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ORIGINAL RESEARCH

In vitro Combined Inhibitory Activities of β-Lactam Antibiotics and Clavulanic Acid Against *bla*$_{KPC-2}$-Positive *Klebsiella pneumoniae*

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Background: The spread of KPC-producing Enterobacteriaceae has triggered a global public health concern, with KPC-2-positive strains being the most prevalent in China. We hereby studied the in vitro combined inhibitory activities of three kinds of β-lactam antibiotics and clavulanic acid at different concentrations against *bla*$_{KPC-2}$-positive *Klebsiella pneumoniae* to explore the antimicrobial characteristics of these combinations and alternative therapeutic regimens for infections caused by *bla*$_{KPC-2}$-positive *K. pneumoniae* strains.

Materials and Methods: In this study, 153 clinically isolated *bla*$_{KPC-2}$-positive *K. pneumoniae strains* from 19 provinces in China were collected from 2016 to 2018. Antimicrobial susceptibility testing of imipenem/clavulanic acid, meropenem/clavulanic acid, ceftazidime/clavulanic acid, and each antimicrobial agent alone was performed by broth microdilution technique according to the CLSI guidelines. The concentration ratios of β-lactam antibiotics to clavulanic acid were as follows: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32. The antimicrobial susceptibility of the combinations was determined according to the breakpoints of Imipenem, meropenem, and ceftazidime established by the CLSI directives for *Enterobacteriaceae*.

Results: The MICs of all three combinations gradually declined with increments in the proportion of clavulanic acid in the regimens, and the most significant decline in the MIC_{50} and MIC_{90} was seen in combinations at the concentration ratio of 1:1 (also 1:2 for meropenem/clavulanic acid). When the concentration of clavulanic acid was restricted to 4 mg/L, the susceptibility of more than 70% of the isolates to the regimens could be restored with imipenem MIC 2–4 mg/L, meropenem MIC 2–8 mg/L or ceftazidime MIC 8mg/L. However, the percentage decreased to 30 to 40% when the initial MIC level was higher.

Conclusion: The highest combined inhibitory activity of β-lactam antibiotics/clavulanic acid at low concentration ratios against *bla*$_{KPC-2}$-positive *K. pneumoniae* may offer a new way to optimize the effects of these antimicrobial regimens.

Keywords: KPC, *Enterobacteriaceae*, combined inhibitory activity, clavulanic acid, β-lactam antibiotic

Background

Being a widely-spread class A carbapenemase, KPC has been found in a number of bacterial genera (Especially *Enterobacteriaceae*)[1–3] across many countries and regions since it was first reported in the United States of America back in 1996.[4,5] In China, after the first KPC-2-producing *Klebsiella pneumoniae* strain was reported in 2004,[6] an increasing number of KPC-positive *K. pneumoniae* have been identified in eastern provinces such as Beijing, Shanghai, and Jiangsu,[7–10]

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with KPC-2 producing strains being the most prevalent.[5] The extensive spread of KPC-positive bacteria was attributed to its location on plasmids and its preference for *K. pneumoniae* since *K. pneumoniae* tends to accumulate and transfer resistance determinants.[11–13]

KPC is notorious for its broad substrate spectrum, including penicillins, cephalosporins, carbapenems, and aztreonam.[12] Although bla_{KPC-2}-positive strains are susceptible to tigecycline and colistin in most cases, the administration of these two drugs is sometimes restricted due to safety concerns, Minimal Inhibitory Concentrations (MICs) increase during administration and high price, which is quite the ordeal for clinicians. In order to come up with optimum therapeutic regimens for bla_{KPC-2}-positive *Enterobacteriaceae*, numerous research on dual β-lactam antibiotics and combinations containing tigecycline or colistin have been carried out and successfully confirmed the lower mortality rate and higher efficiency of combination regimens compared with monotherapy.[14–20] Besides, β-lactam-antibiotic-based combinations were also found to have similar efficacy profiles to tigecycline-based therapies against KPC-producing *Klebsiella pneumoniae* with a lower mortality rate and cost, which confirms the importance and necessity of performing more research on β-lactam-antibiotic-based antimicrobial regimens.[20,21] Previous research on the in vitro combined inhibitory activities of β-lactam antibiotics and clavulanic acid at different concentrations or ratios against *Enterobacteriaceae* isolates have revealed some dose-related inhibitory effects of clavulanic acid, but the limited testing concentrations and strain differences made the results both unclear and incomplete.[22,23] In this study, we widened the testing concentration and ratio of β-lactam antibiotics/clavulanic acid to achieve more comprehensive information on the dose-related effects of β-lactam antibiotics/clavulanic acid against bla_{KPC-2}-positive *Enterobacteriaceae*.

