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ORIGINAL ARTICLE

Synergy Testing by E-Test and Microdilution Checkerboard for Fosfomycin Combined with Tigecycline against KPC-Producing *Klebsiella pneumoniae*

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SUMMARY

Background: **KPC-producing *Klebsiella pneumoniae* (KPC-Kp) has become a serious threat to patients worldwide, as the treatment options are limited. The combination of fosfomycin with other antibiotics has been reported but with inconsistent results. Thus, we performed synergy testing of fosfomycin combined with tigecycline by E-test, which was easy to perform and to determine results, compared with microdilution checkerboard, which is considered to be the "gold standard", to evaluate the agreement between the two methods.**
Methods: **Thirty non-repetitive KPC-Kp isolates from different patients were included in this study. Bacterial identification and routine antibiotic susceptibility testing were performed by a VITEK 2 Compact automated system. The KPC producing isolates were identified by modified Carbapenem Inhibitory Method (mCIM) and PCR amplification of carbapenemase genes. Synergy testing of fosfomycin combined with tigecycline was performed by E-test (E-test stripes were placed at 90° angle), with microdilution chequerboard performed in parallel. Fractional inhibitory concentration index (FICI) was calculated. Statistical analyses were performed by SPSS 18.0 software.**
Results: **All 30 KPC-Kp were mCIM test positive and KPC-2 producing. The susceptibility rates of fosfomycin and tigecycline were 36.7% (11/30) and 63.3% (19/30), respectively. Both checkerboard and E-test results showed that most MICs of fosfomycin and tigecycline decreased in the combination group. FICI showed 13.3% - 16.7% isolates were synergistic, 30.0% - 36.7% were additive, and 50.0% - 53.3% were indifferent. No antagonism was found. There was no significant difference between the two groups ($p > 0.05$), and the overall agreement (with FICI difference ≤ 0.25 in each isolate) between the two methods was 76.7% (23/30).**
Conclusions: **The synergy testing results determined by E-test correlated well with microdilution checkerboard. Thus E-test synergy testing has the potential to be used in routine clinical laboratory.**
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KEY WORDS

synergy testing, fosfomycin, tigecycline, carbapenem resistant *Klebsiella pneumoniae*

INTRODUCTION

Carbapenem resistant *Enterobacteriaceae* (CRE) has become a global health challenge, as infections caused by CRE are associated with high mortality because of the limited therapeutic options. CRE with increasing trends has been reported in recent years from many countries, including China [1-3]. *Klebsiella pneumoniae* Carbapenemase (KPC) producing *Klebsiella pneumo-*

niae (KPC-Kp) is the most prevalent CRE in China [4]. Combination therapies (e.g., fosfomycin, tigecycline, and colistin) are recommended for treating KPC-Kp; however, there is no consensus of which kind of combination is better for recommendation [5,6].

Fosfomycin could inhibit the formation of bacterial cell wall peptidoglycan chains, thus showing a broad spectrum of antibacterial activity [7]. Previous studies showed that a combination of fosfomycin with tigecycline had synergistic effect against KPC-Kp, but with inconsistent results [8,9].

The synergy testing of antibiotic combinations could be performed by microdilution checkerboard which is considered to be the "gold standard"; however, this method is time-consuming and labor-intensive [10]. Alternatively, E-test could also be used to perform the synergy testing [11,12], which was easier to perform and to determine results. To the best of our knowledge, no study compared the synergy testing between E-test and microdilution checkerboard for fosfomycin combined with tigecycline against KPC-Kp. Thus, in this study, 30 KPC-Kp clinical isolates from various specimen types were collected. Fosfomycin combined with tigecycline synergy testing was performed by the two methods in parallel.

