ORIGINAL ARTICLE

Antimicrobial Efficacy of Doripenem Colistin Combination on Carbapenem-Resistant *Acinetobacter baumanii* Isolates by E-test Agar Dilution and Ultrastrastructural Methods

[1]Nevine Fam, [1]Doaa Gamal*, [2]Magda Azmy, [1]Reham Wasfy, [1]Laila Aboul-Fadl, [3]Mohamed Badr, [3]Mervat El-Damarawy

[1]Microbiology Department Theodor Bilharz Research Institute, Giza, Egypt
[2]Clinical pathology & Electron Microscopy Department Theodor Bilharz Research institute, Giza, Egypt,
[3]Intensive care unit Theodor Bilharz Research Institute, Giza, Egypt

*Corresponding Author:
Doaa Gamal
Microbiology Department Theodor Bilharz Research Institute, Giza
E-mail: doaagamal@gmail.com;
Fax: 35408125; Tel: 01005828937

ABSTRACT

Background: *Acinetobacter baumannii (A.baumannii) is a serious nosocomial pathogen and a threat for hospitalized patients especially in intensive care units (ICUs). Infections caused by the bacteria are difficult to treat owing to the frequent development of resistant strains which are mostly multidrug resistant (MDR). Emerging resistance to carbapenems is an alarming issue. As treatment options are limited so the discovery of new therapies, including combination therapy, is required. Doripenem is a new carbapenem, which is more stable against carbapenemases. Colistin remains a last line treatment for MDR A.baumannii and its combined use with cabapenems may present a possible strategy for treatment of serious bacterial infections.* **Objective:** *In this study we evaluated the efficacy and antibacterial activity of doripenem monotherapy versus doripenem combination therapy with colistin against carbapenem-resistant A. baumannii isolates in vitro.* **Methodology:** *Thirty two carbapenem non-susceptible A. baumannii clinical isolates were identified and antimicrobial susceptibility testing (AST) and minimum inhibitory concentration (MIC) determination were done by using VITEK2 compact system (bioMerieux, France). Phenotypic detection of MBLs was assessed using MBL E-test strips. Synergy testing was performed by agar dilution E-test method using doripenem E-test strips and colistin at 1/2 MIC values. Morphological changes were evaluated by scanning electron microscopy (SEM).* **Results:** *All 32 A. baaumannii isolates were resistant to carbapenems including doripenem and 23/32 (72%) were resistant to colistin. The colistin doripenem combination displayed synergy in (27/32; 84%) among carbapenem resistant isolates while antagonism was detected in 5/32 (16%). Metallo-β-lactamase (MBL) production was detected in 24 isolates (75%); however the MBL status did not affect the synergistic activity of the combination therapy. Electron microscopy on selected isolates showed major morphological changes after exposure of A. baumannii to doripenem and colistin combination confirming synergy between the 2 drugs.* **Conclusion:** *This study confirms the superiority of doripenem colistin combination at 1/2 MIC over doripenem or colistin monotherapy in both carbapenem resistant and carbapenem colistin resistant A. baumanni isolates. This combination could be a life-saving alternative for treatment of serious infections that improves the clinical outcome of critically ill patients in ICU. This should be further examined in clinical studies.*

INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) has emerged as one of the most crucial nosocomial pathogens. It is becoming a serious threat for hospitalized patients because of increasing antibiotic resistance rates[1,2]. Infections due to multidrug-resistant *A. baumannii* (MDRAB) present an enormous challenge in health care facilities. The organism has been implicated in a variety of infections, including respiratory tract, bloodstream, skin and soft tissues infections and surgical sites. It is considered to be a particular problem in intensive care units (ICUs) in which vascular catheters and endotracheal tubes are the most frequent sources of infections[3,4]. Emerging resistance to carbapenems, which are still considered as the safest and most efficient therapeutic options towards these bacteria, is increasing[1]. Carbapenem resistance in *A. baumannii* is mainly due to the presence of β-lactamases (class B metallo-β-lactamases or class D

OXA-type β-lactamases) as well as altered permeability and penicillin binding protein (PBP) modifications[5].