Materials and Methods

Strains

All 153 *Enterobacteriaceae K. pneumoniae* strains were collected from 23 hospitals in 19 Chinese provinces from 2016 to 2018. The specimen sources of the isolates were as follows: sputum 70, blood 29, urine 16, abdominal fluid 6, bile 6, cerebrospinal fluid 5, and other specimen sources 21.

Gene Identification

All strains were confirmed to be bla_{KPC-2}-positive by polymerase chain reaction (PCR) and DNA sequencing. Other common β-Lactamases genes were also detected by PCR, including bla_{NDM}, bla_{IMP}, bla_{OXA}, bla_{VIM}, bla_{CTX-M} Group1, bla_{CTX-M} Group2, bla_{CTX-M} Group9, bla_{FOX}, bla_{MOX}, bla_{CMY}, bla_{DHA}, and bla_{ACC} using primers targeting different β-Lactamases gene groups (Table 1).

Antimicrobial Susceptibility Testing

MICs were determined by broth microdilution following the 2019 CLSI guidelines. The in vitro combined inhibitory activities of imipenem/clavulanic acid, meropenem/clavulanic acid, and ceftazidime/clavulanic acid were determined. The inhibitory activities of imipenem, meropenem, ceftazidime, and clavulanic acid were also tested. The ratios of β-lactam antibiotics to clavulanic acid were set as follows: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32. The doubling dilutions of the concentrations of each β-lactam antibiotic (Alone or in combination with clavulanic acid) ranged from 0.06 to 128mg/L; the testing concentration of clavulanic acid in each combination can be calculated using the combination ratio. The concentrations of clavulanic acid ranged from 0.5 to 1024mg/L. The breakpoints of imipenem, meropenem, and ceftazidime established by the CLSI for *Enterobacteriaceae* were used to evaluate the combinations' antibacterial activities: Imipenem, ≤1/≥4mg/L; meropenem, ≤1/≥4mg/L; and ceftazidime, ≤4/≥16mg/L. *E. coli* ATCC 25,922 and ATCC 35,218 were used for quality control.

Results

As expected, the MICs showed that most isolates were resistant to imipenem (MICs ranging from 2 to >128mg/L), meropenem (2 to >128mg/L), and ceftazidime (8 to >128mg/L). Nonetheless, 3 isolates were exclusively susceptible to imipenem, 3 to meropenem and 1 to ceftazidime, with 3 isolates susceptible to both imipenem and meropenem, and 1 isolate susceptible to all the three β-lactam antibiotics tested.

For all three combinations (Imipenem/clavulanic acid, meropenem/clavulanic acid, and ceftazidime/clavulanic acid), the MICs elicited a gradual decline with increments in the proportion of clavulanic acid in the regimens (Table 2). The biggest decline in MIC_{50} and MIC_{90} was depicted in combinations at low ratios. The MIC_{50} of the imipenem/clavulanic acid regimen halved from 32 to