MATERIALS AND METHODS

Bacterial isolates

A total of 30 KPC-Kp isolated from different patients hospitalized at Peking University First Hospital (Beijing, China) from 2016 to 2018 were included in this study. The specimen types of these isolates included sputum (n = 15), urine (n = 8), catheter (n = 3), blood (n = 2), and wounds (n = 2). The bacterial identifications were performed by a VITEK 2 Compact automated system (BioMerieux, France). Then the carbapenemase-producing isolates were identified by modified Carbapenem Inactivation Method (mCIM) following CLSI document M100-S28 [13]. Carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}) were detected by PCR as described previously [14]. Multi-locus Sequencing Typing (MLST) was performed to determine the Sequence Types (STs) of these isolates, following the procedure described at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html.

Antibiotic susceptibility testing

The routine antibiotic susceptibility testing (AST) was performed by a VITEK 2 Compact automated system (BioMerieux, France), and the carbapenem (imipenem or meropenem) minimal inhibitory concentrations (MICs) of the CRE isolates were confirmed by E-test method. The carbapenem MICs of "≥ 4" were defined as "resistant", following the CLSI recommended breakpoints [13]. These KPC-Kp isolates were selected for further AST by broth microdilution for fosfomycin and tigecycline according to CLSI guidelines (M07-A9, 2012) as described previously [4]. As there were no CLSI breakpoints for fosfomycin and tigecycline, European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for them, with fosfomycin MIC > 32 mg/L and tigecycline MIC > 0.5 mg/L defined as "resistant" [15].

Microdilution checkerboard method

The combination of fosfomycin and tigecycline were determined for synergy by microdilution checkerboard as described previously [11]. Glucose-6-phosphate was added to the medium when determining the MICs for fosfomycin. Briefly, serial 2-fold dilutions of each antibiotic were distributed into a 96-well microtiter plate, and the susceptibility of each antibiotic combination was determined in a checkerboard conformation. Each isolate was performed in duplicate to determine the results.

E-test synergy testing

The MIC of each single antibiotic was determined by E-test following the manufacturer's instructions. Tigecycline and fosfomycin E-test stripes were purchased from AutoBio (China) and Liofilchem (Italy), respectively. Synergy assay of the two antibiotic combinations by E-test was described previously [10,12]. Briefly, the E-test strip containing one kind of antibiotic was placed on cation-adjusted Mueller-Hinton agar plates, then the E-test strip containing another kind of antibiotic was crossed at a 90° angle at the intersection between relative MIC for each isolate. Both single and combined MICs after overnight culture at 35°C were determined following the manufacturer's instruction.

Determination of synergy

The effect of the two antibiotic combination was defined by fractional inhibitory concentration index (FICI) as follows: $FICI_{A/B}$ = [MIC_A(combination)/MIC_A(alone)] + [MIC_B(combination)/MIC_B(alone)]. "Synergistic" was defined as "FICI ≤ 0.5". "Additive" was defined as "0.5 < FICI ≤ 1". "Indifferent" was defined as "1 < FICI ≤ 2". "Antagonistic" was defined as "FICI > 2" [16].

Statistical analysis

The FICI results obtained by E-test and microdilution checkerboard were compared by paired *t*-test with SPSS 18.0 software, with $p < 0.05$ defined as statistical significance.

RESULTS

Characteristics of the included KPC-Kp isolates

MLST typing of the 30 included KPC-Kp isolates showed that 26 isolates belonged to ST92, and 4 isolates belonged to ST11. All 30 isolates were mCIM test positive, and PCR detection of carbapenemase genes showed all the isolates were KPC-2 gene positive.

Table 1. MICs and FICIs of fosfomycin (FOS) and tigecycline (TIG).