Doripenem is a newly marketed carbapenem with an in vitro activity against Gram-positive, Gram-negative, and anaerobic bacteria and is also more stable against carbapenamases. It has been considered a valuable agent for the treatment of serious bacterial infections caused by multidrug-resistant Gram-negative bacteria in hospitalized patients[6]. Colistin is considered to be the last treatment option for MDRAB. However, its efficacy, toxicity and resistance are concerns when used as a monotherapy [7]. Heteroresistance to colistin has been observed in vitro and during therapy, raising concerns that colistin alone may lack sufficient killing activity to be used as a monotherapy[3]. Colistin produces rapid bactericidal effects at high concentrations. At lower concentrations, it affects the bacterial outer membrane and increases the permeability of Gram-negative bacteria, which facilitates the penetrative ability of other combined compounds resulting in potent synergistic activity[8]. This study aimed to evaluate the efficacy and antibacterial activity of doripenem monotherapy versus doripenem combination therapy with colistin against carbapenem-resistant *A. baumannii* isolates by E-test agar dilution method and scanning electron microscopy

METHODOLOGY

Isolates collection and identification:

The study was conducted on 32 different clinical microbiological specimens including; respiratory secretions, wound swabs, blood, urine and body fluids that were referred to the Microbiology laboratory from outpatient clinic attendants as well as hospitalized patients from different departments including ICU of Theodor Bilharz Research Institute (TBRI) during the period from November 2014 to December 2015. Identification for isolates was done by API 20E (*bioMérieux, France*) and confirmed using VITEK 2 compact system (*bioMérieux, France*).

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing (AST) was done primarily by using Kirby Bauer disc diffusion method and then minimum inhibitory concentration (MIC) was detected by VITEK2 compact system (*bioMérieux, France*) and results were interpreted according to CLSI[9]. MICs for the following antibiotics were tested: ticarcillin (TIC), piperacillin (PIP), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefepime (CPM), ciprofloxacin (CIP), amikacin (AK), gentamicin (GM), tobramycin (TOB), trimethoprim/sulphamethaxole (SXT), minocycline (MIN), meropenem (MEM) and imipenem (IPM). MIC for doripenem (DOR) was detected by E-test strips following manufacturer's guidelines (*bioMerieux, France*). MIC of colistin (CS) was determined by agar

dilution method according to CLSI guidelines (CLSI)[10] and results were interpreted according to current CLSI breakpoints in which ≥ 4 μg/ml was resistant and ≤ 2 μg/ml was sensitive (CLSI)[9].

Testing for synergy of colistin doripenem combinations:

Antimicrobial synergy testing was performed by agar dilution E-test method as described by by Pongpech et al.[11] Mueller Hinton agar (MHA) plates were incorporated with colistin at one-half the MIC, as detected by agar dilution method, then the plates were inoculated with 0.5 McFarland suspensions of each test isolate. The E-test strip of doripenem was then applied on each MHA plate incorporated with colistin and to another MHA without colistin. Following incubation for 24 hours at 37°C, the E-test MICs results were read and compared.

Synergy interpretation:

The fractional inhibitory concentration index (FICI) was calculated using the following formula: FICA + FICB=FICI, where FICA equals the MIC of doripenem (drug A) in combination with colistin (drug B) divided by the MIC of doripenem alone and FICB equals the MIC of colistin in combination divided by the MIC of colistin alone. The results were interpreted as synergy, additive and antagonism if the FICI were less than 1.0, 1.0 and over1.0, respectively[11].

Phenotypic detection of Metallo-β Lactamases (MBLs):

Phenotypic detection of MBLs was done using E-test MBL strips (*bioMérieux, France*) which contained a double sided seven-dilution gradient of imipenem (IP) (4 to 256μg/ml) and imipenem (1 to 64μg/ml) in combination with a fixed concentration of EDTA (IPI). Interpretation of results was carried out according to the manufacturer's instructions. A reduction in MIC in the presence of EDTA of greater than or equal to eight-fold (IP/IPI ≥ 8) or a phantom zone between IP/IPI or deformation of either ellipse was interpreted as indicating MBL activity.

Detection of morphological changes by scanning electron microscopy (SEM): Scanning electron microscopy was chosen to examine the morphological changes in the selected carbapenem resistant *A. baumannii* isolates. These isolates were taken from culture plates of MHA plates incorporated with colistin at point of intersection with doripenem E-test strips after incubation for 24 hours duration.

- Bacterial colonies were processed according to Glauert[12]. They were first immediately fixed for 2 hours in equal volumes of glutaraldehyde 4% and caccodylate 0.2.M, then washed in equal volumes of Sacchrose 0.4 M and caccodylate 0 .2 M for 2 hours and then post fixed in osmium tetroxide 2% and caccodylate 0.3 M for 1 hour.

