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ORIGINAL ARTICLE

Management of clinical infections of *Escherichia coli* by new β-lactam/ β-lactamase inhibitor combinations

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ABSTRACT

Background and Objectives: *Escherichia coli (E. coli)* is an important member of *Enterobacteriaceae* family involved in severe infections. The increased rate of resistance towards different classes of antibiotics limits their treatment options. The aim of this study was to assess the *in vitro* activity of classical and novel combinations of β-lactam/ β-lactamase inhibitor against *E. coli* clinical isolates.

Materials and Methods: 140 clinical isolates of *E. coli* were collected from clinical specimens from Gastrointestinal Surgery Center (GISC) in Egypt. Extended spectrum β-lactamase (ESBL) was detected by double disk synergy test. Furthermore, the minimum inhibitory concentrations (MICs) for five different combinations were determined using the broth microdilution method including: amoxicillin/clavulanate and ampicillin/sulbactam as an example for classical combinations and cefoperazone/sulbactam, ceftazidime/avibactam, and cefepime/enmetazobactam as an example for new combinations.

Results: The percentage of ESBL production among the tested isolates was 46.4%. Isolates were highly resistant to classical β-lactam/ β-lactamase inhibitor combinations, where (40.7%) and (42.9%) of isolates were resistant to amoxicillin/clavulanate and ampicillin/sulbactam, respectively. While new β-lactam/ β-lactamase inhibitor combinations had promising inhibitory action. The addition of novel β-lactamase inhibitors restored the susceptibility of isolates, where (94.3%) of isolates became susceptible to ceftazidime/avibactam combination, followed by cefoperazone/sulbactam (89.2%) and cefepime/enmetazobactam (85.7%). The synergistic effect seems to be effective where ceftazidime and avibactam were synergistic in 80% of isolates.

Conclusion: The antibacterial activity of some antimicrobial agents can be enhanced by the addition of new β-lactamase inhibitors. Further *in vivo* investigation is needed to confirm their therapeutic efficacy against local isolates.

Keywords: Beta-lactamase inhibitors; *Escherichia coli*; Microbial resistance; Minimum inhibitory concentration; Extended spectrum beta-lactamase production

INTRODUCTION

Escherichia coli is one of the most medically important members of family *Enterobacteriaceae* causing well-defined diseases as well as nosocomial infections like urinary tract infections, gastroenteritis, pneumonia, septicemia, and meningitis (1). Some of these diseases are associated with high mortality rates if not treated properly, so it is important to combat them with highly effective antibiotics (2).

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The most used antimicrobial agents for such infections are β-lactams like penicillins, cephalosporins, monobactams and carbapenems (3). Extensive use of antimicrobials and disinfectants has promoted the rapid development of bacterial resistance (4). Third generation cephalosporins-resistant *Enterobacteriaceae* have been categorized as "critical priority" pathogens and such resistance becomes a global health problem especially in developing countries (5). As a result, novel therapeutic options targeting those species are needed urgently (6).

β-lactamase enzymes are major contributors of cephalosporins resistance, some β-lactamases have activity even against 3rd and 4th generation cephalosporins and monobactams, these are the extended spectrum β-lactamases (ESBLs). So β-lactamase inhibitors (BLIs) such as clavulanic acid, sulbactam, avibactam, tazobactam and enmetazobactam have been developed and joined with β-lactam antibiotics to overcome such resistance (7, 8). Recently, different (β-lactam/ β-lactamase inhibitor) combinations have been approved to exhibit *in vitro* synergistic activities against multidrug-resistant (MDR) organisms, like amoxicillin/clavulanate, ampicillin/sulbactam, cefoperazone/sulbactam, ceftazidime/avibactam and cefepime/enmetazobactam (9, 10).

Clavulanic acid and sulbactam were the first BLIs to be approved for use. However, their BLI profiles are largely limited to class A serine penicillinases (e.g., TEM-1 abd SHV-1) and ESBLs (e.g., CTX-M-15) in addition to some class C and D (e.g., AmpC and OXA-1) β-lactamases (11). Due to their limited spectrum and the spread of antimicrobial resistance especially in Gram-negative pathogens, novel BLI with expanded profiles are required (12).

