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ORIGINAL ARTICLE

In Vitro Assessment of Colistin - Carbapenem Combination Against Multi Drug Resistant *Enterobacteriaceae* Isolates in Suez Canal University Hospitals, Ismailia

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ABSTRACT

Background: *Health crisis of Multi-drug resistant gram negative bacilli (MDRGNB), including* Enterobacteriaceae, *seems overwhelming as its worldwide spread causes clinical failure in the therapeutic care of diseases by these pathogens and results in significant morbidity and mortality. Combination therapy, using two or more drugs, may be the last resort for treatment of these multi-drug resistant (MDR) organisms.* ***Objective:*** *to update the antibiotic policy to improve treatment of MDR Enterobacteriaceae infections and to reduce morbidity and mortality rates due to these infections.* ***Methodology:*** *Out of 219 of* Enterobacteriaceae *strains isolated from different types of infections in Suez Canal University Hospitals (SCUHs), 48 isolates (21.91%) were proved to be MDR, including resistance to imipenem. In vitro assessment of Imipenem-colistin combination on the MDR-*Enterobacteriaceae *strains was performed using the checkerboard technique.* ***Results:*** *The combination had a synergistic effect on 63.04% of the isolates and additive effect on 23.9%. Indifferent effect was shown in 10.8%, while antagonism was shown in 2.1% of the strains. At least, four-fold reduction in imipenem MIC was proved in 86.9% of the strains, 30.43% turned to be imipenem sensitive with drop of their MICs from ≥ 4 to ≤ 1μg/ml, 15.21% changed to intermediate resistance with MIC decrease from ≥ 4 to 2 μg/ml. Three of the 5 strains that showed indifference and the only strain which showed antagonism were colistin resistant strains.* ***Conclusion:*** *High rates of synergy, in addition to reversal of imipenem resistance, were reported by colistin - imipenem combination against MDR-*Enterobacteriaceae, *which may encourage clinical trials of combination therapy in treatment of Hospital acquired infections (HAI) by MDR pathogens.*

INTRODUCTION

The rise in morbidity and mortality rates due to MDR pathogens has been a challenging topic. For gram negative bacteria, especially the *Enterobacteriaceae*, the case is remarkably alarming as the current therapeutic alternatives for these pathogens are deficient, and there is a scarcity of novel effective antibiotics being emerged [1]. So, physicians were forced to increase using carbapenem group of β-lactam antibiotics for treatment of infections caused by MDRGNB2.

Unfortunately, gram negative bacteria, the most clinically relevant being *Enterobacteriaceae ,Acinetobacter baumannii(A. baumannii) and Pseudomonas aeruginosa (P .aeruginosa)* were showing increasing resistance to carbapenems. With the massive spread of resistance to carbapenems, antibiotics of last choice, the problem is considered a life-threatening problem[3]. Carbapenem resistant *Enterobacteriaceae* (CRE) have crucial epidemiological implications due to the facility of horizontal spread of carbapenem-resistant plasmids and due to the high mortality rates they cause[4].

Combination antimicrobial therapy, using combination between two or more drugs, is considered the most viable therapeutic strategy for achieving maximal antimicrobial effects against MDR pathogens. Combination therapy enhances the antibacterial effects of available drugs [5].

The addition of colistin to imipenem is effective in vitro. Colistin is more likely to overcome impermeability and imipenem can exert its action by inhibition of bacterial wall synthesis.

The rationale of this study is it assesses the effectiveness of colistin-carbapenem combination against MDR- *Enterobacteriaceae* infections.

METHODOLOGY

Study population:

This is a quasi experimental study carried out during the period from December 2017 to November 2018. Forty eight carbapenem resistant *Enterobacteriaceae* were isolated from 272 patients admitted to different wards in SCUHs. Patients were of both sex, and from all

age groups.Approval of the study design was signed by the ethical committee of Faculty of Medicine, Suez Canal University.

Sample collection and processing:

Various clinical specimens from patients (urine, sputum, blood, pus and endotracheal aspirate (ETA)) were properly collected under aseptic conditions to be processed to isolate and identify MDR, including resistance to carbapenem, *Enterobacteriaceae* strains.