Table 1 Primers Used for Amplification

Target(s)	Primers	Sequence (5′ to 3′)	Amplified Product (bp)	Reference
NDM	NDM-F	GGTTTGGCGATCTGGTTTTC	621	36
	NDM-R	CGGAATGGCTCATCACGATC		
VIM	VIM-F	GATGGTGTTTGGTCGCATA	390	
	VIM-R	CGAATGCGCAGCACCAG		
OXA	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
IMP	IMP-F	GGAATAGAGTGGCTTAAY*TCTC	232	
	IMP-R	GGTTTAAY*AAAACAACCACC		
KPC	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	
	KPC-R	CTTGTCATCCTTGTTAGGCG		
CTX-M Group1	CTXM1-F	GAATTAGAGCGGCAGTCGGG	588	37
	CTXM1-R	CACAACCCAGGAAGCAGGC		
CTX-M Group2	CTXM2-F	GATGGCGACGCTACCCC	107	
	CTXM2-R	CAAGCCGACCTCCCGAAC		
CTX-M Group9	CTXM9-F	GTGCAACGGATGATGTTCGC	475	
	CTXM9-R	GAAACGTCTCATCGCCGATC		
MOX-1,MOX-2,CMY-1,CMY-8 to CMY-11	MOXM-F	GCTGCTCAAGGAGCACAGGAT	520	38
	MOXM-R	CACATTGACATAGGTGTGGTGC		
DHA-1,DHA-2	DHAM-F	AACTTTCACAGGTGTGCTGGGT	405	
	DHAM-R	CCGTACGCATACTGGCTTTGC		
ACC	ACCM-F	AACAGCCTCAGCAGCCGGTTA	346	
	ACCM-R	TTCGCCGCAATCATCCCTAGC		
FOX-1 to FOX-5b	FOXM-F	AACATGGGGTATCAGGGAGATG	190	
	FOXM-R	CAAAGCGCGTAACCGGATTGG		

Note: *Y = C or T.

16 mg/L at the ratio of 1:1 compared to imipenem alone. Similarly, meropenem/clavulanic acid at the ratio 1:1 halved the MIC_{50} from 128 to 64mg/L compared to meropenem alone, and ceftazidime/clavulanic acid combination reduced the MIC_{50} from >128 to 32mg/L compared to ceftazidime alone. The MIC_{90} demonstrated the same trend; the MIC_{90} halved from 64 to 32mg/L with imipenem/clavulanic acid at the ratio of 1:1 compared to imipenem alone; meropenem/clavulanic acid at the ratio of 1:1 reduced the MIC_{90} from >128 to 128mg/L compared to meropenem alone, and ceftazidime/clavulanic acid administration reduced the MIC_{50} from >128 to 64mg/L compared to ceftazidime alone. Nevertheless, when the ratio was raised to 1:2, meropenem/clavulanic acid became the only combination whose MIC50 and MIC90 halved again; meanwhile the MIC_{50} and MIC_{90} of imipenem/clavulanic acid and ceftazidime/clavulanic acid remained unchanged. The data presented in Table 2 reveals the decline in the inhibitory activities of all three combinations with increments in the ratio of β-lactam antibiotics to clavulanic acid.

Table 2 The MIC Characteristics, Susceptibility and Resistance Rates of the KPC-Producing *Enterobacteriaceae* Strains Tested

Drug or Combination	Ratio	Criteria	MIC Variations (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Susceptibility Rate (%)	Resistance Rate (%)
Imipenem	/	≤1mg/L, susceptible; ≥4mg/L, resistant	0.125->128	32	64	2.0	97.4
Imipenem-clavulanic acid	1:1		0.125->128	16	32	5.9	81.0
	1:2		0.125->128	16	32	5.9	77.8
	1:4		0.125->128	8	16	9.8	75.2
	1:8		0.125–128	8	16	17.6	71.9
	1:16		0.125–64	8	16	22.9	71.2
	1:32		0.125–32	4	8	27.5	66.0
Meropenem	/	≤1mg/L, susceptible; ≥4mg/L, resistant	≤0.06->128	128	>128	2.0	97.4
Meropenem-clavulanic acid	1:1		≤0.06->128	64	128	7.8	81.7
	1:2		≤0.06->128	32	64	11.8	81.0
	1:4		≤0.06–128	32	64	14.4	78.4
	1:8		≤0.06–128	16	32	16.3	75.8
	1:16		≤0.06–128	16	32	19.6	73.2
	1:32		≤0.06–128	8	16	24.2	70.6
Ceftazidime	/	≤4mg/L, susceptible; ≥16mg/L, resistant	4->128	>128	>128	0.7	97.4
Ceftazidime-clavulanic acid	1:1		0.5->128	32	64	7.2	85.0
	1:2		0.5->128	32	64	9.2	77.8
	1:4		0.5->128	32	64	15.0	71.2
	1:8		0.25–128	16	32	24.8	68.6
	1:16		0.25–64	8	16	28.8	46.4
	1:32		0.25–32	8	16	42.5	15.0

Note: Data of intermediate isolates was not shown in this table.