Ceftazidime/avibactam combination was approved by the US Food and Drug Administration (FDA) in 2015 for the treatment of complicated urinary tract infections. It is highly potent against *Enterobacteriaceae*. The spectrum of activity of ceftazidime/ avibactam is attributable to avibactam's ability to inhibit class A, C, and some D β-lactamases, including KPC and OXA-48 carbapenemases (10).

Regarding cefepime/enmetazobactam combination; cefepime is a fourth-generation cephalosporin stable against AmpCs and OXA-48 with well-documented efficacy in serious Gram-negative infections (13). Enmetazobactam (formerly known as AAI101) is a novel ESBL inhibitor having potent inhibitory activity towards CTX-M, TEM, SHV, and other class A β-lactamases. The combination of cefepime/enmetazobactam has met the European Medicines Agency and the FDA pre-specified primary endpoint in the phase 3 clinical trial (9). The aim of this study is to assess the *in vitro* activity of different classical and new β-lactam/ β-lactamase inhibitor combinations against local *E. coli* clinical isolates.

MATERIALS AND METHODS

Ethical approval and consent to participate. All experimental tests were carried in the Faculty of Pharmacy, Delta University according to approval number (FPDU13/2022-June 2022).

Isolation and identification of isolates. A total of 140 *Escherichia coli* isolates were included in the present study. The isolates were recovered from 350 clinical specimens from the laboratory of the Gastrointestinal Surgery Center (GISC) in Mansoura, Egypt during the period from December 2020 to June 2021. The isolates were identified biochemically. Specimens were streaked on nutrient agar (Oxoid, Hampshire, UK) and MacConkey agar (Oxoid, Hampshire, UK) as a selective and differential medium, on which *E. coli* colonies revealed pink colonies. Eosin methylene blue agar was used for identification of *E. coli* by production of the highly characteristics green-black colonies with metallic sheen. For long term storage, all isolates were preserved in tryptone soya broth (TSB, Oxoid, Hampshire, Uk) with 20% glycerol and stored at -80°C (14).

MIC of β-lactam/ β-lactamase inhibitor combinations. The MICs for five different combinations were determined according to the broth microdilution method described by the Clinical and Laboratory Standards Institute (15). Using amoxicillin/clavulanate and ampicillin/sulbactam as an example for classical β-lactam/ β-lactamase inhibitor combinations and cefoperazone/sulbactam, ceftazidime/avibactam, cefepime/enmetazobactam as examples for new combinations.

The tested antibiotics (amoxicillin, ampicillin, cefoperazone, ceftazidime and cefepime) were obtained from Sigma-Aldrish (Darmstadt, Germany). While β-Lactamase inhibitors (clavulanic acid, sulbactam, avibactam and enmetazobactam) were purchased

from MedChemExpress; MCE (Monmouth Junction, USA). All powders were stored in sealed containers at 4°C.

Using a 96-well microtiter plate, each well contained 100 µL of double strength Mueller-Hinton broth (Oxoid, Hampshire, UK) with antibiotic solutions singly and in combination with β-lactamase inhibitors, including a growth control well and a sterility control (uninoculated) well. β-lactamase inhibitors were added by the following ratios: amoxicillin/clavulanic acid at (2:1) ratio (16), ampicillin/sulbactam (1:2) ratio (17), cefoperazone/sulbactam (1:2) ratio (9), ceftazidime/avibactam (4:1) ratio (18), while for cefepime/enmetazobactam combination, enmetazobactam was used at a fixed concentration of 8 µg/mL (13). The antibiotics and β-Lactamase inhibitors were added at twice the desired final concentration, twofold serial dilution was performed.

E. coli strains were initially grown on nutrient agar for 24 h at 37°C, and isolated colonies were used to prepare a saline suspension with a turbidity equivalent to a 0.5 McFarland standard (1.5×10^8 CFU/mL). Finally, 100 µL of bacterial saline suspension were added to each well except for sterility control wells.