- The samples were then washed with distilled water and finally dehydrated in ascending grades of ethyl alcohol for 5 min each (30%.50%.70%.90%)then absolute alcohol 100% for 10 min for 3 times.
- Specimens were examined with environmental scanning electron microscope (Inspect S; FEI, Holland) operated at 10–30KV, at the Electron Microscopy Unit of TBRI.

RESULTS

Carbapenem non-susceptible *A. baumannii* isolates were mostly isolated from urine and sputum specimens (10/32; 31%) and mainly from ICU (9/32; 28.12%). All isolates were resistant to carbapenems tested including DOR by E-test strips showing MIC>32ug/ml **(Fig 1a).** Most of the isolates showed resistance to multiple classes of antibiotics including; β-lactams; TIC (97%), PIP (100%), TZP (100%), CAZ (97%), CPM (100%), aminoglycosides; AK (69%), GM (81%), and TOB (66%), quinolones; CIP (97%) and trimethoprim / sulphamethoxazole (91%). Seventeen isolates (53%) were sensitive to MIN; 12 (38%) were intermediate and only 3 isolates (9%) were resistant. MICs of CS by agar dilution method showed that only 9 (28%) were within the susceptible range, while 23 isolates (72%) were resistant, both MIC_{50} and MIC_{90} of the isolates were 4 μg/ml **(Table 1).**

Table 1: Antimicrobial susceptibility patterns for 32 *A. baumannii* test isolates in terms of MICs, MIC_{50} and MIC_{90}

Isolate/MIC_{50} /MIC_{90}	MIC of Antibiotics (ug/ml)														
	TIC	PIP	TZP	CAZ	CPM	IPM	MEM	AN	GM	TOB	CIP	MIN	SXT	DOR*	CS**
R18	128	128	128	64	64	16	16	64	16	16	4	2	320	≥ 32	2
R13	128	128	128	64	64	8	8	8	4	8	4	8	320	≥ 32	4
R14	128	128	128	64	64	16	16	64	1	2	4	1	320	≥ 32	4
R9	128	128	128	64	64	16	16	2	1	1	4	8	320	16	4
R11	128	128	128	64	64	16	16	4	16	16	4	16	320	≥ 32	2
R8	128	128	128	64	64	8	16	64	16	16	0.5	2	320	≥ 32	4
R2	128	128	128	64	64	16	16	8	16	16	4	16	320	≥ 32	4
R7	128	128	128	64	64	8	8	2	16	8	4	1	320	≥ 32	2
R12	64	128	128	8	32	16	16	64	16	16	4	2	80	≥ 32	1
R10	128	128	128	64	32	4	8	16	16	8	4	1	20	≥ 32	2
R28	128	128	128	64	64	16	16	32	16	8	4	1	320	≥ 32	4
R31	128	128	128	64	64	16	16	16	4	16	4	8	320	≥ 32	4
R34	128	64	128	64	64	8	8	64	16	16	4	2	320	≥ 32	4
R41	128	128	128	64	64	16	16	64	16	16	4	4	20	≥ 32	4
R45	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	2
R46	128	128	128	64	64	16	16	64	16	16	4	2	320	≥ 32	8
R50	128	128	128	64	64	16	16	32	16	8	4	1	320	≥ 32	4
R51	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	4
R58	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	4
R60	128	128	128	64	64	16	16	16	1	1	4	1	320	≥ 32	2
R44	128	128	128	64	64	16	16	64	16	16	4	16	320	≥ 32	4
R36	128	128	128	64	64	16	16	64	16	16	4	1	320	≥ 32	2
R22	128	128	128	64	64	16	16	64	16	4	4	8	20	≥ 32	8
R24	128	128	128	64	64	16	16	32	16	8	4	2	320	≥ 32	4
R77	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	4
R72	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	2
R75	128	128	128	64	64	16	16	64	8	8	4	2	320	≥ 32	4
R76	128	128	128	64	64	16	16	32	16	16	4	8	320	≥ 32	4
R71	128	128	128	64	64	16	16	64	16	16	4	4	320	≥ 32	4
R74	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	4
R73	128	128	128	64	64	16	16	64	16	16	4	4	320	≥ 32	8
R80	128	128	128	64	64	16	16	64	16	16	4	8	160	≥ 32	4
MIC50	128	128	128	64	64	16	16	64	16	16	4	8	320	≥ 32	4
MIC90	128	128	128	64	64	16	16	64	16	16	4	16	320	≥ 32	4

MIC: Minimum inhibitory concentration, detected by VITEK2. TIC: Ticarcillin. PIP: Piperacillin. TZP: Tazobactam/ Piperacillin. CAZ: Ceftazidime. CPM: Cefipime. IPM: Imipenem. MEM: Meropenem. AN: Amikacin. GM: Gentamicin. TOB: Tobramycin. CIP: Ciprofloxacin. MIN: Minocycline. SXT: Trimethoprim/Sulphamethaxole. DOR: Doripenem. CS: Colistin. *MIC was detected by E-test. **MIC was detected by agar dilution.
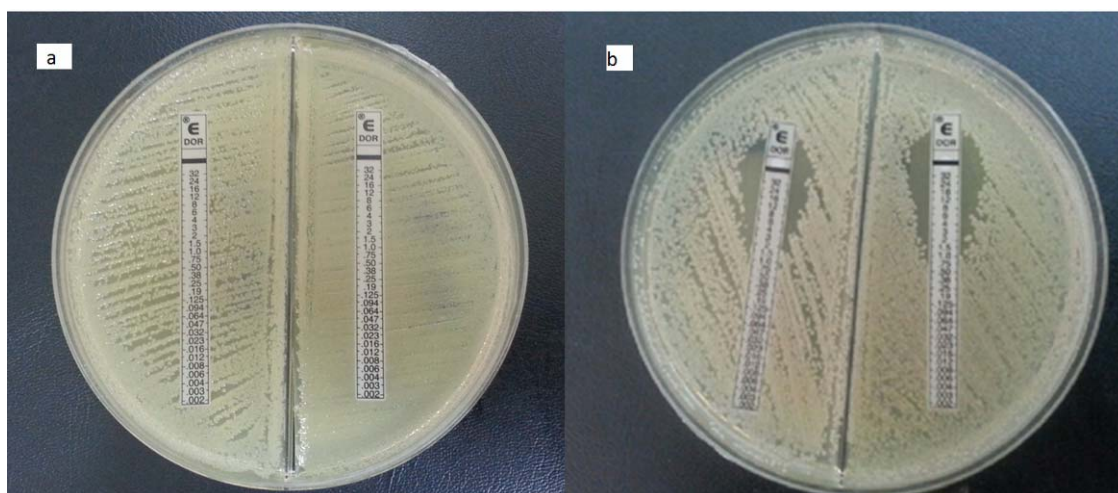
Fig. 1: (a) Doripenem MIC by E-test showing 2 carbapenem resistant isolates (R75; R76) with MIC ≥ 32μg/ml (a). (b) Synergy testing by E-test agar dilution showing reduction in doripenem MIC in presence of colistin at ½ MIC (FICI=0.562 and 0.546 respectively).
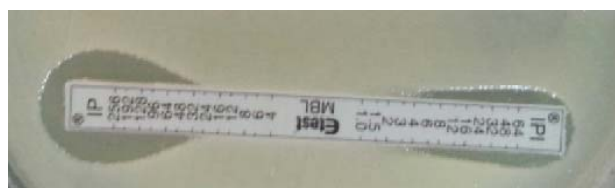
Results of synergy testing showed reduction in DOR MIC values in agar plates incorporated with ½ MIC of CS in 27 isolates (84%) denoting synergy between the 2 drugs, while antagonism was detected in 5 isolates (16%). **(Table 2) (Fig 1: a, b).** Among colistin resistant isolates, 91% showed synergistic results to doripenem colistin combination, whereas only 2 isolates (9%) showed antagonistic result **(Table 3)**. MBL production was detected in 24 isolates (75%) **(Fig 2)**; 19 (79%) of these isolates showed synergy and 5 (21%) showed antagonism. **(Table 3).**

Fig. 2: MBL IP/IPI E-test showing 8 folds reduction in imipenem (IP) MIC in the presence of EDTA (IPI) (from 32 to 4 μg/ml), indicating positive MBL production.

Table 2: Antimicrobial effects of colistin doripenem combination against carbapenem resistant *A. baumannii* isolates.

| Doripenem colistin combination results	Synergy (FICI <1)	Additive (FICI=1)	Antagonism (FICI >1)
	27/32 (84%)*	0	5/32(16%)

Table 3: Antimicrobial effects of colistin doripenem combination against carbapenem colistin resistant *A. baumannii* isolates.