When the concentration of clavulanic acid was restricted to 4mg/L, the susceptibility of some strains to β-lactam antibiotics with low β-lactam antibiotic MIC levels was restored (Table 3). Imipenem/clavulanic reduced the MIC of all five isolates from imipenem MICs of 2 mg/L and 4 mg/L to no more than 1 mg/L, but the rate sharply dipped to 8/24 when the imipenem MIC value rose to 8mg/L. Meropenem/clavulanic and ceftazidime/clavulanic acid delineated similar efficacy. Meropenem/clavulanic acid reduced the MIC of ten out of the thirteen isolates from meropenem MICs of 2 mg/L, 4 mg/L, and 8 mg/L to 1 mg/L or less, and was effective for approximately 40% (9/21) of the strains with meropenem MIC of 16mg/L. Ceftazidime/clavulanic acid only revealed high efficacy to isolates with ceftazidime MIC of 8mg/L, with the MIC of all three strains reduced to no more than 4 mg/L, and limited efficacy (5/15) to strains with ceftazidime MICs of 16 mg/L.

Moreover, PCR confirmed the existence of other β-lactamase genes in the isolates (Table 3). For meropenem/clavulanic acid, eight isolates regaining susceptibility contained more than one β-lactamase genes, while eleven isolates contained only bla_{KPC-2}; meanwhile, six isolates failing to regain susceptibility contained at least two β-lactamase genes, while nine isolates contained only

Table 3 The β-Lactam Antibiotic MIC Values and β-Lactamase Genes of Part of the Isolates Tested (Clavulanic Acid ≤4mg/L)

Combination	MIC of β-Lactam Antibiotic(mg/L)	Efficacy*	Isolates Regaining Susceptibility		Isolates Failing to Regain Susceptibility	
			bla$_{KPC-2}$ Only	*bla*$_{KPC-2}$ Along with Other Genes**	*bla*$_{KPC-2}$ Only	*bla*$_{KPC-2}$ Along with Other Genes
Imipenem/clavulanic acid	2	1/1	8	5	8	8
	4	4/4				
	8	8/24				
Meropenem/clavulanic acid	2	0/1	11	8	9	6
	4	3/3				
	8	7/9				
	16	9/21				
Ceftazidime/clavulanic acid	8	3/3	3	5	7	3
	16	5/15				
Total	/	19/39	11	8	11	9

Notes: *Efficacy=the number of the isolates regaining susceptibility/the total number of the isolates with β-lactam antibiotic MIC value shown in the second column. **the gene combinations include the following: *bla*$_{KPC-2}$+*bla*$_{CTX-M}$-Group1, *bla*$_{KPC-2}$+*bla*$_{CTX-M}$-Group1+ *bla*$_{CTX-M}$-Group9, *bla*$_{KPC-2}$+*bla*$_{CTX-M}$-Group1+*bla*$_{DHA}$, *bla*$_{KPC-2}$+ *bla*$_{CTX-M}$-Group9, *bla*$_{KPC-2}$+ *bla*$_{DHA}$, *bla*$_{KPC-2}$+ *bla*$_{DHA}$+ *bla*$_{CTX-M}$-Group9, *bla*$_{KPC-2}$+ *bla*$_{DHA}$+ *bla*$_{NDM}$.

bla$_{KPC-2}$. Imipenem/clavulanic acid displayed a similar trend. However, ceftazidime/clavulanic acid illustrated different characteristics than the other two groups; more isolates regaining susceptibility to ceftazidime/clavulanic acid carried two or more β-lactamase genes. For all three combinations, eleven out of twenty two isolates containing only *bla*$_{KPC-2}$ with low MICs regained susceptibility to the combinations, while eight out of seventeen isolates carrying two or more β-lactamase genes regained susceptibility to the combinations.