Isolate No.	FOS MICs				TIG MICs				FICIs		
	E-test		Checkerboard		E-test		Checkerboard		E-test	Checker-board	MICs decreased ≥ 4 times in checkerboard
	single	combined	single	combined	single	combined	single	combined			
1	16	8	16	16	1	1	1	0.5	1.5	1.5	Not applicable
2	4	2	4	4	0.5	0.25	0.5	0.25	1.0	1.5	Not applicable
3	8	4	8	8	1	1	1	1	1.5	2	Not applicable
4	64	8	64	16	0.5	0.5	0.5	0.25	1.125	1.25	FOS
5	2	1	2	2	0.25	0.25	0.5	0.25	1.5	1.5	Not applicable
6	64	16	32	8	0.25	0.0625	0.25	0.125	0.5	0.75	FOS
7	4	1	4	1	0.5	0.5	0.25	0.125	1.25	1.0	FOS
8	128	32	128	64	2	0.5	2	0.25	0.5	0.75	TIG
9	32	8	64	16	0.5	0.125	0.25	0.125	0.75	0.75	FOS
10	16	4	16	2	0.25	0.125	0.5	0.125	0.75	0.5	FOS + TIG
11	128	16	64	16	1	0.25	1	0.25	0.375	0.5	FOS + TIG
12	64	8	32	4	1	0.5	2	1	0.625	0.625	FOS
13	64	8	32	8	0.5	0.25	0.5	0.25	0.625	0.75	FOS
14	2	0.5	2	0.5	0.125	0.064	0.5	0.125	0.75	0.5	FOS + TIG
15	32	4	32	8	1	0.25	1	0.5	0.375	0.5	FOS
16	64	8	64	4	0.25	0.25	0.5	0.25	1.125	0.5625	FOS
17	256	256	128	128	0.5	0.25	0.5	0.5	1.5	2.0	Not applicable
18	16	16	16	16	0.5	0.25	0.5	0.125	1.5	1.25	TIG
19	64	8	16	8	4	2	4	2	0.625	1.0	Not applicable
20	128	64	128	128	0.25	0.25	0.125	0.125	1.5	2.0	Not applicable
21	64	16	64	32	1	1	2	1	1.25	1.0	FOS
22	256	256	128	128	2	2	2	1	2.0	1.5	Not applicable
23	64	16	32	16	1	0.5	1	0.5	0.75	1.0	Not applicable
24	64	16	64	64	0.5	0.5	0.5	0.25	1.25	1.5	FOS
25	128	16	64	16	0.25	0.125	1	0.25	0.625	0.5	FOS + TIG
26	64	32	64	64	0.5	0.5	0.5	0.25	1.5	1.5	Not applicable
27	128	64	128	128	2	2	4	1	1.5	1.25	TIG
28	1	1	1	1	0.125	0.0625	0.25	0.125	1.5	1.5	Not applicable
29	64	32	64	64	0.5	0.5	2	0.5	1.5	1.25	TIG
30	128	64	64	64	0.25	0.125	0.25	0.125	1.0	1.5	Not applicable

Table 2. Synergy testing effects performed by E-test and Checkerboard.

	E-test				Checkerboard			
	Synergistic	Additive	Indifferent	Antagonistic	Synergistic	Additive	Indifferent	Antagonistic
Percentage % (n/n)	16.7% (5/30)	30.0% (9/30)	53.3% (16/30)	0% (0/30)	13.3% (4/30)	36.7% (11/30)	50.0% (15/30)	0% (0/30)