Doripenem colistin combination results	Synergy (FICI <1)	Additive (FICI=1)	Antagonism (FICI >1)
	21/23 (91%)*	0	2/23 (9%)

Electron microscopy on selected colistin resistant isolates showed no significant differences between bacterial cells exposed to colistin alone compared to unexposed controls showing uniform structure and intact cell wall suggesting that colistin alone did not disrupt the *A. baumannii*. Cases with synergy showed different morphological changes starting from ballooning and distention of cells to disruption and release of cellular material after their exposure to doripenem and colistin at 1/2 MIC **(Figs 3 and 4).**
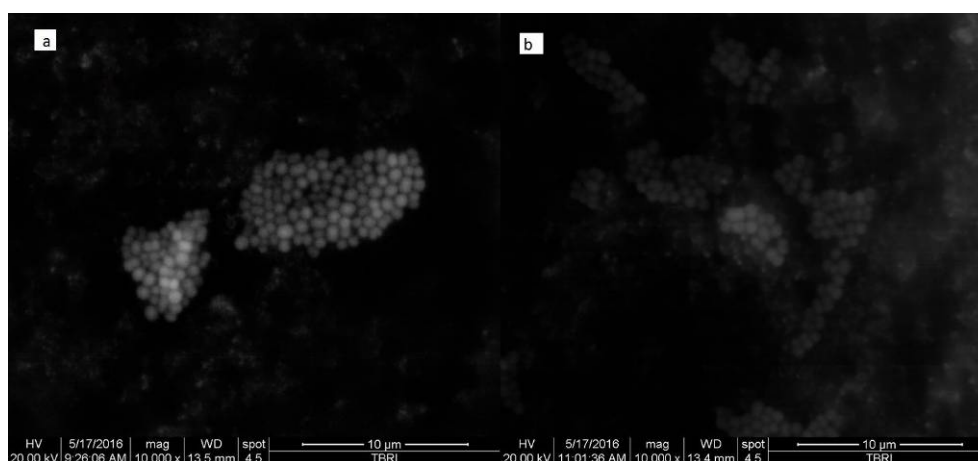
Fig. 3: (a) Scanning electron micrographs of *A. baumannii* unexposed controls with uniform structure and intact cell wall. (b) Colistin resistant-exposed bacterial cells showing no clear difference between exposed and unexposed controls suggesting that colistin did not disrupt the *A. baumannii*.
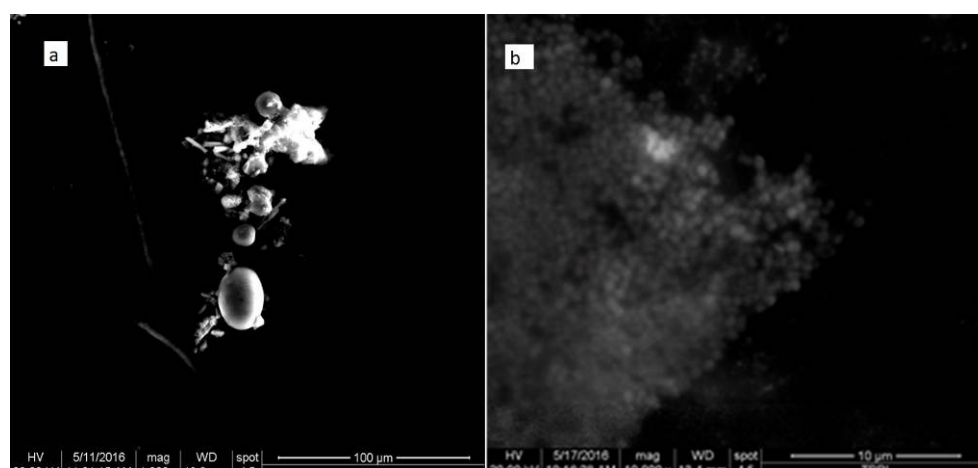
Fig. 4: (a) Scanning electron micrographs of *A. baumannii* showing ballooning of the bacteria due to increased permeability of cell. (b) Cell wall destruction, cellular disruption and release of intra-cellular material after 24 hours exposure to combination of doripenem colistin at 1/2 MIC.

DISCUSSION

A. baumannii is an important pathogen implicated in various nosocomial infections for which treatment options are very limited. The emergence of carbapenem resistance further limits these therapeutic options and drive the reuse of polymyxins in combinations with carbapenems. Combination therapy with colistin is a common strategy used to minimize the potential emergence of resistance and improve the clinical effectiveness of colistin in treatment of MDR *A. baumannii* infections [13, 14].

