

# In vitro Effect of the Combination of Aztreonam and Amoxicillin/Clavulanic Acid Against Carbapenem-Resistant Gram-Negative Organisms Producing Metallo- $\beta$ -Lactamase

This article was published in the following Dove Press journal:  
Infection and Drug Resistance

Qian Xu<sup>1,2,\*</sup>

Ying Fu<sup>3,\*</sup>

Jingshu Ji<sup>3</sup>

Xiaoxing Du<sup>1,2</sup>

Yunsong Yu<sup>1,2</sup>

<sup>1</sup>Department of Infectious Diseases, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, 310016, People's Republic of China; <sup>2</sup>Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, Zhejiang Province, 310016, People's Republic of China; <sup>3</sup>Department of Clinical Laboratory, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, 310016, People's Republic of China

\*These authors contributed equally to this work

**Introduction:** Antibiotics for treating infectious diseases caused by carbapenem-resistant Gram-negative pathogens (CR-GNOs) are very limited in clinical practice. We aim to provide supportive evidence by revealing the combined effect of aztreonam (ATM) and amoxicillin/clavulanic acid (AMC) against GNOs with carbapenem resistance mediated by metallo- $\beta$ -lactamase (MBL).

**Methods:** All isolates were identified by the VITEK system and EDTA inhibitory assays. PCR followed by sequencing was conducted to confirm the genotypes of MBL and extended spectrum  $\beta$ -lactamase (ESBL). Time kill assay was performed to clarify the bactericidal effect of drug combination.

**Results:** A total of 59 MBL-producing CR-GNOs (33 *Enterobacteriaceae* spp. isolates and 26 Pseudomonadales isolates) were identified and there found three MBL genes, namely, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub> and *bla*<sub>VIM</sub>, with ratios of 76.2%, 11.8% and 11.8%, respectively. The *Enterobacteriaceae* spp. isolates were commonly positive for the ESBL genes, including *bla*<sub>TEM</sub> (18 isolates), *bla*<sub>SHV</sub> (20 isolates) and *bla*<sub>CTX-M-1</sub> (8 isolates), while the *P. aeruginosa* isolates were positive for *bla*<sub>OXA-10</sub> (11 isolates). The checkerboard microdilution assay was used to detect combination effect of ATM and AMC, which showed synergy (97.0%) and partial synergy (3.0%) in *Enterobacteriaceae* spp. isolates, and partial synergy (42.3%) and indifference (34.6%) in the Pseudomonadales isolates. Four *Enterobacteriaceae* spp. isolates were selected for a time-kill assay, and rapid bactericidal effects were observed in the combination groups compared to the control and mono-ATM groups; these effects began in the first hour and continued to the sixth hour, yielding a 5- to 7-fold reduction in Log<sub>10</sub> CFU/mL.

**Discussion:** The combination of ATM and AMC would be an available option to control infections caused by MBL-producing CR-GNOs, especially *Enterobacteriaceae* spp. isolates that coproduce ESBLs, and exhibit significant synergic effects in vitro.

**Keywords:** gram-negative pathogens, carbapenem resistance, metallo- $\beta$ -lactamase, extended spectrum  $\beta$ -lactamase, aztreonam, amoxicillin/clavulanic acid, combination

## Introduction

The production of metallo- $\beta$ -lactamase (MBL) is an important mechanism of carbapenem resistance in Gram-negative pathogens (GNOs).<sup>1</sup> It has placed significant burden on healthcare system since infections caused by MBL-producing GNOs (MBL-GNOs) have distributed globally during the last two decades and are always associated with increased

Correspondence: Yunsong Yu  
Qingchun East Road No. 3, Hangzhou  
City, Zhejiang Province, 310016, People's  
Republic of China  
Tel/Fax +86-571-86006142  
Email yys119@zju.edu.cn

morbidity and mortality.<sup>2</sup> The human public health is seriously threatened due to very few effective antibiotics that were available for clinical treatment.<sup>3</sup>