MIC was defined as the lowest concentration at which no visual bacterial growth. It was detected by using resazurin dye obtained from Sigma-Aldrish (Darmstadt, Germany) that undergoes colorimetric change in response to cellular metabolic reduction producing pink, fluorescent resofurin product (19). The dye had a high tinctorial power and the color changes were easily judged.

Phenotypic detection of ESBL production. According to clinical and laboratory standard institute (CLSI), clinical isolates which had shown inhibition zone less than 25 mm for ceftriaxone, 17 mm for cefpodoxime and/or 27 mm for aztreonam were considered as potential ESBL-producing isolates (15). ESBL production was confirmed by double disk synergy test (DDST20). Briefly, disks representing third generation cephalosporins including ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), and cefotaxime (CTX, 30 µg) along with fourth generation cephalosporin cefepime (FEB, 30 µg) were placed at a distance of 20mm from amoxicillin-clavulanate disk (AMC, 20/10 µg) which placed in the center of MHA plate inoculated with tested isolate. All antibiotic disks used in DDST20 were provided from Oxoid, Hampshire, UK. Plates were incubated at 37ºC for 16-18 hrs. Any enhancement or distortion of the inhibition zone of any antibiotic disc towards amoxicillin-clavulanic acid indicates ESBL production.

Statistical analysis. One way ANOVA test was used for the statistical analysis using GraphPad Prism software (version 7.0, GraphPad Software Inc., La Jolla, CA, USA). P-values of <0.05 considered statistically significant. All data were expressed as mean ± SEM.

RESULTS

Susceptibility of *E. coli* clinical isolates to different antibiotic combinations by broth microdilution method. The data presented in Table 1 showed the susceptibility of *E. coli* isolates to different cephalosporins singly and in combination with various β-lactamase inhibitors as interpreted according to CLSI guidelines (15). The data revealed that isolates were highly resistant to tested third generation cephalosporins: cefoperazone (52.9%), ceftazidime (43.5%) and the fourth generation cefepime (32.1%) when used alone. Also, they were resistant to classical BL/ BLI combinations like amoxicillin/clavulanate (40.7%) and ampicillin/sulbactam (42.9%).

On the other hand, the addition of new β-lactamase inhibitors restored the susceptibility of *E. coli* isolates, where (94.3%) of isolates became susceptible to ceftazidime/avibactam combination, followed by cefoperazone/sulbactam (89.2%) and cefepime/enmetazobactam (85.7%). Around 2% only of total isolates remained resistant.

As shown in Fig. 1, representative *E. coli* isolate (No. 101) was resistant to amoxicillin/clavulanate (64 µg/mL), and ampicillin/sulbactam (16 µg/mL) as example for classical BL/BLI combinations, also it had high resistance to single cephalosporins like cefoperazone (128 µg/mL), ceftazidime (64 µg/mL), and cefepime (128 µg/mL). After using new BL/BLI, the isolate restored the susceptibility with cefoperazone/sulbactam (16 µg/mL), ceftazidime/avibactam (2 µg/mL), and cefepime/enmetazobactam (0.5 µg/mL).

The addition of new β-lactamase inhibitors results in a great decline in the MICs of β-lactams alone (Table 2). Fifty-four isolates were highly resistant to cefoperazone alone with MIC ≥ 128 µg/mL, after combining with sulbactam this number dropped dramatically to only one isolate and the number of sensitive isolates

Table 1. Susceptibility patterns of *E. coli* isolates towards different β-Lactams singly and in combination with β-Lactamase inhibitors

Combinations	*E. coli* isolates (n=140)					
	Resistant		Intermediate		Sensitive	
	N	%	N	%	N	%
Amoxicillin/Clavulanate	57	40.7%	32	22.9%	51	36.4%
Ampicillin/ Sulbactam	60	42.9%	31	22.1%	49	35%
Cefoperazone	74	52.9%	18	12.9%	48	34.2%
Cefoperazone/Sulbactam	3	2.2%	12	8.6%	125	89.2%
Ceftazidime	61	43.5%	18	13%	61	43.5%
Ceftazidime/Avibactam	3	2.1%	5	3.6%	132	94.3%
Cefepime	45	32.1%	34	24.3%	61	43.6%
Cefepime/Enmetazobactam	4	2.9%	16	11.4%	120	85.7%

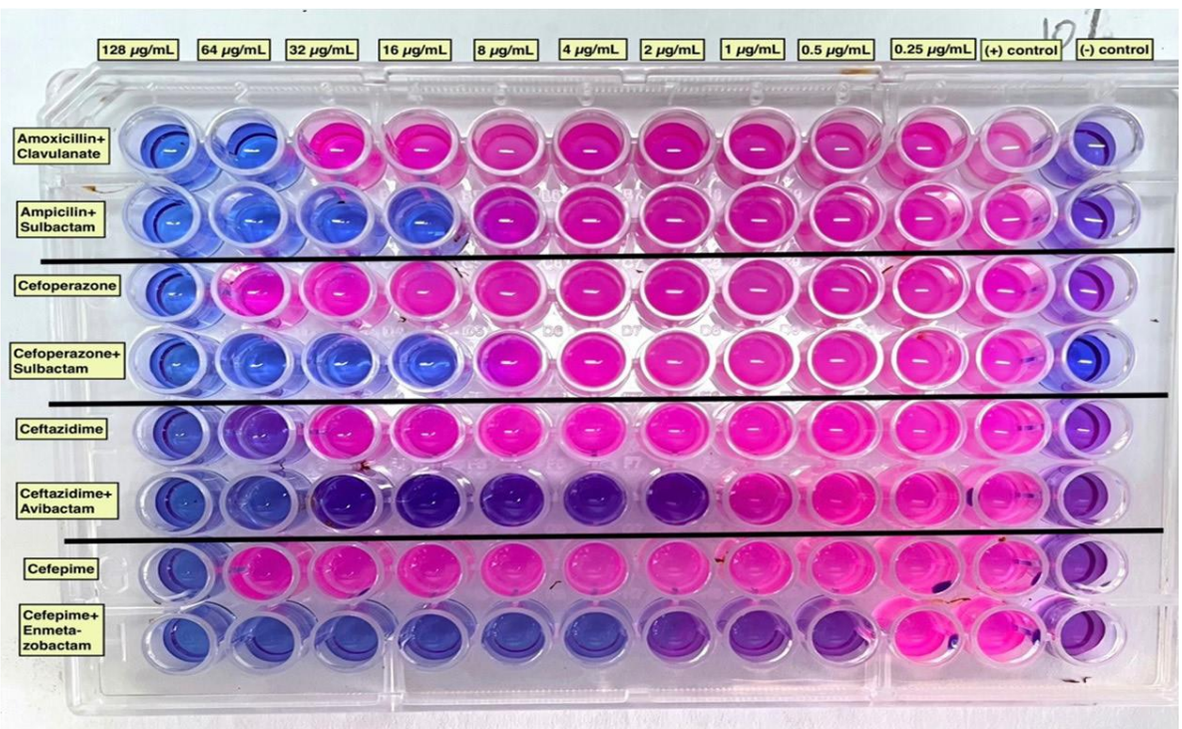

Fig. 1. MICs of β-lactams (alone) and in combination with β-lactamase inhibitors against the representative *E. coli* isolate No. 101. β-lactams with β-lactamase inhibitors were tested in concentrations ranging from (128 to 0.25 µg/mL)

(MIC ≥ 0.25 µg/mL) was duplicated. Furthermore, with avibactam-ceftazidime combination, none of the isolates showed MIC ≥ 64 µg/mL and the number of isolates with MIC ≥ 0.25 µg/mL raised from 34 to 79 isolates. The same observation was recorded with cefepime/ enmetazobactam combination where 74 isolates showed MIC ≥ 0.25 µg/mL compared to 4 isolates only remained resistant with MIC ≥16 µg/mL. After applying one way ANOVA test, the lowest P-value was recorded with cefoperazone/sulbactam (0.0001) followed by ceftazidime/avibactam (P-value of 0.005) then cefepime/ enmetazobactam combination (P-value of 0.02) with P-values of <0.05 considered statistically significant figure (2). These results revealed that β-lactam/ β-lactamase inhibitor combinations were totally effective against most *E. coli* clinical isolates and the MICs values of these three combinations were greatly declined.

Table 2. MICs distribution patterns of cephalosporins singly and in combination with new β-lactamase inhibitors against *E. coli* clinical isolates

MIC range (µg/mL)	≥0.25	0.5	1	2	4	8	16	32	64	128	512	1,024
Cefoperazone	14	5	5	3	8	6	6	18	21	23	17	14
Cefoperazone/Sulbactam	28	4	11	17	26	21	18	13	1	1	-	-
Ceftazidime	34	6	5	5	11	18	16	13	16	9	2	5
Ceftazidime/Avibactam	79	9	6	20	18	5	2	1	-	-	-	-
Cefepime	40	4	8	10	12	21	10	14	10	11	-	-
Cefepime/Enmetazobactam	74	28	9	7	7	11	2	-	2	-	-	-

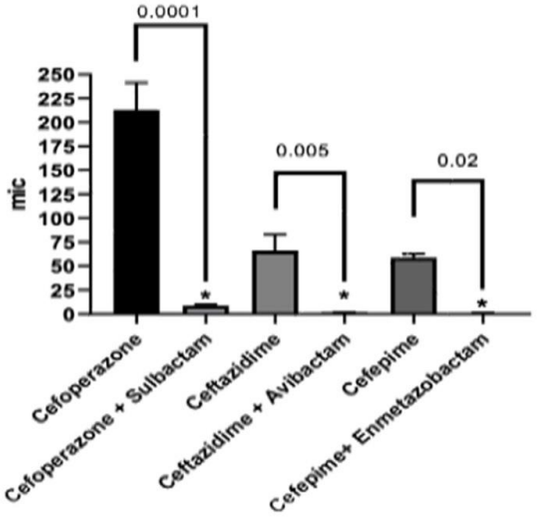

Fig. 2. MICs distribution patterns of cephalosporins singly and in combination with new β-lactamase inhibitors against *E. coli* clinical isolates. One way ANOVA test. P-values of <0.05 considered statistically significant. All data were expressed as mean ± SEM.

Synergy testing. Obviously, if an isolate is susceptible to combination and resistant to the β-lactam alone, then synergy can be assumed (20). The interpretation of the antimicrobial combination interactions was defined as: synergy, if the decrease in the MIC was ≥ 4-fold; additive if the decrease in the MIC was 2-fold; indifference if the interactions did not meet the above criteria and were not antagonistic; and antagonism if the increase in the MIC was ≥ 4-fold.

Table 3 demonstrated the percentage of isolates with synergistic, partially synergistic, additive, and indifferent results for the three tested combinations. Ceftazidime and avibactam were synergistic in 80% (63/79) of isolates, partially synergistic in 6% (5/79), additive in 4% (3/79), and indifferent in 10% (8/79). Cefepime/enmetazobactam combination was synergistic in 76% (60/79), and partially synergistic in 8% (6/79), while cefoperazone/sulbactam combination was synergistic in 63% (58/92) and partially synergistic in 21% (19/92).

ESBL production test. Out of the one hundred and forty *E. coli* isolates, only sixty-five isolates were confirmed to be ESBL producer with percentage of 46.4% of the total *E. coli* isolates (Fig. 3).