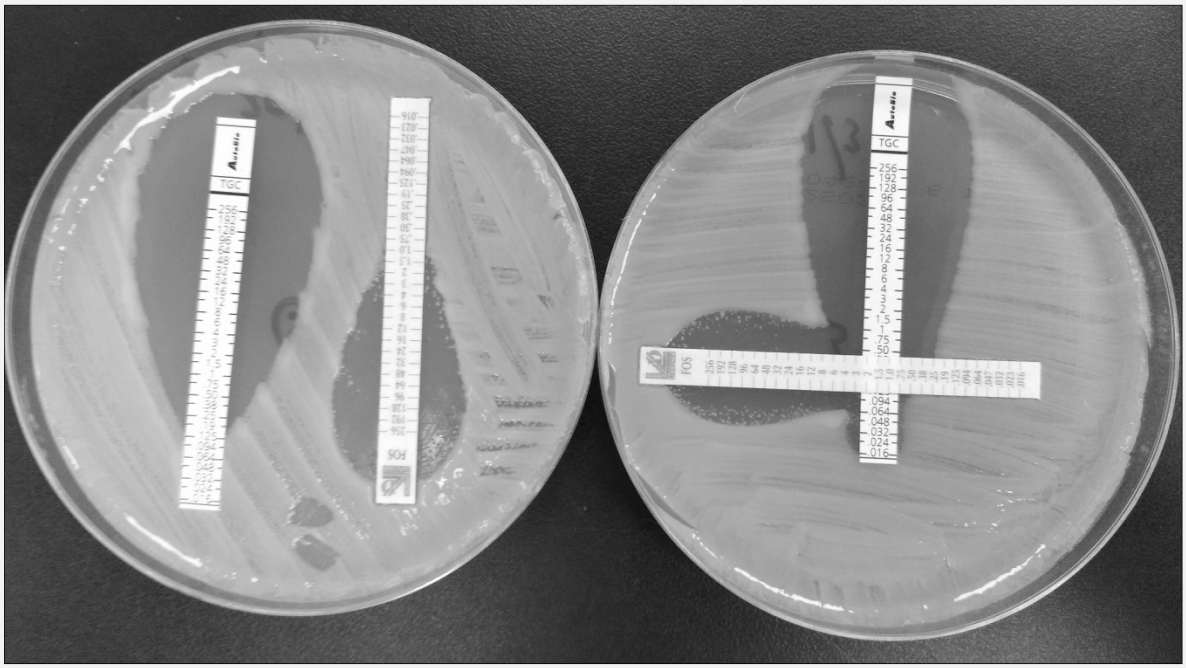

Figure 1. E-test synergy testing results of the isolate No. 14.

FICI = 0.75, indicating "additive" effect.

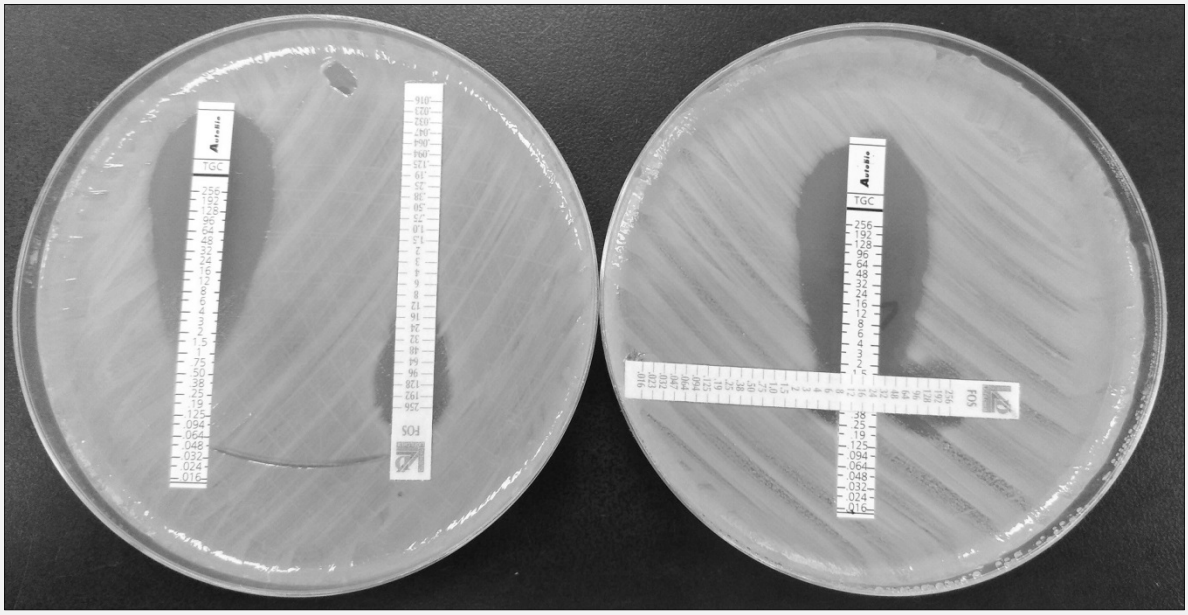

Figure 2. E-test synergy testing results of the isolate No. 15.

FICI = 0.375, indicating "synergistic" effect.

