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Effect of Antibiotic Combinations on the Sensitivity of Carbapenem Resistant *Acinetobacter baumannii* Strains

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Abstract

Acinetobacter baumannii is one of the predominant pathogens involved in hospital acquired infections worldwide. Despite intensive surveillance and preventive measures, these infections are major cause of high mortality rates. In this study a total of 375 *A. baumannii* isolates were isolated from a variety of clinical collected from 5 different hospitals in Great Cairo. These isolates were identified as *A. baumannii* biochemically, by API20E system and genetically by detection of 16S rRNA gene. All isolates showed positive results and confirmed for the presence of the suspected gene by PCR.

The antibiotic susceptibility patterns for the isolated *A. baumannii* were also evaluated. The detection of the presence of *OXA-type* (OXA 23, 24, 51 and 58) carbapenemases-encoding genes which are responsible for carbapenem resistance was also performed. I observed the presence of *OXA 23* gene in 84% of the isolates. While 35.2% were positive to *OXA 24* gene and 87.2% were positive to *OXA 51* gene. No isolates showed positive results for the presence of *OXA 51* gene.

They were also evaluated for the effectiveness of drug combination of carbapenems and colistin against 30 isolates. Regarding imipenem and colistin combination, 13.3% of the strains showed synergy, while 86.7% showed additive results. For the combination of meropenem and colistin, 66.7% of the strains showed synergy, while 33.3% showed additive results. The antibacterial effect of both combinations on *A. baumannii* showed commonly synergistic or additive results. To a significant extent meropenem and colistin showed a superior synergy compared to imipenem and colistin.

Keywords: *Acinetobacter baumannii*; Carbapenem; Colistin; Carbapenemases; Synergy

Introduction

Healthcare associated infections (HAIs) are a type of infections caused by prolonged hospital stay and it accounts for a major risk factor for serious health issues leading to death [1]. About 75% of the burden of these infections is present in developing countries [2].

Healthcare-associated infections are a worldwide dilemma, with significant mortality. For decades, Gram-negative bacilli (GNB) sustained their share of HAIs; however Acinetobacter is the main GNB associated with consistently increasing proportions of HAIs. *A. baumannii* is expected to become a chief cause of HAIs in Egyptian hospitals due to the extensive use of antimicrobials in intensive care units (ICUs), in addition to its inconceivable ability to acquire resistance [3].

A surveillance program conducted in Egypt showed high incidence of ICU-onset HAIs and a high resistance rates among organisms causing nosocomial infections, considered a main risk to patient safety [4]. Non-lactose fermenting bacteria such as *A. baumannii* species are major causes of hospital-acquired infections [5]. The prevalence of MDR *A. baumannii* isolates from intensive care units among hospitals in Egypt is increasing rapidly [6].

Multiple mechanisms have been implicated in the resistance of *Acinetobacter* spp. and it is considered one of the most virulent MDR pathogens. Acinetobacter has been isolated in food and inanimate objects and can colonize humans and live in dry or moist conditions [7].

Farid et al. [5] stated that one of the predominant Gram-negative bacteria causing HAIs in Egyptian hospitals is *A. baumannii*. Their infections are difficult to treat due to limited susceptibility to antimicrobial drugs and the appearance of antibiotic resistance during therapy. Multidrug resistance, which is produced by a variety of resistance mechanisms, leaves inadequate alternatives for treatment in many patients. For this reason there are not many choices available for treatment and control of such dangerous organism in Egyptian hospitals. Combination therapy is one of the supreme effective methods for treatment as it reduces adverse effects and resistance to the antibiotics used. The aim of this study is detection of *A. baumannii* isolates from intensive care units in Egypt. Furthermore, examine their antimicrobial susceptibility patterns and their major resistance mechanisms. Also, determine the effect of combination therapy on treating such pathogen.

Materials and Methods

Bacteriological examination

A total of 375 *A. baumannii* isolates were isolated from various clinical specimens including 73 blood, 67 throat swaps, 57 urine, 49 pus, 46 wound, 24 Nasogastric tube, 22 endotracheal tubes, 22 sputum and 15 nasal swaps collected from 5 health care institution in Cairo and Giza (National Cancer Institute, Al-Kasr eleiny Hospital, Al-Zahraa Hospital, Al-Demerdash Hospital, Al-Galaa Hospital). These isolates identified biochemically by using catalase test and ability to grow at 44°C [8] and were confirmed by API 20E test kit (BioMèrieux, France).

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DNA extraction

DNA was extracted from the microbial colonies using the QIAmp DNA mini kit (Qiagen Inc.) according to the producer's recommendation. The DNA concentration and purity were determined by assessing the absorbance at 260 nm and by computing the ratio of absorbance at 260 nm to that at 280 nm using a spectrophotometer (U.V-VIS.), U.V 2500 (Labomed. Inc.) [9]. DNA was used directly or stored at −20°C for future use.

Detection of *A. baumannii* group specific 16S rRNA gene and *OXA* genes (*OXA 23, OXA 24, OXA 51* and *OXA 58*) responsible for carbapenem resistance by Multiplex PCR.

Amplification and detection of *A. baumannii* group specific gene was performed according to the technique formerly described by Misbah et al. [10]. All oligonucleotides were synthesized in Bio Basic Inc. (Canada). The sequences of the primers used for detection of *A. baumannii* group specific gene "16S rRNA" is: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-TACCAGGGTATCTAATCCTGTT-3′. The sequences of the primers used for detection of the *OXA 23, 24, 51* and *58* genes are: 5'-GATCGGATTGGAGAACCAGA-3' and 5'-ATTTCTGACCGCATTTCCA-3' for *OXA 23*, 5'-GGTTAGTTGGCCCCCTTAAA-3' and 5'-AGTTGAGCGAAAAGGGGATT-3' for *OXA 24* and 5'-TAATGCTTTGATCGGCCTTG-3' and 5'-TGGATTGCACTTCATCTTGG-3' for *OXA 51* and 5'-AAGTATTGGGGCTTGTGCTG-3' and 5'-CCCCTCTGCGCTCTACATAC-3' for *OXA 58* gene primers (Kapa, USA) [11].

The PCR was made in a total volume of 25 µl reaction mixtures containing 150-200 ng of DNA as template, 0.5 µM of each primer and 1x of PCR master mix (Taq Master/High yield, Jena Bioscience) which provides 2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate, 1x PCR buffer (with 1.5 mM-$MgCl_2$). The amplification cycles were performed out in a programmable heating block, (Primus Thermal Cycler, MWG Biotech, Germany). Reaction conditions were optimized to be 94°C for 3 min as initial denaturation, followed by 30 cycles of 95°C for 30 s (55°C for 60 s and 52°C for 40 s for detection of *A. baumannii* and *OXA* genes, respectively). A final extension step at 72°C for 5 min was followed. Negative control (no template) and positive control (reference strain) were included. Amplification products were electrophoresed in 2% agarose gel in 0.5x TBE (Tris-borate-EDTA) at 70 V for 60 min and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 1500 bp DNA ladder was run simultaneously as a marker. Presence of 750 bp, 501 bp, 249, 353 and 599 bp DNA fragment indicated positive sample of *A. baumannii* group specific *OXA 23, 24, 51* and *58* genes, respectively.

Antimicrobials susceptibility testing

A total of 375 *A. baumannii* isolates were examined for their sensitivity to several antimicrobial agents by disc diffusion method according to Clinical laboratory standards institute [12]. Isolates were cultured on Mueller-Hinton agar and tested for their susceptibility to 12 antimicrobial agents.

The following antimicrobial discs were used: piperacillin (100 µg), piperacillin/tazobactam (75/10 µg), ampicillin- sulbactam (10/10 µg), ceftazidime (30 µg), cefepime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10), colistin (50 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). All antimicrobial discs were purchased from Oxoid Chemical Co. UK.