DISCUSSION

Escherichia coli is one of the most frequent bacteria involved in human infections. The problems of empirical treatment, self-medication and the misuse of antimicrobial agents which present in many countries have a great impact in increasing the local resistance rates with increasing the risk of therapy failure (21). β-lactams such as penicillins, cephalosporins, and carbapenems are considered appropriate choices for treatment of such infections. Unfortunately, the increased local resistance rate limits their role in treatment (22). This study investigated the *in vitro* activity of different classical and novel β-lactam/ β-lactamase inhibitor combinations against *E. coli* clinical isolates and identified several significant findings.

In the current study, high resistance rates were observed towards different third generation cephalosporins like cefoperazone (52.9%) and ceftazidime (43.5%), followed by the fourth generation cefepime (32.1%) which came in agreement with previous studies (23, 24). Furthermore, high resistance rates were noticed with classical BL /BLI combinations like amoxicillin/clavulanate and ampicillin/sulbactam (40.7% and 42.9%) respectively. Our results were in context with another study performed in Egypt,

Table 3. Outcomes of antimicrobial interactions in combinations against *E. coli* isolates

Combination	Synergy		Partial Synergy		Additive		Indifference	
	N	%	N	%	N	%	N	%
Cefoperazone/Sulbactam	58	63%	19	21%	12	13%	3	3.3%
Ceftazidime/Avibactam	63	80%	5	6%	3	4%	8	10%
Cefepime/Enmetazobactam	60	76%	6	8%	4	5%	9	11%

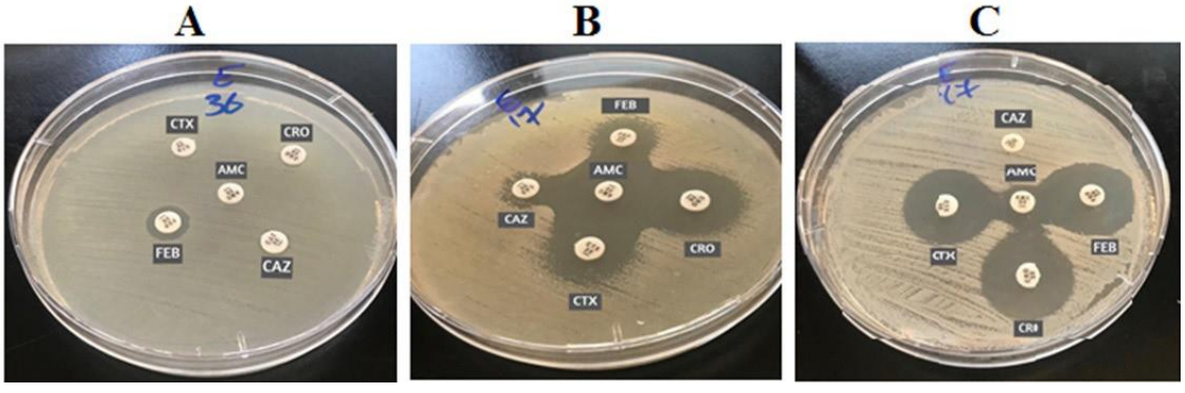

Fig. 3. Double disk synergy test (DDST): amoxcilin-clavulanic acid disc was placed in the center at 20 cm center to centre of ceftazidim, cefatriaxone ,cefotaxime and cefepime discs. A: *E. coli* (isolate 36): represents no synergy between cephalosporin discs and amoxacilin clavulanic acid disk. B: *E. coli* (isolate 17): represents synergy of four cephalosporin discs with amoxicillin clavulanic disc. C: *E. coli* (isolate 27): represents synergy of ceftriaxone, ceftazidium and cefotaxime with amoxacilin clavulanic acid disc.

Ali et al. (25) confirmed the high resistance rate of diarrheagenic *E. coli* isolates to amoxicillin/clavulanate by 60.7%

During the past decade, significant advances were made in the development of new BL /BLI combinations for treatment of Gram-negative infections. The latest advances include cefoperazone/sulbactam, ceftazidime/avibactam, and cefepime/enmetazobactam.