Although MBL is capable of inhibiting a broad range of  $\beta$ -lactams, including penicillin, cephalosporins and carbapenems, it lacks the ability to hydrolyse monobactams, such as aztreonam (ATM).<sup>4</sup> Therefore, antibiotic treatments based on ATM are considered a useful option for controlling GNOs that produce MBL.<sup>5</sup> However, it is not suggested that ATM be used alone in clinical practice because of the complexity of resistance mechanisms in CR-GNOs. Increasing evidence has shown that resistance mechanisms, such as extended spectrum  $\beta$ -lactamases (ESBLs) and efflux pumps, that would directly lead to the failure of ATM widely exist in clinical CR-GNOs.<sup>6</sup> Recently, ATM combined with  $\beta$ -lactam/ $\beta$ -lactamase inhibitors (BL/BLIs) is becoming a prospective choice for treatment.<sup>7</sup>

The combination of ATM and BLIs or BL/BLIs has generated more interest in the medical community; among these inhibitors, ceftazidime/avibactam and amoxicillin/clavulanic acid (AMC) are the most promising options.<sup>6,8</sup> It was reported that ATM combined with ceftazidime/avibactam or AMC could be an attractive but unconventional treatment for infections with MBL-producing *Enterobacteriaceae*.<sup>8,9</sup> Nevertheless, compared to ceftazidime/avibactam, AMC is less expensive, which contributes to the relief of the economic burden.<sup>10,11</sup> To provide more evidence for clinical treatment in our country, we study multiple species of CR-GNOs that produce MBL that were isolated from different hospitals and evaluate the combined effect of ATM and AMC in vitro by using the checkerboard microdilution method and time kill assays.

## Materials and Methods

### Species Identification and Antimicrobial Susceptibility Test

Carbapenem-resistant Gram-negative bacteria were collected from several tertiary hospitals in China between 2011 and 2016. The VITEK system (bioMérieux, France) was used for species identification and antimicrobial susceptibility testing (AST). The minimal inhibitory concentrations (MICs) of meropenem, aztreonam (Sigma Aldrich, USA) and amoxicillin/clavulanic acid (4:1, Sigma Aldrich, USA) were determined with the broth microdilution method according to the standard procedure of the Clinical & Laboratory Standards Institute (CLSI) guideline.<sup>12</sup> Since the ratio of amoxicillin and clavulanic acid in our study was different from that recommended by

the CLSI, the MIC of AMC was recalculated according to the 4:1 ratio, and the MICs of both amoxicillin and clavulanic acid were obtained. The breakpoint of amoxicillin in AMC in the CLSI was used to explain the result of AMC by using the MIC of amoxicillin recalculated as objectives. Considering no interpretation breakpoint of *P. aeruginosa* to AMC and of *A. baumannii* to ATM and AMC in the CLSI guideline, the breakpoint of *P. aeruginosa* to ATM was used to explain the AST of *A. baumannii*, while the breakpoint of *Enterobacteriaceae* spp. to AMC was used for both *P. aeruginosa* and *A. baumannii*.<sup>12</sup>

### Phenotypic Detection of MBLs and ESBLs

The MBL phenotype was confirmed by EDTA inhibitory assay, and a  $\geq 5$  mm increase in the zone diameter for meropenem vs the zone diameter for meropenem plus EDTA demonstrated MBL positivity.<sup>13</sup> The phenotypic detection of ESBL production was detected following the procedures of the double-disk synergy test (DDST).<sup>14</sup>

### Resistance Genes Encoding MBLs and ESBLs

Carbapenem-resistance genes, including *bla*<sub>NDM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SIM</sub> and *bla*<sub>SPM</sub>, were screened by PCR and sequencing.<sup>15,16</sup> The genotypes of the ESBL families, such as *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-1</sub> group, *bla*<sub>CTX-M-2</sub> group, *bla*<sub>CTX-M-8</sub> group, *bla*<sub>CTX-M-9</sub> group, *bla*<sub>OXA-10</sub> and *bla*<sub>PER</sub>, were confirmed by PCR and the sequencing primers listed in [Supplemental Table 1](#).

### Checkerboard Microdilution Assay

The checkerboard microdilution assay was used to determine the combined effect of ATM and AMC in vitro against isolates that produce MBL according to a previous study.<sup>17</sup> The fractional inhibitory concentration index (FICI) was calculated, and the interpretation was proposed as follows: FICI  $\leq 0.5$ , synergy;  $>0.5$  to  $<1$ , partial synergy; 1, additive;  $>1$  to  $<4$ , indifference; and  $\geq 4$ , antagonism.

### Time-Kill Assay

Four isolates of MBL-producing *Enterobacteriaceae* spp., including three *K. pneumoniae* (Kpn1, Kpn6 and Kpn8) and one *E. cloacae* (Ecl6), were selected for the time-kill assay. The bactericidal activity of the ATM and AMC combination in vitro was tested as previously reported with minor modifications.<sup>18</sup> Briefly, bacteria were shaken in cation-adjusted Mueller-Hinton broth for 2–4 hours until the

OD<sub>600nm</sub> reached 0.6~0.8. Then, the cell density was adjusted to 0.5 McFarland standard (approximately 10<sup>8</sup> CFU/mL) and then diluted 100 times with cation-adjusted Mueller Hinton broth until the initial inoculum density was theoretically approximately 5×10<sup>6</sup> CFU/mL. The bacterial numbers (CFU/mL) were counted on Luria-Bertani agar plates at the 0, 1, 2, 4, 6 and 24 hour time points after shaking. Each isolate was tested in four replicates, and each experiment included five tested groups with different antibiotic combinations, including the control group, 1× MIC ATM group, 1× MIC ATM plus 1/2× MIC AMC group, 1× MIC ATM plus 1× MIC AMC group, and 1× MIC ATM plus 2× MIC AMC group. When the colonies were below the limit of detection, a minimal value of nine was used.

## Results and Discussion

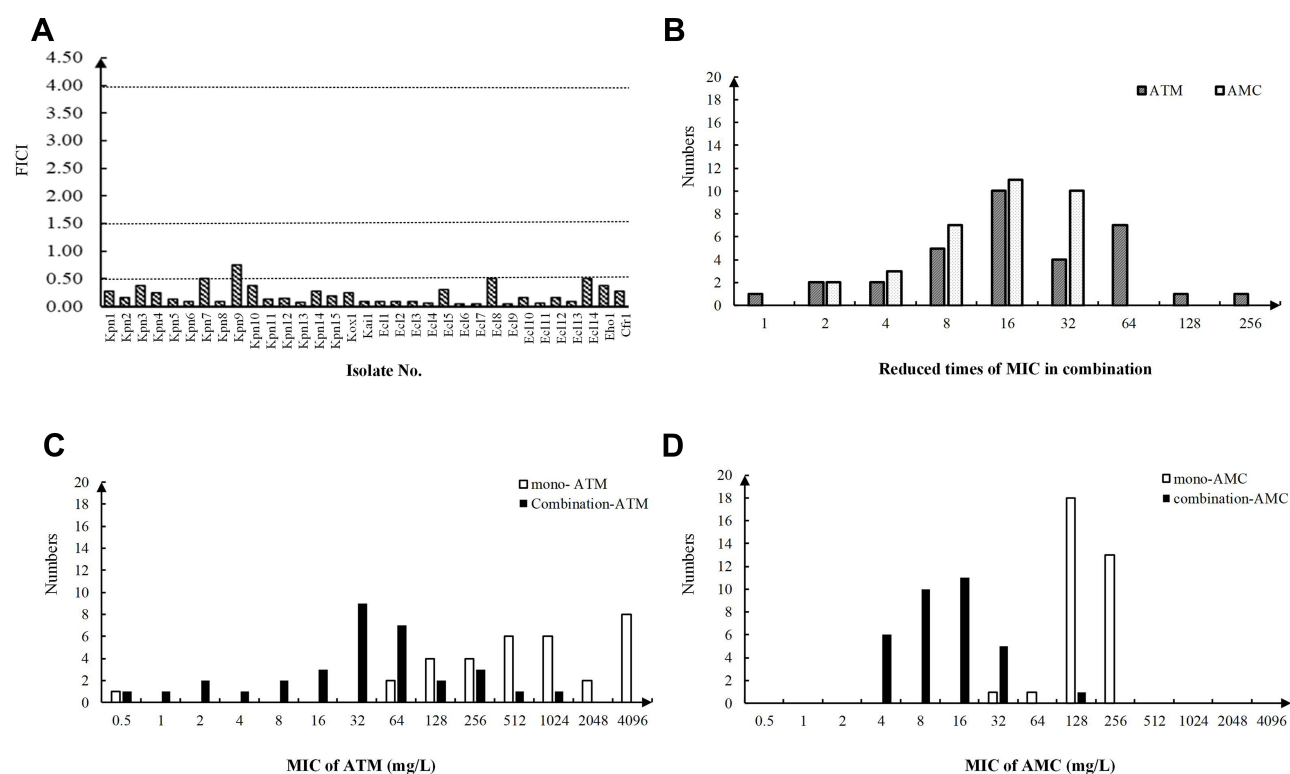
MBLs are one of the most dominant mechanism of carbapenem resistance and can be produced by diverse species of Gram-negative organisms (GNOs), such as *Enterobacteriaceae* spp. (especially *Klebsiella pneumoniae* and *Escherichia coli*), *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.<sup>4,19–21</sup> We acquired a total of 59 MBL-positive GNOs, including 33 *Enterobacteriaceae* spp. (15 *K. pneumoniae*, one *K. oxytoca*, one *K. oxytoca*, 14 *Enterobacter cloacae*, one *Enterobacter hormaechei* and one *Citrobacter freundii*), 22 *P. aeruginosa* and 4 *A. baumannii* isolates in this study (Supplemental Table 2 and Supplemental Figure 1), and we found that *K. pneumoniae*, *E. cloacae* and *P. aeruginosa* were the predominant hosts that harbour MBL genes; among these species, *E. cloacae* was not commonly reported.<sup>22</sup>

To date, over ten types of MBLs have been reported, among which New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and Imipenemase (IMP) are the top three ones throughout the world.<sup>4,15,21,23,24</sup> We first screened the MBL genes by PCR and observed that 45 isolates were positive for *bla*<sub>IMP</sub> (76.2%), 7 for *bla*<sub>VIM</sub> (11.9%) and 7 for *bla*<sub>NDM</sub> (11.9%) (Table 1). Further sequencing results confirmed that *bla*<sub>IMP</sub> have five different genotypes, namely, *bla*<sub>IMP-4</sub>, *bla*<sub>IMP-25</sub>, *bla*<sub>IMP-26</sub>, *bla*<sub>IMP-69</sub> and *bla*<sub>IMP-45</sub>, and *bla*<sub>VIM</sub> have two genotypes, namely, *bla*<sub>VIM-36</sub> and *bla*<sub>VIM-2</sub>, while *bla*<sub>NDM-1</sub> was the only genotype of *bla*<sub>NDM</sub> (Supplemental Figure 1). *bla*<sub>IMP-4</sub> and *bla*<sub>IMP-25</sub> were the most dominant MBL genes in clinical isolates of *Enterobacteriaceae* spp. and *P. aeruginosa*, respectively.

ATM is thought to be an effective antimicrobial agent against MBL-GNOs, but it remains controversial to use ATM

**Table 1** Characteristics of the Drug Resistance Genes and Results of 59 MBL-Producing GNOs in the Checkerboard Microdilution Assay [Number (Rate%)]

Group	Number	MBL Genes			ESBL Genes						FICI Interpretation			
		<i>bla</i> <sub>NDM</sub>	<i>bla</i> <sub>IMP</sub>	<i>bla</i> <sub>VIM</sub>	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>SHV</sub>	<i>bla</i> <sub>CTX-M-1</sub> Group	<i>bla</i> <sub>CTX-M-9</sub> Group	<i>bla</i> <sub>OXA-10</sub>	<i>bla</i> <sub>PER</sub>	Synergy	Partial Synergy	Additive	Indifference
Total	59	7 (11.9%)	45 (76.2%)	7 (11.9%)	18 (30.5%)	20 (33.9%)	8 (13.6%)	3 (5.1%)	11 (18.6%)	2 (3.4%)	34 (57.6%)	12 (20.3%)	4 (6.7%)	9 (15.3%)
<i>Enterobacteriaceae</i>	33	3 (9.1%)	30 (90.9%)	0 (0%)	18 (54.5%)	20 (60.6%)	8 (24.2%)	3 (90.9%)	0 (0%)	0 (0%)	32 (97.0%)	1 (3.0%)	0 (0%)	0 (0%)
<i>Pseudomonadales</i>	26	4 (15.4%)	15 (57.7%)	7 (26.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	11 (42.3%)	2 (7.7%)	2 (7.7%)	11 (42.3%)	4 (15.4%)	9 (34.6%)



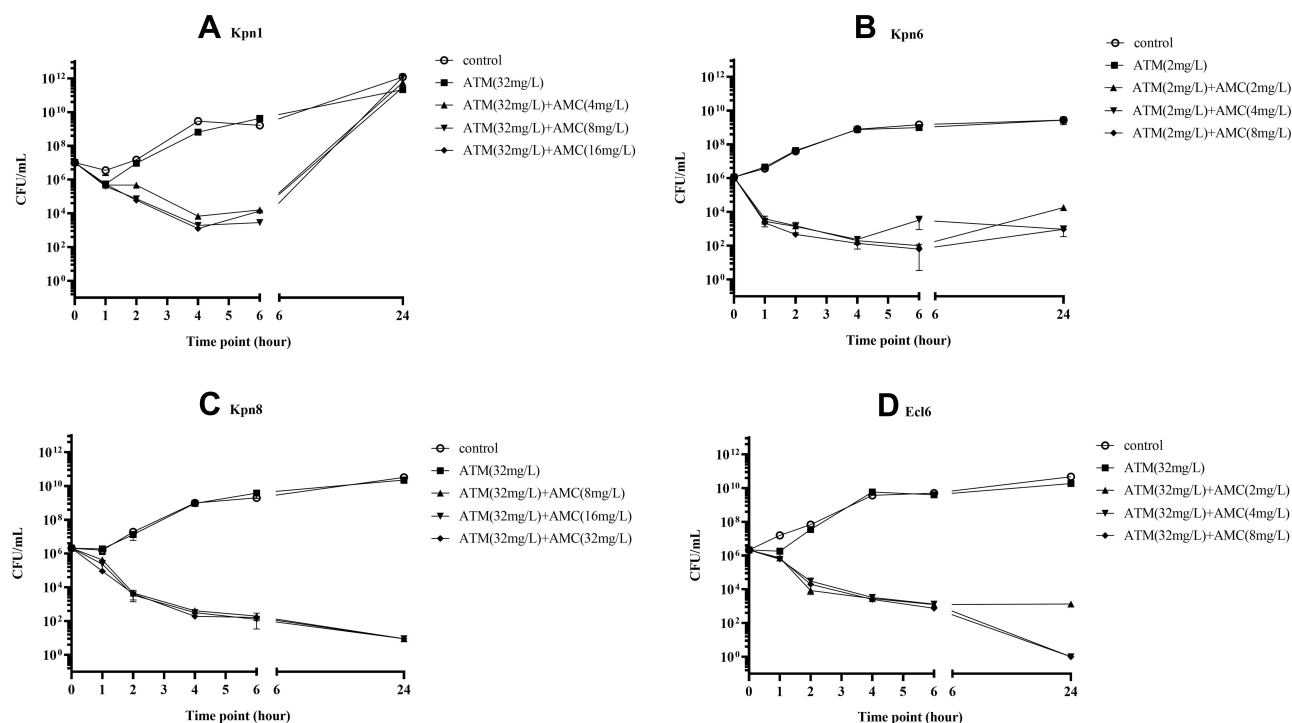
**Figure 1** Results of the *Enterobacteriaceae* isolates in the checkerboard microdilution assay. **(A)** FICI values of *Enterobacteriaceae* isolates. The interpretation of the FICI was as follows:  $\leq 0.5$ , synergy;  $>0.5$  to  $<1$ , partial synergy;  $=1$ , additive;  $>1$  to  $<4$ , indifference. The dashed line is the line that divides the different interpretations. **(B)** Reduced times of the MIC<sub>ATM</sub> and MIC<sub>AMC</sub> in combination compared to the mono-antibiotic treatments. **(C)** MIC range of aztreonam in both the mono-ATM group and the combination group. **(D)** MIC range of amoxicillin/clavulanic acid both in the mono-AMC group and in the combination group.

alone because an increasing number of clinical isolates show resistance.<sup>25</sup> All of our strains except for Kpn9 were resistant to ATM, with MICs ranging from 64 mg/L to 4096 mg/L for *Enterobacteriaceae* spp. isolates and from 8 mg/L to 256 mg/L for Pseudomonadales isolates ([Supplemental Table 2](#)). Similar to ATM, AMC is also ineffective in most cases, with MICs ranging from 32 mg/L to 256 mg/L for *Enterobacteriaceae* and from 64 mg/L to 2048 mg/L for Pseudomonadales ([Supplemental Table 2](#)). Our results, as well as those of other studies, suggested that most of the MBL-producing GNOs have developed potential mechanism to destroy ATM and render it useless when it was used as monotherapy. As a result, producing ESBLs was one of the most important mechanism<sup>22</sup>

According to a recent report in China, the positive ratio of ESBL genes, including *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub>, was approximately 88.2% in CRE isolates.<sup>21</sup> Taking the epidemic genotypes of ESBLs in our country into consideration, we screened six types of ESBL genes in *Enterobacteriaceae* isolates and *Pseudomonadales* isolates. There were four ESBL genotypes identified in 25 *Enterobacteriaceae* isolates with rates of 54.5% (*bla*<sub>TEM</sub>), 60.6% (*bla*<sub>SHV</sub>), 24.2% (*bla*<sub>CTX-M-1</sub>)

group) and 90.9% (*bla*<sub>CTX-M-9</sub> group), and 12 Pseudomonadales isolates harboured *bla*<sub>OXA-10</sub> or *bla*<sub>PER</sub> (Table 1, Supplemental Table 2).

AMC has been widely used in clinical practice and it is active against GNOs that produce ESBLs or AmpC  $\beta$ -lactamase due to the presence of clavulanic acid.<sup>26</sup> Here, we combined AMC with ATM and used checkerboard microdilution assay to evaluate the effect of ATM and AMC in vitro with the fractional inhibitory concentration index (FICI). It was shown that the combination was synergistic against 32 *Enterobacteriaceae* isolates (97%), with one exception against which the combination exhibited partial synergy (3%) (Table 1, Figure 1A). Interestingly, the combination led to a significant MIC reduction in both ATM and AMC (Figure 1B). Compared to mono-antibiotic, the MICs of both ATM and AMC in combination generally decreased by 4- to 64-fold and shifted entirely to the left lower MIC side (Figure 1B–D). The susceptibilities of ATM and AMC were restored in 4 (12.5%) and 16 (50%) isolates of the 32 *Enterobacteriaceae* isolates, respectively (Supplemental Table 2). Then, four isolates (Kpn1, Kpn6, