefotaxime, ceftazidime and piperacillin-tazobactam. Resistance to ciprofloxacin, amikacin and tetracycline was 96, 88 and 54 per cent, respectively. Overall, 78 (39/50) and 88 per cent (44/50) of the 50 CRKP isolates exhibited synergy by TKA for meropenem-colistin and imipenem-tigecycline, respectively. No synergy was detected for levofloxacin-polymyxin B combination. The best combination among the three was that of imipenem and tigecycline followed by meropenem-colistin.**

***Interpretation & conclusions*: Of the three combinations tested, imipenem and tigecycline followed by meropenem-colistin were found to be best. No synergy was detected for levofloxacin-polymyxin B combination.**

Key words Antibiotic synergy - carbapenem-resistant *Klebsiella pneumoniae* - combination therapy - microdilution checkerboard method - time kill assay

The prevalence of severe infections due to multidrug-resistant *Klebsiella pneumoniae* strains resistant to nearly all currently available antibiotics has increased over the past decade, especially in the intensive care units (ICUs)[1]. Carbapenem-resistant *Enterobacteriaceae* has become a public health threat and economic burden due to lack of effective antibiotics, wide transmission and high mortality rate of up to 50 per cent in bloodstream infections[2]. Since the first report of carbapenemase-producing *K. pneumoniae*

strains in North Carolina in 1996, these highly resistant organisms have been isolated with increasing frequency[3]. Carbapenemases enzymes are expressed by genes on transmissible plasmids, leading to widespread resistance[4]. As a result, the polymyxins that were long abandoned due to their high toxicity profile found use in the treatment of carbapenem-resistant *K. pneumoniae* (CRKP). However, reports of polymyxin-resistant strains have been alarmingly increasing worldwide[5]. Combination antibiotic treatment may offer comparative advantage over monotherapy regarding mortality in critically ill patients with severe infections with CRKP[6]. Likewise, combined treatment with two or more drugs with *in vitro* activity, especially those including a carbapenem, has been found to be more effective than monotherapy regimens for CRKP bloodstream infection[6]. With resistance towards carbapenem, given the scarcity of novel agents and limited data on combination therapy for CRKP, the treatment has become challenging. The present study was performed to evaluate the antimicrobial resistance profile of CRKP clinical isolates and to test three two-drug combinations of antimicrobials for synergy.

Material & Methods

The study was conducted from January 2017 to June 2018 in the department of Microbiology, Government Medical College and Hospital, Chandigarh, India. A total of 50 non-repeat clinical isolates of *K. pneumoniae* were obtained from various specimens (blood, pus, urine, sputum, pleural fluid, ascitic fluid, tracheal aspiration, tissue, cerebrospinal fluid and endometrial curretings) submitted to the microbiology laboratory. Ethical clearance for the study was taken from the Ethical Committee of Government Medical College and Hospital, Chandigarh.

Antimicrobial susceptibility testing: *K. pneumoniae* was identified using standard microbiological methods described by Mackie and McCartney[7]. Antimicrobial susceptibility testing was done using Kirby Bauer disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2017[8]. Criteria for performance of extended spectrum beta-lactamase **(**ESBL) test was done by standard disc diffusion procedure[8]. Ceftazidime (30 µg) and cefotaxime (30 µg) were used to perform disk diffusion method of screening for ESBL production[8]. If screening test was positive, cefotaxime zone size ≤ 27 mm and ceftazidime ≤22 mm, phenotypic confirmation methods was used to diagnose ESBL[8]. For ESBL confirmation, disc diffusion method was performed using ceftazidime (30 µg) and ceftazidime-clavulinic acid (30/10 µg) along with cefotaxime (30 µg) and cefotaxime/clavulanic acid (30/10 µg) antimicrobial agents[8]. A ≥5 mm increase in the zone of either antimicrobial agent tested in combination with clavulanate compared to the zone diameter of the agent when tested alone is diagnosed as ESBL.

Modified carbapenem inactivation (mCIM) method for suspected carbapenemase production: This test is not recommended for routine use and is done for epidemiological or infection control purposes[8]. Meropenem disk (10 µg, Oxoid, United Kingdom) inactivation test method[8] was used. For each isolate to be tested, 1 µl loop full of bacteria from an overnight incubated blood agar plate was emulsified in 2 ml tube with trypticase soy broth (TSB). Then, a 10 µg meropenem disk was added to each tube using sterile forceps and incubated at 35°C±2°C in ambient air for four hours ± 15 minutes. Just before completion of the TSB-meropenem disk suspension incubation, Mueller–Hinton agar (MHA) plate was inoculated with 0.5 McFarland suspension of *Escherichia coli* ATCC 25922 (HiMedia) After incubation, meropenem disk was removed and placed on MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922. Plate was incubated at 35±2°C in ambient air for 18-24 h[8].

Interpretation: As per the CLSI 2017 guidelines[8]

(*i*) Carbapenemase positive: Zone 6-15 mm or presence of colonies within a 16-18 mm zone. If the test isolate produces carbapenemase, the meropenem in the disk will be hydrolyzed, and there will be no inhibition or limited growth inhibition of the meropenem-susceptible *E. coli* ATCC 25922.

(*ii*) Carbapenemase negative: Zone of 19 mm. If the test isolate does not produce carbapenemase, the meropenem in the disk will not be hydrolyzed and will inhibit growth of the meropenem-susceptible *E. coli* ATCC 25922.

(*iii*) Indeterminate: Zone 16-18 mm. The presence or absence of a carbapenemase cannot be confirmed.

Minimum inhibitory concentration (MIC) determination: For all carbapenem (imipenem)-resistant *K. pneumoniae* isolates by disk diffusion method, minimum inhibitory concentration (MIC) values

were determined by epsilometric test (Etest) (BioMérieux, Solna, Sweden) and broth microdilution checker-board (MCB) method[9].

The MIC determination was done for the following antimicrobials used in combinations: colistin, meropenem, tigecycline, imipenem, polymyxin B and levofloxacin. The antimicrobials were labelled as resistant/sensitive or wild type/non-wild type based on their breakpoints given by the CLSI 2017[8]. Test methods were based on the standard CLSI M7A9 broth dilution susceptibility methods[9]. MCB breakpoints given by the European Committee on Antimicrobial Susceptibility Testing were used for tigecycline[10].

An isolate was labelled as carbapenem resistant if it showed imipenem or meropenem resistance by disk diffusion MIC Etest and MCB methods.

Synergy testing: All CRKP isolates were tested for *in vitro* synergy testing of various antimicrobial agents (AMAs) combinations by practicing Etest, MCB and time kill assay (TKA) as gold standard. The various AMAs combinations tested were: (*i*) meropenem+colistin sulphate, (*ii*) imipenem+tigecycline and, (*iii*) levofloxacin+polymyxin B (all from Sigma-Aldrich, USA).

Synergy testing by epsilometric test: MHA (Hi Media, India) was inoculated with 0.5 McFarland suspensions of the study isolates. This method was performed conceding to the method interpreted by Laishram *et al*[11], the fixed ratio epsilometric method. All results were confirmed by MCB which was taken as reference method[8].

MCB method: Antimicrobial stock solutions were prepared according to the manual given in CLSI M7-A9 in the appropriate diluents[9]. Number of concentrations tested were prepared according to the requirement for each combination. Inoculum prepared was matched with optical density 0.5 McFarland. The MIC tray was incubated at 35° ± 2°C. Reading was taken as MIC of single-drug alone and in combination after 24 h incubation.

Interpretation of Etest and MCB method: For the interpretation of results of both Etest and MCB, the fractional inhibitory concentration (FIC) is calculated for each antibiotic at a given concentration combination by the following formula[11]:

FIC of antimicrobial agent A (FIC_A) = MIC of antimicrobial agent A in combination (MIC_{AB})/MIC of antimicrobial agent A alone (MIC_A)

FIC of antimicrobial agent B (FIC_B) = MIC of antimicrobial agent B in combination (MIC_{BA})/MIC of antimicrobial agent B alone (MIC_B).

The cumulative FIC index = FICA + FICB. Synergy is interpreted when the FIC index is ≤0.5, addition corresponds to >0.5 to ≤1, indifference corresponds to the FIC index >1 to ≤4 and antagonism when the FIC index is >4.0[11].

Synergy testing by time-kill assay (TKA): Time kill assay (TKA)[12] was used as a good standard to confirm the results. TKA was performed only on those CRKP isolates which were found to be synergistic to antimicrobial combinations by Etest and MCB methods. To perform TKA, the MIC of the each antimicrobial drug in combination for the CRKP isolate of interest was calculated by an MCB method[12]. Inoculum-matched 0.5 McFarland was prepared and added to glass tube containing single drug or combined drugs and incubated at 37°C with intermittent shaking for different timings and then sub-cultured with their respective timings, *i.e*. 0, 4, 6 and 12 h[12].

Interpretation: Results were interpreted as individual agent MIC and combined MICs. The difference in $\log_{10}$ colony forming unit (cfu)/ml was determined between individual agent at one-fourth MIC and the combination of antimicrobial agents A and B at one-fourth or one-eighth MIC. Synergism is defined as a ≥2-$\log_{10}$ cfu/ml decrease by the combination compared with the most active single agent. Antagonism is defined as a ≥2-$\log_{10}$ cfu/ml increase by the combination compared with the most active single agent[11].

Quality control strains: For modified carbapenem inactivation (mCIM) method for suspected carbapenemase production, *K. pneumoniae* ATCC BAA-1705 (carbapenemase-positive) and *K. pneumoniae* ATCC BAA-1706 (carbapenemase-negative) quality control strains were included. For MIC determination methods, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 quality control strains were included in the study.

Statistical analysis: The statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA, and version 22.0 for Windows).

Table. Comparison of synergy results by all methods in all combinations for 50 clinical isolates of carbapenem resistant *Klebsiella pneumoniae*

AMA combinations	Meropenem and colistin (n=50), n (%)	Imipenem and tigecycline (n=50), n (%)	Levofloxacin and polymyxin B (n=50), n (%)
Etest	41 (82)	44 (88)	20 (40)
MCB	44 (88)	48 (96)	11 (22)
TKA	39 (78)	44 (88)	0

AMA, antimicrobial agents; Etest, epsilometric test; MCB, microdilution checker-board; TKA, time kill assay

Categorical variables were described as percentages. Proportions were compared using Chi-square test; Pearson's Chi-square test (significant two-sided).

Results

Majority of CRKP isolates were obtained from adults (n=48, 96%, females > males), and only two isolates (4%) were from children. Most isolates were from ICUs (68%, 34/50), remaining being from different wards. Majority were obtained from pus and urine samples (28/50, 56%) from the ICU setup. Carbapenemase detection was observed in 100 per cent isolates by mCIM method. All isolates (100%) in addition to being carbapenem resistant were ESBL producers and were completely resistant to amoxicillin-clavulanic acid, cefepime, cefotaxime, ceftazidime and piperacillin-tazobactam. Resistance to ciprofloxacin, amikacin and tetracycline was 96, 88, and 54 per cent, respectively. The overall mortality rate observed in these patients was 56 per cent (28/50). The common regimen given to patients infected with CRKP in the ICUs was meropenem and colistin which was also chosen for synergy testing.

MIC determination by Etest: All of the 50 isolates were resistant to meropenem with MIC ≥32 µg/ml and for imipenem with MIC ≥4 µg/ml. Of these, 20 isolates (40%) were resistant with MIC range of ≥32 µg/ml. All isolates were resistant to levofloxacin by Etest [40/50 (80%)] having MIC range of ≥32 µg/ml. For colistin, tigecycline and polymyxin B, MIC was observed to be in susceptible range (wild type for colistin) by Etest. All results were confirmed by reference MCB method.

MIC determination by MCB method: Based on MCB test, for meropenem, 66 per cent of isolates showed MIC range ≥64 to <128 µg/ml, for imipenem, 56 per cent gave results between MIC range ≥8 and <32 µg/ml and for levofloxacin, 48 per cent of isolates had MIC range ≥64 to <128 µg/ml. Almost all isolates with all three antimicrobials depicted high MIC, and none of the isolates had MIC <8 µg/ml. The remaining three antimicrobials, with sensitive results on Etest, were also within sensitive range by MCB test (wild type for colistin).

Synergy determination

Meropenem and colistin: Based on Etest methodology, 82 per cent (41/50) isolates resulted in synergistic results (Table) and 18 per cent were interpreted as additive for this combination. None of the isolates showed indifference. On MCB testing, 88 per cent (44/50) isolates were interpreted as synergistic with FIC value <0.5, eight per cent depicted additive results with FIC 0.75 and four per cent (2/50) isolates resulted with FIC >1 considered as indifferent results. When the results of Etest were compared with MCB test, two isolates showed synergistic results with Etest but indifferent results with MCB test. Similarly, five isolates, which were showing additive results by Etest, were synergistic by MCB test. None of the isolates had antagonistic (FIC>4) results in this combination. Overall, a total of 39 of 50 isolates (78%) had similar synergistic results by both methods, which was confirmed by TKA test.

For all 39 isolates, at baseline, full growth was observed at all concentrations. After four hours, growth was completely inhibited at all time intervals and no growth was seen even at the lowest MIC, *i.e*. 1/8 of both AMAs. Results were the same at six hours, except for a few colonies observed at 1/8 MIC. Synergy was maintained after 12 and 24 h, except <50 colonies observed at 1/4 and 1/8 MIC of both meropenem and colistin (Fig. 1). Indifference and antagonism results were not observed by TKA method for this combination.

Imipenem and tigecycline: For both imipenem and tigecycline, by Etest, 88 per cent (44/50) had synergistic results and only 12 per cent (6/50) had additive results. By MCB test, 96 per cent (48/50)

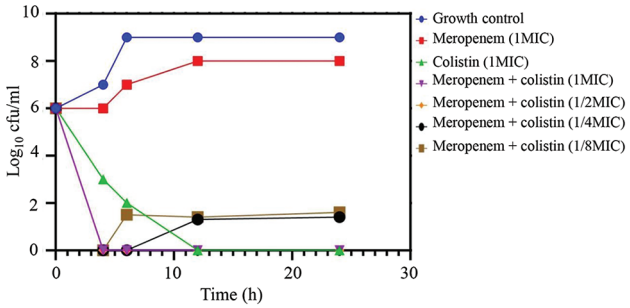

Fig. 1. Time kill curve for carbapenem-resistant *Klebsiella pneumoniae* isolate for meropenem and colistin at 1 MIC alone and in combination at 1, 1/2, 1/4 and 1/8 MIC at different time intervals. MIC, minimum inhibitory concentration.

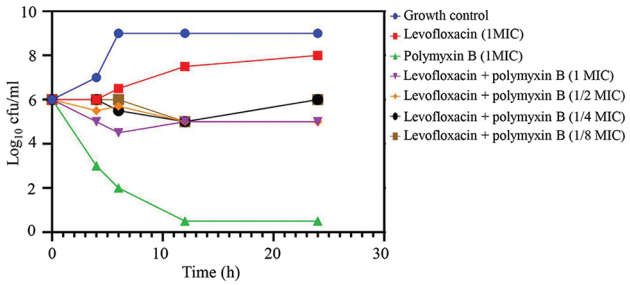

Fig. 3. Time kill curve for carbapenem-resistant *Klebsiella pneumoniae* isolate for levofloxacin and polymyxin B at 1 MIC alone and in combination at 1, 1/2, 1/4 and 1/8 MIC at different time intervals. MIC, minimum inhibitory concentration.

isolates showed synergy results and only four per cent (2/50) had additive results. When the results of both tests were compared, it was found that four isolates interpreted as additive on Etest were synergistic on MCB. All 44 isolates showed synergy by TKA method. At baseline, >100 colonies were observed at all concentrations. However, at 4 and 6 h, growth was completely inhibited and not a single colony was observed. After 12 and 24 h, synergy was maintained except a few colonies (<20 cfu) observed at 1/4 and 1/8 MIC (Fig. 2). The overall synergistic activity in imipenem-tigecycline combination (88%) was higher than meropenem and colistin combination (78%).

Levofloxacin and polymyxin B: On Etest, only 40 per cent (20/50) isolates showed synergy, and the remaining 60 per cent (30/50) were observed to have additive results. By MCB test, only 22 per cent (11/50) isolates exhibited synergy whereas 58 per cent showed additive effect with FIC index of 0.625 and 0.75, respectively. Further, 20 per cent isolates showed indifference. Of the total isolates showing synergy results with both tests, only 10 were found common to both tests, which were put for TKA method for

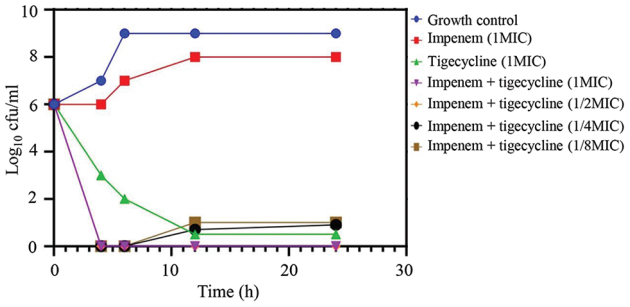

Fig. 2. Time kill curve for carbapenem-resistant *Klebsiella pneumoniae* isolate for imipenem and tigecycline at 1 MIC alone and in combination at 1, 1/2, 1/4 and 1/8 MIC at different time intervals. MIC, minimum inhibitory concentration.

further confirmation of synergy results. Contrary to above combinations, in all 10 isolates, synergy was not detected at various concentrations (1, 1/2, 1/4, 1/8 MIC) at different time intervals 0, 4, 6, 12 and 24 h) (Fig. 3). More than 100 colonies were observed, and in none of the culture plates, growth was inhibited. Overall, no synergy was observed for this combination.

On comparison of synergy results obtained of all three combinations, by our reference method, TKA, significant difference was obtained for imipenem-tigecycline and meropenem-colistin (P<0.001). However, it was not observed on comparison of TKA synergy results of imipenem-tigecycline with levofloxacin and polymyxin B and meropenem-colistin with levofloxacin and polymyxin B as synergy was not detected in any of the isolates by levofloxacin and polymyxin B combination.

Discussion

The antimicrobial susceptibility of clinical isolates of CRKP was determined against many antimicrobial agents. High resistance to ciprofloxacin (96%), amikacin (88%) and tetracycline (54%) was seen which was in concordance with the published literature[13]. All of the 50 isolates were found to produce carbapenemases by mCIM method. The detection of carbapenemases can be made by several phenotypic methods with varying sensitivity and specificity[14].

High synergistic results were obtained with MCB method. However, in MCB method, there are increased chances of carry over contamination and preparation of several intermediate concentrations of antibiotics is laborious. Another issue with MCB assays is the use of different criteria to interpret the test[11]. Thus, TKA was also preformed as done by Sopirala *et al*[15]; TKA was performed on the antibiotic

combinations found to be synergistic or additive by MCB and/or Etest methods.

In our study, best combination among three was that of imipenem and tigecycline. Tigecycline is used for treatment of complicated skin and skin structure infections, complicated intra-abdominal infections and community-acquired pneumonia[16]. Keeping aside the gastrointestinal side effects, tigecycline may still be one of the best drugs available[17]. There were several issues with *in vitro* susceptibility testing for tigecycline. Disk diffusion gives inconsistent results, attributed to changes in cations within the medium[17]. The difference of results for imipenem and tigecycline by Etest and MCB method could be due to limitations of Etest. The synergy by TKA method was best observed at 4 and 6 h intervals as no growth was observed even at 1/8th MIC of both imipenem and tigecycline combination. Yim *et al*[18] reported tigecycline and imipenem to be the most active combination against *K. pneumoniae* by TKA synergy studies. Similar to their study, no antagonism was observed in our study. In contrast to a study by Cha[19], no synergy and no antagonism were observed with imipenem combinations.

For meropenem-colistin combination, increased percentage of synergism was found by MCB method (88%) as compared to Etest (82%). Krishnappa *et al*[20] also detected higher synergism by the MCB method (48%) than Etest (35%). Results by TKA method were significant on comparison with MCB and Etest. In a study by Manohar *et al*[21], for the similar combination, synergy in 50 per cent of isolates was observed by TKA method and of which two isolates were of *K. pneumoniae*.

In our centre, due to increased usage and increased resistance of levofloxacin from various clinical isolates as also observed in a study by Magesh *et al*[22] levofloxacin in combination with polymyxin B was used. Neither synergy nor addition results were observed with TKA method for this combination. Borjan *et al*[23] observed lack of improved bactericidal activity when polymyxin B was combined with other antibiotics. Zhang *et al*[24] assessed synergistic effects of levofloxacin and other antibiotics such as colistin, meropenem and amikacin with tigecycline by TKA. In their study, for tigecycline and levofloxacin combination, only one CRKP isolate showed synergistic results[24].

The limitation of our study was that synergism observed could not be compared with molecular basis of resistance. The various mechanisms of resistance such as β-lactamases production (including New Delhi metallo-β-lactamase, NDM) and efflux pump production or porin loss were not studied genotypically.

To conclude, best synergy results were obtained by imipenem and tigecycline followed by meropenem and colistin combination. Since synergistic activity depends on the bacterial strains and susceptibility testing methods, it must be emphasized that evaluating the efficacy of these combinations by *in vitro* testing is essential to guide the *in vivo* treatment appropriately for use in the clinical trials.

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