Culture was done on blood and MacConkey's type I agar plates and incubated overnight at 35–37°C. The isolates were preliminarily identified on the basis of their morphology, cultural characteristics and biochemical profile.

Antibiotic susceptibility testing:

Antibiotic susceptibility testing of individual strains was performed by Kirby-Bauer disc diffusion method on Mueller- Hinton agar (Oxoid, UK) according to **CLSI (2019)** [6] guidelines. The following antimicrobial agents were included: Amoxacillin-clavlanate (20/10 µg), Ampicillin-sulbactam (10/10µg), Cefotaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), ceftriaxone (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Gentamycin (10 µg), Amikacin (30µg), Imipenem (10µg), Meropenem (10µg), Trimethoprim-sulfamethoxazole (1.25/3.75µg), Aztreonam (30µg), Nitrofurantoin (300µg) (Oxoid, Basingstoke, UK).

Isolates proved to be carbapenem sensitive were excluded. Isolates were considered MDR when resistance occurred to three or more antibiotics in three different groups and those MDR isolates were included for further detection of effect of colistin-carbapenem combination.

Testing for the effect of carbapenem-colistin combination by Checkerboard technique [7]:

Standard powder forms of colistin methanosulphate and imipenem (Sigma-Aldrish Company/Germany) were stored at 4°C until use. Preparation of the antibiotics stock solution was done by weighing and subsequently dissolving adequate quantities of the antibiotic drugs in the proper solvents (distilled water for colistin and phosphate buffer solution for imipenem) to obtain concentration of 1000µg/ml in sterile Cation Adjusted Muller Hinton broth (CAMHB).

Preparation of the bacterial inoculum was done by subculture of the stored isolates on blood agar plates to obtain fresh cultures. Three to five colonies were touched with sterile swab and then transferred to 5 ml of sterile MHB to make 0.5 McFarland standard (1.5 x 10^8 CFU/ml). Dilutions were done in order to match a final concentration of ($5x10^5$ CFU/ml).

Testing for the effect of combination was done by preparation of 96-well microtiter plates with their wells carrying concentrations of the antimicrobials alone and in combination **(Fig.1)**. Determination of the MIC of the combinations was done using the broth microdilution technique as recommended by the **CLSI** [6] 100µl of the serial dilutions of the individual drugs were added, imipenem concentrations with their range from 0.5 to 128 µg/ml starting from column 9 up to column 1 and colistin concentrations ranged from 0.25 to 16 µg/ml starting from row G up to row A. Column 10 contained serial dilutions of colistin only while row H contained those of imipenem only. So, Minimum inhibitory concentration (MIC) of colistin and imipenem were determined from column 10 and row H respectively.

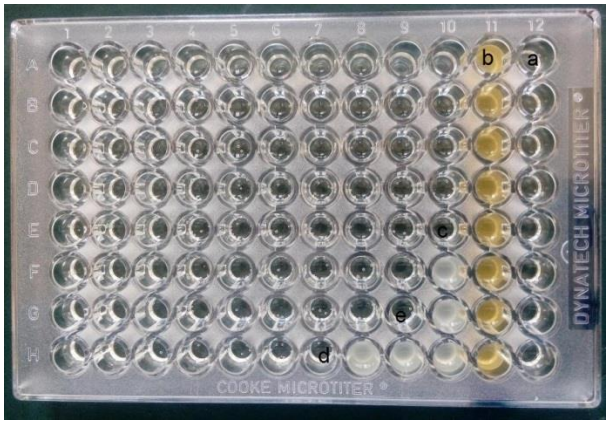

Fig. 1: Checkerboard technique to detect the effect of imipenem-colistin combination on MDR *K. pneumoniae* strain where **a** represents sterility control, **b** represents growth control, **c** represents MIC of colistin, **d** represents MIC of imipenem and **e** represents well showing synergism (lowest MIC of imipenem-colistin combination that inhibit the growth of the tested organism).

Plates were inoculated with each test organism. On each plate, wells of column 11 had no antibiotic to be used as a positive growth control and those of column 12 were used as sterility control. Inoculum verification plate was prepared on blood agar to check presence or absence of contamination. The plates were incubated for 24 hr at 37°C. The MICs of each antibiotic alone and in combination were estimated as the lowest concentration of the antibiotic alone and in combination that completely inhibited the growth of the organism with no turbidity as judged by naked eye.

***Calculation of fractional inhibitory concentration index (FICI)*[8]:**

- FIC of colistin = MIC of colistin in combination with imipenem /MIC of colistin alone.
- FIC of imipenem = MIC of imipenem in combination with colistin /MIC of imipenem alone.
- FICI = FIC colistin + FIC imipenem.

***Interpretation of results*:**

- FICI ≤ 0.5…..synergistic.
- FICI > 0.5 and ≤ 1.0…additive.
- FICI > 1.0 and ≤ 4.0…. indifferent.
- FICI > 4.0…..antagonistic.

RESULTS

Among the collected clinical specimens, *Enterobacteriaceae* were isolated at a rate of 80.5%; *Klebsiella spp*. were isolated at the highest rate (54.79%), followed by *Escherichia coli(E. coli*) (36.07%), *Enterobacter cloacae* (1.82%), and *Proteus mirabilis* (*P. mirabilis*) (6.3%), while *Serratia marcescencs* showed the lowest rate of isolation (0.91%)**.**

Resistance to carbapenems was detected according to **CLSI 6 .**Forty eight (21.91%) of the *Enterobacteriaceae* isolates were carbapenem resistant. Carbapenem sensitive strains were excluded and *P. mirabilis* strains were also excluded due to intrinsic resistance of *Proteus* to colistin antibiotic. Carbapenem resistant *Enterobacteriaceae* isolates were subjected to antimicrobial susceptibility testing for detection of MDR isolates. All the strains were proved to be MDR as each was resistant to at least one member of three or more different antibiotic groups. The MDR isolates were then tested for the effect of carbapenem-colistin combination using the Checkerboard technique.

Effect of imipenem-colistin combination on the imipenem MIC of MDR isolates is shown in **(Table1) ;** Two isolates (4.34%) had no changes in MIC, 4(8.69%) showed two-fold decrease, 24 (52.17%) showed four-fold decrease, 14 (30.43%) showed an eight-fold decrease, while only 2 (4.34%) showed a decrease of MIC to 1/16 of its value before combination. So, 40 isolates (86.9%) showed at least a four-fold reduction in imipenem MIC by imipenem/colistin combination and 14 isolates (30.43%), turned to be imipenem sensitive with a reversal of MICs from ≥ 4 to ≤ 1µg/ml, while 7 isolates (15.21%) changed to intermediate resistance with change of MIC from ≥ 4 to 2 µg/ml.

Table 1: Effect of imipenem-colistin combination on the imipenem MIC of MDR isolates:

Imipenem MIC before combination (µg/ml)	No. of isolates	Imipenem MIC changes in combination with colistin				
		No change	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC
64	**4**	**1**		**1**		**2**
32	**10**	**1**	**1**	**5**	**3**	
16	**12**		**2**	**8**	**2**	
8	**9**		**1**	**5**	**3**	
4	**11**			**5**	**6**	
Total	**46**	**2 (**4.3%**)**	**4** (8.6%)	**24**(52.1%)	**14**(30.4%)	**2 (**4.3%**)**

- 14 isolates (30.4%), shadowed in pale blue, had a reversal of imipenem resistance with a change of MICs from ≥ 4 to ≤ 1µg/ml.
- 7 isolates (15.2%), shadowed in violet, changed to intermediate resistance with change of MIC from ≥ 4 to 2 µg/ml.