This study aimed to evaluate the *in vitro* efficacy of doripenem monotherapy versus doripenem combination with colistin in 32 carbapenem resistant MDR *A. baumannii* isolates. MICs revealed that resistance among isolates to all β-lactam agents ranged from 97% in (e.g. TIC) to 100% (e.g. CAZ), followed by CIP

(97%), then SXT (90%) and aminoglycosides; TOB (66%) and GM (81%). Our results were comparable to those found in Pakistan by Sohail et al.[15], in which resistance to ceftazidime was (99.2%), followed by the resistance to GM (93.6%), and to IPM (90.9%). Our isolates showed moderate rate of susceptibility to MIN (53%), this was lower than that reported from China (74.2%)[16] and higher than that reported from Jordan (26.7%) [17].

Resistance to colistin was found in 72% by agar dilution method, which was higher than that found in Iran (57%) by broth microdilution test[18]. Other parts of the world from Asia, Europe, North America and South America reported recent increase of colistin-resistant *A. baumannii* ranging from <7% to 40.7% in Spain [7,15,16,17].

Mechanisms of resistance to colistin is not yet clear, but some studies confirm that it might be related

to a loss of LPS or/and the PmrAB two-component system[7].

The use of colistin in feeding animals especially in developing countries, as in Egypt, has been suggested as a possible main cause in the spread of plasmid-mediated colistin resistance MCR-1. MCR-1 is a member of the phosphoethanolamine transferase enzyme family, its expression results in the addition of phosphoethanolamine to lipid A thus resulting in colistin resistance. It has first been described in Enterobacteriaceae isolated from animals, food and human beings in China, afterwards it has been reported in other countries in Asia, Europe and North America. Recent reports from Egypt further denote the dissemination of this mechanism [19,20,21].

Colistin, as the last resort for treatment of MDR *A. baumannii*, has received much attention in recent years and recognized as treatment option especially in combination with other antibiotics. However, the clinical use of colistin is hindered by its side effects, mainly nephrotoxicity, in addition to unclear optimal dosing. So it has been suggested that combination therapy of colistin carbapenem might be among the best strategies against colistin-resistant as well as carbapenem resistant *A. baumannii*[7,22]. The main rationale for this combination lies in the existence of in vitro synergy. In our study, we used E-test agar dilution method, previously described and validated, to assess synergy between the 2 drugs [23].

Our results showed that antibacterial activity of doripenem colistin combination against carbapenem resistant and carbapenem colistin resistant *A. baumannii* isolates gave synergy results in 84% and 91% respectively, that was superior over single monotherapy regarding both drugs. Our results were comparable to previous studies from USA in which the combination of colistin doripenem was synergistic and bactericidal against 83% of colistin resistant *A. baumannii* (5/6) and 75% (4/6) of the isolates, respectively assessed using time kill assays[24]. Whereas in Korea colistin doripenem showed the highest synergy rate in both the extensive drug-resistant (XDR) (53.7%) and MDR (53.6%) *A. baumannii* isolates compared to other antibiotic combinations including tigecycline colistin/ doripenem[14]. This synergistic effect was revealed by E test agar dilution method and by the morphological bacterial changes shown by SEM. It may be related to subsequent weakening of cell wall or membrane due to the action of doripenem and colistin as seen by electron microscopy, same findings were previously described [11].

MBL activity was detected in 75 % of the isolates. Although these results were not confirmed by molecular methods, it correlated with the high prevalence of MBLs (specifically the NDM like) previously reported in *A. baumanni* isolates from Egypt[25]. MBL production was considered uncommon in *Acinetobacter* spp [11] , but it is now emerging as main mechanism of carbapenem

resistance in our region. Similarly, MBL was detected phenotypically in 43/96 (45%) of carbapenem resistant isolates in Iran[18] .

On examining the synergy results regarding the MBL status we found that synergy was high regardless of MBL activity detected in the isolates, other authors suggested that high synergy may be linked to the absence of MBL activity. Further evaluation is required on larger sample size and confirmed with molecular detection for MBL genes (Table 4). [11]

In conclusion, the present *in vitro* study indicated the superiority of the combination of doripenem with ½ MIC of colistin than doripenem or colistin monotherapy in carbapenem resistant *A. baumanni* isolates. Without elevation of colistin dosage, this combination could be a life-saving alternative for the treatment of infections due to these strains. This may reduce the toxicity of colistin and improve the clinical outcome in critically ill patients. Further clinical studies are needed to confirm our findings.

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