Assessment of combination therapy by checkerboard assay for treatment of multi-drug resistant bacterial isolates

The minimum inhibitory concentration (MICs) of colistin (COL), imipenem (IMP) and meropenem (MERO) for the bacterial isolates were determined by the broth microdilution method, according to the CLSI [13]. The synergy study for COL/IMP and COL/MERO combinations was carried out for thirty isolates, using the checkerboard method. The checkerboard test was done on 96-well microtiter plates. Most of the strains were resistant or intermediate to carbapenems and susceptible to colistin, the concentration of colistin ranged from 1/32x MIC to 32x MIC while that of the carbapenem (imipenem or merpenem) ranged from 1/8x MIC to 8x MIC. The initial bacterial inoculum was adjusted to 10^6 CFU/ml. The plate was incubated for 18 h at 35°C. The fractional inhibitory concentration index (FICI) was calculated using the concentrations in the first non-turbid (clear) well in each row and column along the turbidity/non-turbidity interface and then averaged. The results were then classified as: synergy for Σ FIC $\leq$ 0.5; additive for Σ FIC between 0.5 and 1.5 and indifference for values of Σ FIC between 1.5 and 2; Antagonism was linked to values above 2 [14-16].

Statistical Methods

Statistical analysis of checkerboard assay results was made using the chi-square test. Differences were considered significant when $p \leq 0.05$. The data was coded and entered using the statistical package SPSS version 15 (IBM, New York, United States).

Results

Three hundred and seventy five isolates of *A. bumannii* were identified and confirmed morphologically, by different biochemical tests. All isolates reacted positively to catalase and ability to grow on 44°C. All isolates were confirmed by API 20E system showed 90.32% identification. All isolates were confirmed by PCR assay using *A. bumannii* specific primers. Peaks for positive samples appeared at (750 bp) as shown in Figure 1.

The antimicrobial susceptibility patterns of three hundred and seventy five isolates were examined for antibiotics resistance. Hundred percent of *A. bumannii* isolates were sensitive to colistin, followed by tobramycin (30.9%), gentamicin (22.4%), trimethoprim/sulfamethoxazol (20%), tetracycline (18.7%), piperacilin-tazobactam (13%), levofloxacin (10.7%), amikacin (9.3%), ciprofloxacin (9.3%), meropenem (9.3%), ampicillin-sulbactam (6.7%), imipenem (5.6%), piperacillin (5%), ceftazidime (4%), cefepime (4%), ceftriaxone (1.9%) and 100% resistant to ceftizoxime.

PCR reactions for confirmed *A. bumannii* isolates were done for detection of *OXA 23, 24, 51* and 58 genes that are responsible for carbapenem resistance. Three hundreds and fifteen isolates (84%), 132 isolates (35.2%), 327 isolates (87.2%) showed positive results to the presence of *OXA 23, OXA 24* and *OXA 51*, respectively and confirmed for the presence of the genes by showing bands on 501, 249, 353 bp. No isolates show positive results to the presence of *OXA 58* as illustrated in Figure 2.

Minimum inhibitory concentrations determination of colistin, imipenem and meropenem against *A. bumannii*

Thirty strains of *A. bumannii* of different susceptibility profiles

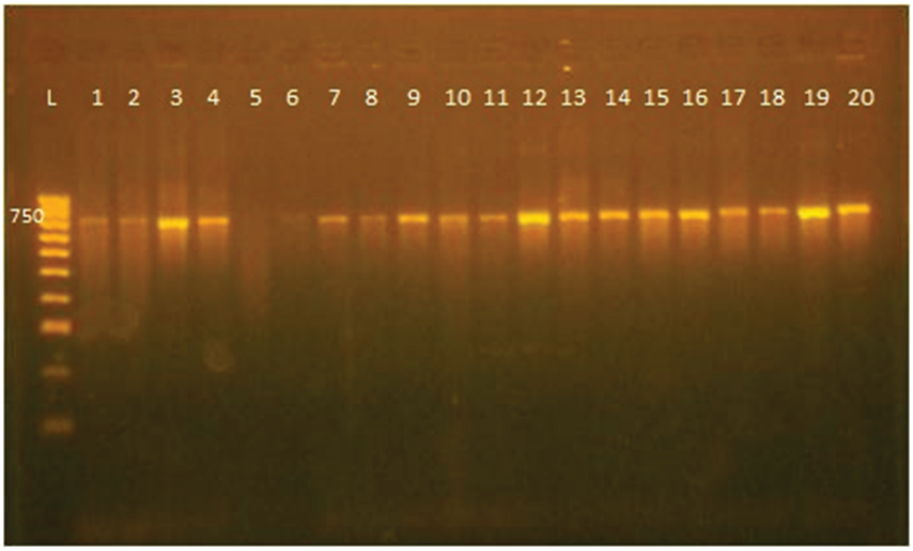

Figure 1: PCR amplification with *16S rRNA* gene primers. A 750 bp of *16S rRNA* gene. Lane (L), DNA molecular size marker (1500 bp ladder) and Lanes (1-4) and (6-20) show positive result with positive bands of 750 bp. Lane (5) shows negative result.