Discussion

The spread of carbapenemase-producing *K.pneumoniae* has aroused worldwide concerns. Researchers have carried out numerous studies to identify alternative therapeutic regimens since colistin and/or tigecycline-resistant strains have been reported and will probably become more popular with the frequent prescription of these antibiotics.[24–26] KPC-2 has become the most prevalent class A carbapenemase amongst *bla*$_{KPC}$-positive *Enterobacteriaceae*, which makes finding optimal therapeutic regimens against *bla*$_{KPC-2}$-carriers more crucial than ever.

As a member of class A carbapenemases, KPCs are inhibited by clavulanic acid and tazobactam.[13] Some research on the in vitro combined inhibitory activity of β-lactam antibiotics combined with clavulanic acid at different concentrations or ratios against *K.pneumoniae* and *Escherichia coli* isolates have revealed some dose-related inhibitory effects of clavulanic acid. The 1:1 ratio was proven to be more effective than the 3:1 ratio against *bla*$_{TEM}$-positive *Escherichia coli* when imipenem was combined with clavulanic acid.[22] Another research also reported that a ten-fold increase in the concentration of clavulanic acid can raise the efficacy of β-lactam antibiotics/clavulanic acid combinations against *K.pneumoniae* and *E. coli*.[23] Therefore, in order to obtain more comprehensive information on the dose-related effects of β-lactam antibiotics/clavulanic acid regimens, we chose to widen the testing concentration range. Furthermore, the concentration of the clavulanic acid when combined with β-lactam antibiotics was set at a maximum of 4mg/L in most in vitro antimicrobial susceptibility tests, which is close to the mean peak serum concentration when administrated at 125 mg to healthy volunteers according to the literature.[27,28] We also evaluated the efficacy of all three combinations when clavulanic acid concentration was less than 4 mg/L as a reference for β-lactam-antibiotic-containing therapy against *bla*$_{KPC-2}$-positive *Enterobacteriaceae*.

The dose-dependent antimicrobial effect of all combinations tested was obvious from the gradually declining MIC_{50} and MIC_{90} with increments in combination ratios (Higher

clavulanic acid proportions). As a classical β-lactamase inhibitor, clavulanic acid strongly binds β-lactamases and turns inactive in the process to protect β-lactam antibiotics from being hydrolyzed, which can perfectly rationalize the dose-dependent phenomenon. Notwithstanding, the biggest and steepest decline in the MIC_{50} and MIC_{90} was observed at the ratio of 1:1 in all three combinations; in meropenem/clavulanic acid the ratio of 1:2 was equally efficient. This finding implied the following: 1) There was a slight difference in terms of the most efficient ratio of β-lactam antibiotics/clavulanic acid for different β-lactam antibiotics. 2) There may be a new insight to optimize the therapeutic effects of β-lactamase/clavulanic acid. The β-lactamase/clavulanic acid in wide use now contains limited proportions of clavulanic acid and a small rise in its dose could be both sensible and helpful. Despite the fact that some case reports have indicated the higher incidence of drug-induced hepatitis, pancreatitis, and other gastrointestinal adverse effects caused by amoxicillin/clavulanic acid compared to amoxicillin monotherapy,[29–31] information on the toxicity profile of clavulanic acid monotherapy or with other β-lactam antibiotics is still vacant. Studies on healthy volunteers have shown that the serum concentration of clavulanic acid is maintained above 3 mg/L for around one hour and above 2 mg/L for approximately two hours after oral ingestion of 125 mg clavulanic acid.[27,28] Another one-week study on ICU patients showed that the serum concentration of clavulanic acid can be kept above 10 mg/L when administrated at a high dose with amoxicillin.[32] Highly variable pharmacokinetics of clavulanic acid were reported in some research articles.[33,34] Further studies on the combined inhibitory activities, pharmacokinetics, and safety of β-lactam antibiotics/clavulanic acid are essential to determine the optimal combination of β-lactam antibiotics/clavulanic acid and the best combination ratio for treatment of infections.

Some research articles recommend that clinicians should take the MIC levels of carbapenems into consideration when developing treatment plans. A study in Greece found that for patients with carbapenemase-producing *K. pneumoniae* bloodstream infections, the mortality rate was only 19.3% when carbapenems MICs ≤8mg/L, which is much lower than the mortality rate of 35.5% when carbapenems MICs >8mg/L, if treated by carbapenem-containing combinations.[20] A review including 63 studies in four continents (most studies were in the United States and Europe) suggested that for patients infected with KPC-producing *Klebsiella pneumoniae* with meropenem MICs lower than 16mg/L, carbapenem-containing regimens can be administrated in high-dosed, prolonged infusions under therapeutic drug monitoring (TDM), whereas the usage of carbapenems should be avoided for strains with meropenem MICs higher than 16mg/L.[35] In this study, we collected 153 clinical isolates from 19 Chinese provinces and discovered that when the concentration of clavulanic acid was restricted to 4 mg/L, the susceptibility of more than 70% of the isolates with imipenem MICs ranging from 2 to 4 mg/L, meropenem MIC levels of 2–8 mg/L or ceftazidime MIC levels of 8 mg/L can be restored; Contrastingly, the percentage plummeted to 30–40% when the β-lactam MIC level of the isolates continued to increase. This suggests that more studies on β-lactam-antibiotic-containing treatment regimens against bla_{KPC}-carrying *Enterobacteriaceae* should be conducted to determine if administration of combination regimens is applicable to strains with lower initial β-lactam antibiotic MICs in China compared to other regions.

The results of antimicrobial susceptibility testing corroborated the escalating additive inhibitory effects of the three combinations of β-lactam antibiotics/clavulanic acid evaluated in this study, produced by increments in the proportion of clavulanic acid in the regimens. With all ratios included, ceftazidime/clavulanic acid impressively got the most significant efficacy against both *K. pneumoniae* and non-*K. pneumoniae*. A study reported a similar phenomenon stating that the MIC of cefotaxime reduced more obviously than that of imipenem when combined with the same concentration of inhibitors against KPC-2-producing *Enterobacteriaceae*, which was explained by the lower k_{cat}/K_m ratio of cefotaxime than imipenem according to the KPC-2 kinetic parameters.[17] The k_{cat}/K_m ratio is used to evaluate the catalytic efficacy of enzymes for substrates. Furthermore, the k_{cat}/K_m ratio of ceftazidime was proven to be lower than those of both imipenem and meropenem for KPC-2.[12] However, this cannot perfectly explain the results obtained in our study, because imipenem holds the highest k_{cat}/K_m ratio whereas the MICs of meropenem reduced least. Further studies need to be carried out to find more about the catalytic efficacy of KPC for β-lactam antibiotics under the protection of inhibitors.

The same number of isolates carrying only bla_{KPC-2} succeeded or failed in restoring susceptibility to the combination regimens when the concentration of clavulanic acid was 4 mg/L or less. A similar phenomenon was observed in isolates carrying two or more β-lactamase

genes. More types of β-lactamase genes and isolates should be tested to confirm whether the inhibitory activities of the combination regimens are independent of the β-lactamase genes for isolates with low β-lactam MICs.

In conclusion, the most prominent efficacy of β-lactam antibiotic/clavulanic acid at low ratios towards *bla*$_{KPC-2}$-positive *Enterobacteriaceae* may offer a new insight to optimize the effect of β-lactam antibiotics/clavulanic acid combination regimens. Additionally, clinicians in China should consider the possibility that β-lactam-antibiotic-containing treatment regimens against *bla*$_{KPC-2}$-positive *Enterobacteriaceae* infections are feasible for strains with lower β-lactam MICs in China compared to other regions.

Compliance with Ethical Standards

The study is conducted on already available data. Ethical approval was approved by the Institutional Review Board of Huashan Hospital, Fudan University.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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