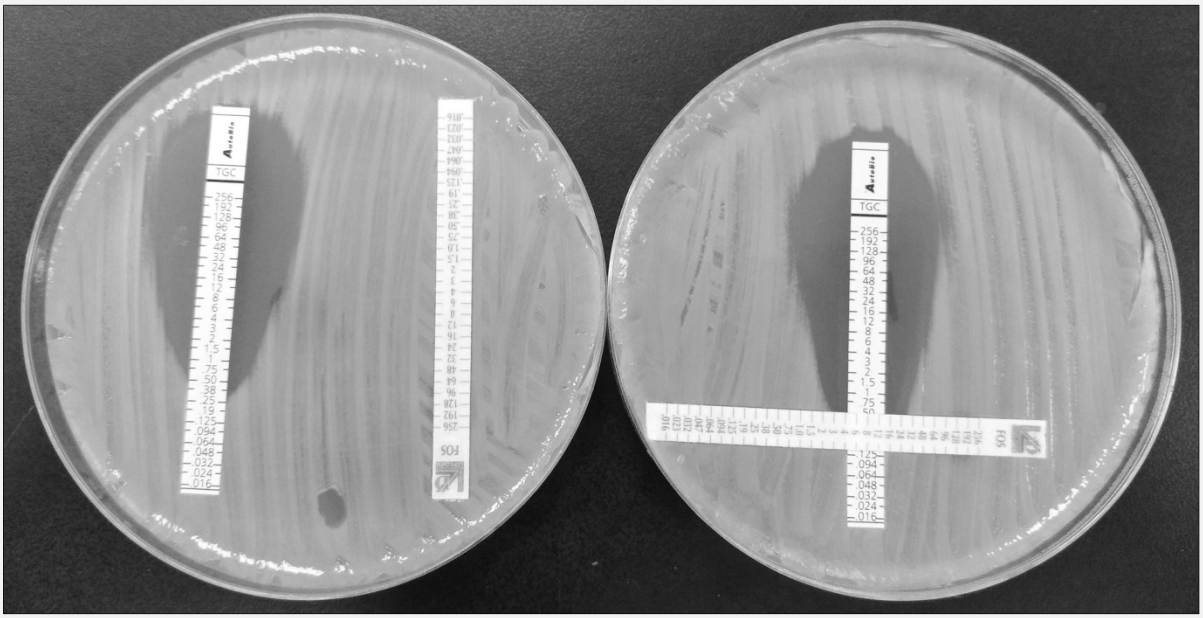

Figure 3. E-test synergy testing results of the isolate No. 17.

FICI = 1.5, indicating "indifferent" effect.

AST profile of included isolates

All 30 KPC-Kp isolates were resistant to imipenem and/or meropenem (4 ≤ MIC ≤ 128 mg/L) and multi-drug resistant or pan-drug resistant to other antibiotics. The susceptibility rates of fosfomycin and tigecycline in these KPC-Kp isolates were 36.7% (11/30) and 63.3% (19/30), respectively.

Synergy testing by E-test and microdilution checkerboard

MICs of the single drug and MICs of the two-drug combination, determined by E-test and microdilution checkerboard were all summarized in Table 1. The MICs of most isolates decreased in the combination group. The main factors (MICs of fosfomycin, tigecycline, or both decreased ≥ 4 times) in checkerboard synergy testing results were also summarized in Table 1, with 10 isolates due to fosfomycin, 4 isolates due to both fosfomycin and tigecycline, 4 isolates due to tigecycline, and 12 isolates not applicable (both fosfomycin and tigecycline decreased < 4 times). Among the 15 isolates showing synergistic and additive effects in checkerboard synergy testing, 93.3% (14/15) of the isolates were susceptible to fosfomycin, and the susceptibility rate of tigecycline increased from 46.7% (7/15) to 80.0% (12/15).

FICIs were calculated to determine the synergistic, additive, indifferent, or antagonistic effect, and showed that 13.3% - 16.7% of the isolates were synergistic, 30.0% - 36.7% were additive, and 50.0% - 53.3% were indifferent. No antagonism was found. The percentages of each effect by the two methods were summarized in Table 2. Figure 1, Figure 2, and Figure 3 were the representative isolates standing for additive, synergistic, and indifferent effect, respectively. There was no statistically significant difference between the E-test and checkerboard groups ($p > 0.05$). The overall agreement (with FICI difference ≤ 0.25 in each isolate) between the two methods was 76.7% (23/30).

DISCUSSION

KPC-Kp accounts for the largest percentage of CRE and is the fastest growing species in China [4]. Due to its resistance to carbapenems and often multidrug resistant to other antibiotics at the same time, the therapeutic options are rather limited. Various combination therapies (e.g., fosfomycin, tigecycline, colistin) are the recommended options; however, there is still no consensus [5, 6]. Colistin was not available in China mainland and could only be purchased from aboard, thus fosfomycin and tigecycline were the most used antibiotic combinations to treat KPC-Kp in China mainland.

Previous study showed fosfomycin could interfere with the initial formation of the bacterial cell wall, which made it easier for the second antibiotic (e.g., tigecy-

cline) to enter the cell and kill the bacteria. A synergy effect has been observed for the combination of fosfomycin and tigecycline [17]. The susceptibility rates of the two antibiotics varied in different studies, ranging from 8.3% to 42.8% for fosfomycin and from 58.3% to 81.6% for tigecycline [8-10]. Thus, the application of synergy testing could provide valuable information for physicians when a combination therapy was needed. Synergy testing with microdilution checkerboard is considered to be the "gold standard"; however, it is time-consuming and labor-intensive making it unsuitable for routine clinical laboratory [10]. In contrast, synergy testing by E-test method is easy to perform and to determine results with low workload, and it has a low risk of contamination. It has been used to evaluate the combination of colistin and sulbactam for multidrug resistant *Acinetobacter baumannii* compared with microdilution checkerboard [12], although some disagreement existed between the two methods. Our results showed that the overall agreement rates between the 2 methods was 76.7% ($p > 0.05$), which was higher than in a previous study [12]. Moreover, our synergy testing results showed the susceptibility rate of tigecycline increased from 46.7% (7/15) to 80.0% (12/15), indicating the therapeutical value of fosfomycin and tigecycline combination regimen. Thus, it is hopeful that E-test synergy testing has the potential to be performed routinely in clinical laboratory to help physicians to determine whether the combination is synergistic, additive, indifferent, or antagonistic. This method could also be used in other antibiotic combinations, although further studies are still needed to evaluate these combinations.

A recent study by Yu et al. [10] evaluated the combination of tigecycline and fosfomycin by checkerboard method, and found this combination was less pronounced than other combinations, with the synergistic, additive, indifferent, and antagonistic rates of 1.5%, 83.1%, 14.0%, and 1.5%, respectively. Their results were different from ours mainly in the synergistic group and additive group. This is probably due to different included isolates and geographic variation. Thus, further multi-center studies with KPC-Kp from various regions and specimen types should be conducted to verify our results.

This study had several limitations. First, this was a preliminary study with only 30 KPC-2 producing *Klebseilla pneumoniae* isolates included and the sample size was small, thus further studies with larger sample size and CRE isolates producing different carbapenemase types are still needed. Second, only fosfomycin and tigecycline were evaluated for synergy testing by E-test, other antibiotics (e.g., colistin, and amikacin) with the potential synergistic/additive effect in treating CRE should also be evaluated in our future studies.

CONCLUSION

Our findings indicated that, fosfomycin combined with tigecycline showed mainly an indifferent effect, some additive and synergistic effect, and no antagonistic effect in our included KPC-Kp isolates. The synergy testing results determined by E-test correlated well with microdilution checkerboard. Thus E-test method may have the potential to be used in routine clinical laboratory for synergy testing, as it is easier to perform and to determine results.

Ethics:
This study was approved by the ethics committee of Peking University First Hospital. Informed consent was not required, as all specimens were collected as part of routine clinical care.

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Declaration of Interest:
There is no conflict of interest.

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