In this study, several significant findings were observed while investigating the *in vitro* activity of some β-Lactams alone and after addition of new β-Lactamase inhibitors. The antimicrobial activity of cefoperazone against Gram-negative bacteria including *E. coli* can be enhanced after addition of sulbactam (26). In the current study, cefoperazone resistance rate was declined from 52.9% to 2.2% after adding sulbactam. This is consistent with a previous study by Kuo et al. (27), which demonstrated that addition of sulbactam to cefoperazone can significantly enhance the antimicrobial activities against *Serratia marcescens, Enterobacter cloacae,* ESBL-*K. pneumoniae* and *A. baumannii*. Furthermore, this study suggested that cefoperazone/sulbactam combination was synergistic in 63% and partially synergistic in 21% of *E. coli* isolates.

Current commercial products containing cefoperazone/sulbactam were made using the fixed ratio of 1:1. Our findings indicated that adding more sulbactam to the current formulations could enhance their *in vitro* activity against some MDR organisms, including ESBL-producing *E. coli*, and the activity is greatest at a 1: 2 ratio which came in agreement with Alfei & Schito (9). The outcomes of our study and other *in vitro* studies indicated that the addition of sulbactam can improve cefoperazone's activity against MDR-*Enterobacteriaceae* (27, 28).

Based on this study, a high number of clinical isolates 61/140 (43.5%) of *E. coli* exhibited reduced susceptibility towards ceftazidime. While, ceftazidime/avibactam combination was largely inhibitory to our collection of *E. coli* isolates, where 94.3% (132/140) of isolates restored their susceptibility to ceftazidime by the addition of avibactam. Recently, Yang et al. (29) reported that a total of 78% of isolates were susceptible to ceftazidime/avibactam. Avibactam is currently marketed in combination with ceftazidime

and has demonstrated high rates of activity against *P. aeruginosa*, carbapenemases and ESBLs producing *Enterobacteriaceae* (10).

The present study found that enmetazobactam restored the activity of cefepime, from 43.6% to 85.7%. Morrissey et al. (30) showed that applying the CLSI breakpoint for cefepime to cefepime/enmetazobactam, enmetazobactam at a fixed concentration of 8 µg/mL lowered the cefepime MIC_{90} from 16 to 0.12 µg/mL for *E. coli*, from 64 to 0.5 µg/mL for *Klebsiella pneumoniae*, from 16 to 1 µg/mL for *Enterobacter cloacae*, and from 0.5 to 0.25 µg/mL for *Enterobacter aerogenes.* The results of this study suggested that cefepime/enmetazobactam may prove to be a valuable option for empirical treatment of serious Gram-negative infections. Furthermore, the synergistic effect seems to be similar between the combination of ceftazidime/avibactam (80%) and cefepime/enmetazobactam (76%) as previously reported (31, 32).

ESBL enzyme production was considered one of the main mechanisms involved in resistance to β-lactams, so it was important to detect its production by different tests (33). In the current study, among one hundred and forty candidate isolates, from routine susceptibility testing, sixty-five isolates were confirmed to be ESBL-producers. Our results came in agreement with other studies performed in Egypt on members of *Enterobacteriaceae*. A recent study showed that 50% of uropathogenic *E. coli* isolates were ESBL producer (34).

The increased rate of ESBL production in Egypt is mainly due to selective overuse of beta-lactamse antibiotics, besides misuse of these antimicrobials agent (35). We found that the prevalence rates of ESBL production in this study are higher than that reported in other studies. In Germany, the percentage of confirmed ESBLs *E. coli* and *Klebsiella* isolates was 20% only (36).

CONCLUSION

In conclusion, significant progress was made in the past decade to develop BL-BLI combinations that target some of the most formidable Gram-negatives, including a combination that completed phase III of development in addition to some approved agents. After these latest advances in BL-BLI combinations being reviewed, our study confirmed that despite the lower susceptible rate to third generation cephalosporins, the antibacterial activity of such ancient antimicrobial agents can be enhanced by the addition of β-lactamase inhibitors. The synergistic effect of such combinations seems to be effective against most multidrug-resistant *E. coli* isolates and warrants further *in vivo* investigation to confirm their therapeutic efficacy.

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