The different effects of imipenem-colistin combination on the MDR isolates after the application of Checkerboard equation of FICI is illustrated in(**Table 2**); 29 isolates (63.04%) had synergistic effects with FICI values ≤ 0.5 (*p* value<0.05) and 11 isolates (23.9%) were additive, 5 isolates (10.8%) had indifferent effects while antagonism was shown in only one isolate(2.1%).Three of the 5 isolates that showed indifferent effect and the only strain which showed antagonism were the colistin resistant strains.

Table 2: Effects of imipenem-colistin combination on the MDR isolates:

	No. of isolates	%	*p*- value
Synergism	**29**	**63.04%**	**0.021***
Additive	**11**	**23.9%**	
Indifference	**5**	**10.8%**	
Antagonism	**1**	**2.1%**	

Synergism: FICI ≤ 0.5, **additive**: FICI >0.5 and ≤ 1.0, **indifference**: FICI>1.0 and ≤ 4.0, **antagonism:** FICI >4.0.
***Statistically significant result ($p < 0.05$)

The effect of imipenem-colistin combination on the isolates according to their degree of resistance is illustrated in (**Table 3)**. Among the highly resistant isolates with MIC 64µg/ml, 3 of 4 strains showed synergism with FICI ≤ 0.5, and among the 10 isolates with MIC 32µg/ml, 4 showed synergism. Among the 12 isolates with imipenem MIC of 16µg/ml, 7 isolates showed synergism, among the 9 Isolates with MIC of 8µg/ml, 6 isolates showed synergism, 3 of them became imipenem sensitive (MIC 1µg/ml). While among the 11 isolates with MIC of 4 µg/ml, 9 isolates showed synergism and 2 had additive effect and all the 11 had turned to be imipenem sensitive (MIC 0.5-1µg/ml).

Table 3: Effect of imipenem-colistin combination on the isolates according to their degree of resistance:

Imipenem MIC before combination (µg/ml)	No of isolates	No (%) of isolates showing synergism
64	**4**	**3 (75%)**
32	**10**	**4 (40%)**
16	**12**	**7 (58.3%)**
8	**9**	**6 (66.66%)**
4	**11**	**9 (81.8%)**
Total	**46**	**29 (63.04%)**

DISCUSSION

One of the most serious widespread health threats of our time is MDR. Organisms showing MDR are emerging worldwide, challenging the clinicians, public health professionals, and hospital infection-control teams 9. The selective pressure originated due to the excessive use of antimicrobials in human medical practices is one of the main causes of emergence and expansion of MDR organisms on a global level and their association with significant morbidity and mortality in infected individuals.

In our study; a total of 219 *Enterobacteriaceae* isolated from 272 patients admitted to different wards in SCUHs in Ismailia, Egypt. *Enterobacteriaceae* were isolated at a rate of 80.5 %, while other organisms were isolated at a rate of 14.8% from. Multi drug resistance, including carbapenem resistance, was detected in 48 (21.9%) out of the total *Enterobacteriaceae* isolates. *Klebsiella pneumoniae*(*K. pneumoniae*)showed the highest rate of MDR (31.25%), followed by *K. ozanae* (20%), *P. mirabilis* (14.28%) and *E. coli* (10.1%).These findings are consistent with (Kotb *et al.*,2019) who reported that the highest resistance rate was detected among *klebsiella species* (57.1%) followed by *Enterobacter species(* 25%) and *E.coli* (9.9%)[10] .

In agreement with our study, another study conducted at Suez Canal university Hospitals Kishk *et al.*, which reported high prevalence of both carbapenem resistance (47.5%) and ESBL production (39.1%) among *K. pneumonia*e strains3. Other studies in Egypt also revealed high rates of MDR among GNB; Mahmoud *et al.* [11] reported that 46.1% of *E. coli*, 26.2% *K. pneumoniae*, and 10.7% of *P. aeruginosa* isolates were MDR and that 50.8% of total isolates were carbapenem resistant, Helmy *et al.* [12], reported that 84.75% of their GNB isolates were MDR with the highest rate recorded in *E. coli*, followed by *K. pneumoniae* and *A. baumannii*, Tohamy*et al.* [13] detected MDR in 38.6% of the *E.coli* isolates, followed by *K. pneumoniae* (34.3%), *A.baumannii* (12.8%), *Enterobacter cloacae* (5.7%), *P. aeruginosa* (2.8%) and *K. oxytoca* (2.8%). Probably, this high prevalence is associated with the antibiotics abuse in Egypt and the improper application of the infection control measures by the hospital personals [3]. Studies all over the world reported high rates of MDR among GNB[14, 15].

The whole world is facing a real health problem due to increase and spread of the MDRGNB. Added to that is the shortage of therapeutic options. The problem necessitates a global cooperation to strictly follow the measures of infection control, to continuously update the antibiotic policy and to search for new therapeutic options.

In the current study we assessed the *in vitro* effect of imipenem-colistin combination against MDR *Enterobacteriaceae species*. Our results showed that out of the 46 MDR isolates,40 isolates (86.9%) showed at least a four-fold reduction in imipenem MIC, 14 isolates (30.43%) turned to be imipenem sensitive with a reversal of MICs from ≥ 4 to ≤ 1μg/ml. Imipenem-colistin combination was proved by applying the Checkerboard equation to have synergistic effect on 29 (63.04%) of the MDR strains with FICI values ≤0.5 and an additive effect on11 isolates (23.9%), indifferent effect on 5 isolates (10.8%) while antagonism was shown in only one isolate (2.1%) Three of the 5 strains that showed indifferent effect and the strain which showed antagonism were all colistin resistant strains.

Zusman *et al.* [16] in their systemic review and meta-analysis study reported that the combination therapy showed synergy in 77% of *A. baumannii* isolates, 50% of *P. aeruginosa*, and 44% of *K. pneumoniae* with low antagonism rates for all isolates. Leu *et al.* [17] reported that among the isolates tested with the combination, 57.3% had at least a four-fold drop in imipenem MICs and 74.6% exhibited reversal of imipenem resistance.

Shah *et al.*[18], in their clinical trial of colistin-carbapenem combination, showed that overall clinical success rate of 60.6% was observed in their patients.

In Turkey**,** Batirel *et al.* [19], in their clinical trial, study compared the efficacy of colistin monotherapy and colistin- carbapenem combinations. Results showed that the rates of complete response/cure and 14-day survival were relatively significant in the combination group in comparison with the monotherapy group (46.3 % vs. 30.6 % and 68.2 % vs. 55.5 %, respectively). The bacterial clearance was significantly much elevated in the combination group than the monotherapy group (79.9 % vs. 55.6 %). Also, the in-hospital mortality rate showed significantly low level in the combination group compared to the monotherapy group (52.3 % vs. 72.2 %). Those enhanced colistin-based combinations are of great clinical interest, given the failure of colistin monotherapy in addition to the emergence and spread in both plasmidic and chromosomal colistin resistance. Even if the combination may not work on all colistin-resistant isolates, yet combination therapy represents an alternative to be implemented for certain MDR cases [20].

Depending on a series of retrospective analyses and in vitro synergy susceptibility tests, researchers have claimed that regimens including carbapenems might be inapplicable as definitive therapeutic options if high MIC levels (over 32 μg/ml) were detected among MDR isolates. However, our study revealed that some isolates with highest imipenem MIC levels (64 and 32 μg/ml), showed synergism.

Further investigations, including other species causing hospital acquired infections, different experimental conditions, and other antibiotic combinations in addition to clinical trials are recommended to better understand the impact of antibiotic combination in treatment of MDR bacteria.

CONCLUSION

Imipenem and colistin combination against MDR-*Enterobacteriaceae* is supported in vitro by high synergy and the reversal of imipenem resistance with low antagonism, which may encourage clinical trials of combination therapy in treatment of HAIs by MDR pathogens, especially in critically ill patients. However, and due to various pharmacokinetic and pharmacodynamic effects of the antibiotic in the host and difference in bacterial and drug concentrations in the specific site of infection, clinical trials should be accompanied by strict follow up of patients for the rates of cure, clearance of the causative pathogen and any signs or symptoms of drug toxicity.

Conflicts of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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