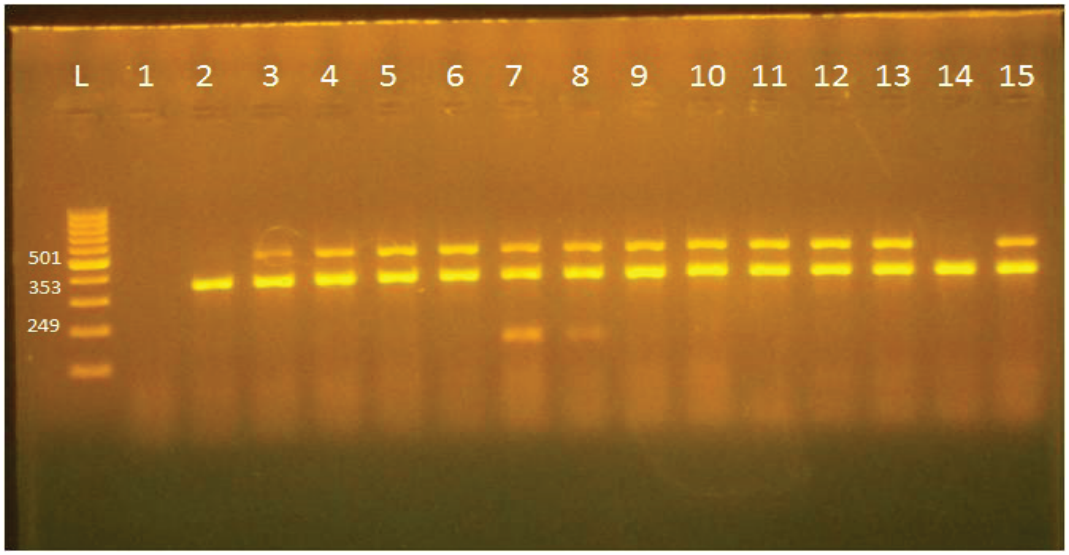

Figure 2: PCR multiplex PCR amplification with OXA genes primers. OXA 23 at 501 bp, OXA 24 at 249 bp, OXA 51 at 353 bp and OXA 58 at 599 bp. Lane (L), DNA molecular size marker (1500 bp ladder) and Lanes (2-15) show positive results for OXA 51 at 353 bp, Lanes (3-13) and (15) show positive results for OXA 23 at 501, Lane (7 and 8) shows positive results for OXA 24 at 249 bp.

were used in this test. Most of the strains were resistant or intermediate to carbapenems and susceptible to colistin. The MICs obtained for each antibiotic are shown in Tables 1 and 2. All *A. bumannii* strains were sensitive to colistin. 12 strains were resistant, 12 strains were intermediate and 6 strains were sensitive to imipenem, 10 strains were resistant, 16 strains were intermediate and 4 strains were sensitive to meropenem.

Results (MIC done in triplet) by broth macrodilution for colistin (COL) for 30 *A. bumannii* strains shows 100% sensitive, as 24 isolates showed MIC of 2 and 6 isolates showed MIC of 1. Colistin MIC Interpretive Criteria (≤ 2=Sensitive and ≥ 8=Resistant) [13].

Results (MIC done in triplet) for 30 *A. bumannii* strains by broth macrodilution for imipenem (IMP) showed 20% sensitive, 40% intermediate and 40% resistant. While for meropenem (MERO) showed 33.3% sensitive, 53.3% Intermediate and 13.4% resistant as shown in Tables 1 and 2.

Checkerboard results

Tables 2 shows the Fractional inhibitory concentration (FICs) calculated for all *A. baumannii* strains using the 2 combinations of antibiotics. For the combination of imipenem and colistin 4 of the 30 strains showed synergy with a percentage of 13.3%. While 26 strains showed additive results with a percentage of 86.7%. For the combination of meropenem and colistin on *A. baumannii*, 20 of the

Imipenem			Meropenem		
No. of Isolates	**MIC ug/ml (Median)**	**Interpretation**	**No. of Isolates**	**MIC ug/ml (Median)**	**Interpretation**
5	32	R	2	16	R
3	16	R	4	32	R
4	32	R	4	32	R
12	8	I	16	8	I
2	0.125	S	1	0.125	S
3	0.25	S	2	0.25	S
1	0.125	S	1	0.125	S

Imipenem MIC Interpretive Criteria (≤ 4=Sensitive, 8=Intermediate and ≥ 16=Resistant), meropenem MIC Interpretive Criteria (≤ 4=Sensitive, 8=Intermediate and ≥ 16=Resistant) (CLSI, 2016)

Table 1: MICs results by broth macrodilution of imipenem (IMP) and meropenem (MERO) for 30 *A. bumannii* strains, (MIC done in triplet).

Colistin and Imipenem combination			Colistin and Meropenem combination		
No. of isolates	**Percent**	**Effect**	**No. of isolates**	**Percent**	**Effect**
4	13%	Synergism	20	66.7%	Synergism
26	87%	Addition	10	33.3%	Addition

Table 2: The FICs calculated for *A. baumannii* strains using different combinations of colistin and imipenem.

30 strains showed synergy with a percentage of 66.7% while 10 strains showed additive results with a percentage of 33.3%.

The antibacterial effect of both combinations on *A. baumannii* showed mostly synergistic or additive results. To a significant extent meropenem and colistin showed a better synergy when compared to imipenem and colistin.

Discussion

Hospital-Acquired Infections (HAIs) are a major cause for high mortality rates worldwide and considered a prominent dilemma in health care facilities. The highest rates of HAIs are highly noticed in ICUs [17].

In the current study a total of 375 non-replicated clinical isolates were collected from various clinical specimens. The antimicrobial susceptibility testing of the isolates was performed using the Kirby Bauer disk diffusion method following the definition of the Clinical and Laboratory Standards Institute using antibiotic discs [13]. Results are consistent with results obtained by AlBshabshe et al. [18] and Altun et al. [19] where all isolates were susceptible to colistin. While in a previous study by Ahanjan et al. [20] showed that susceptibility for colistin was (65%) and resistance was seen for cefotaxime (100%) and ceftazidim (100%). In a previous hospital study presented by Gupta et al. [21] resistance rate among *A. baumannii* showed high incidence of resistance was recorded for piperacillin (55%), followed by ceftriaxone (46%) and ceftazidime (46%). This difference may came from the excessive use of colistin in some hospitals that lead to the emergence of colistin resistant strains of *A. baumannii* [21].

Carbapenems are considered the drug of choice for the treatment of infections caused by Multi drug resistant Gram-negative bacilli [22]. In recent years, Egypt has been considered among the countries that reported high rates of antimicrobial resistance [23]. However, carbapenem resistance has been observed repeatedly in non-fermenting bacilli (*A. baumannii*). Resistance to carbapenem is principally due to carbapenem hydrolyzing enzymes carbapenemases. In the last decade, several classes of *β*-lactamases have been detected in *A. baumannii* [24,25]. The carbapenemases found are mostly belonging to the carbapenem-hydrolyzing OXA-type class D ß-lactamase (CHDL) [26].

Different families of class D ß-lactamase (CHDL) have been reported from numerous geographical areas so far. The most common type of enzymes capable of hydrolyzing carbapenems have been reported in *A. baumannii*, belonging to class D (*bla*OXA-23-like, *bla*OXA-24-like, *bla*OXA-51-like, *bla*OXA-58-like) [27]. The present study revealed that (87.2%) of the carbapenem resistant *A. baumannii* isolates were confirmed for the presence of *OXA 51* gene, (84%) for *OXA 23* gene, (35.2%) for *OXA 24* gene. No isolates have revealed the presence of *OXA 58* gene. While Al-Hassan et al. [27] revealed that three acquired class D carbapenemases (*OXA-23* in 72%, *OXA-40* in 4%, *OXA-58* in 20%) were identified among studied carbapenem-resistant *A. baumannii* strains in two Egyptian centers. Also, our results were nearly confirmed by Gao et al. [28] who confirmed that (100%) of the carbapenem resistant isolates showed positive results for the presence of *OXA 51* gene, (94%) for *OXA 23*, and the other two resistance genes (*OXA 24* and *OXA 58*) were not detected in any of the strains. Same results were revealed by Rolain et al. [29] in Hamad hospital in Qatar, that conveyed that (100%) of *A. baumannii* isolates were positive for *OXA-23;* none were positive for *OXA-24, OXA-58* [30].

In Saudi Arabia, *OXA-23, OXA-24* and *OXA-58* were detected in 72.5, 45 and 37.5%, respectively in isolated *A. baumannii* strains [31]. *OXA-23* was detected in Riyadh and the Eastern Province, with 53 and 79.5% respectively among non-susceptible *A. baumannii* [32]. In the United Arab Emirates, one study found *OXA-23* gene in all isolates under study. In Abu Dhabi, it was found that the OXA-23 gene was detected in 73.6% of all strains involved in the study by Mugnier et al. [33].

Due to the deficiency or ineffectiveness of infection control programs in many clinics, random/extensive use of antibiotics and many other reasons, resistance greatly appeared within these pathogens and they became acknowledged as highly resistant organisms [32]. Carbapenem resistant *A. baumannii* strains are currently broadly spread.

Carbapenems were considered as the choice of extreme cases of resistance. With the rise in resistance against carbapenems, most of the available antimicrobial agents are becoming inadequate [34,35]. Physicians have imperfect solution for the treatment of such infections. The old antibiotic colistin, in spite of its toxicity and side effects, is considered today as the last means when these multi-drug organisms are observed [36,37]. The idea of combining colistin with other antibiotics pursuing a synergistic activity, and possibly a less toxicity, looks encouraging.

Combination therapy limits and decrease microbial resistance, lowers antibiotic toxicity, covers a wide range of pathogens with greater effectiveness and leads to synergy [38,39]. One of the aims of this study was to assess the antibacterial activity of the combination of two antibiotics: colistin and a carbapenem (meropenem or imipenem) on *A. baumannii* using Checkerboard technique.

Most *A. baumannii* strains are becoming multidrug resistant; the main concern is being resistance to carbapenems [40]. All the strains used in this study were resistant or intermediate to carbapenems however susceptible to colistin. It has been detected that meropenem has greater in vitro efficacy than imipenem against *A. baumannii* [13].

The present study showed that (66.7%) of *A. baumannii* isolates showed synergistic effect to meropenem/colistin combination and (33.3%) showed an additive effect. On the other hand, (13%) of *A. baumannii* isolates showed synergistic effect to imipenem/colistin combination and (87%) showed an additive effect. These results are consistent with results obtained by Daoud et al. where (54.5%) of *A. baumannii* isolates showed synergistic effect to meropenem/colistin combination and (45.5%) showed an additive effect [41]. While, (11%) of *A. baumannii* isolates showed synergistic effect to imipenem/colistin combination and (89%) showed an additive effect.

Conclusion

The best synergy rate and consequent highest antibacterial activity were revealed for the combination of meropenem and colistin. This proposes that the combination could be a good alternative for the treatment of *A. baumannii* infections until the successful development of a better antibiotic agent. The cause for the increased synergy with meropenem than imipenem might be that most OXA carbapenemases target with better affinity imipenem as compared to meropenem [42,43]. Besides, the synergistic or additive effect might be affected by the ability of colistin to disrupt the bacterial outer membrane and raise its permeability for carbapenems and therefore cease the cross linking of the new produced polymers [44,45].

Another benefit for combination therapy is delaying the emergence of bacterial resistance and specifically the promptly developing resistance toward colistin [7]. It must be declared that not only synergy is considered as a benefit for the therapy but also additive result is by itself beneficial, because even a miniature increase in the antibacterial activity using the combination therapy may help clinical success and recovery.

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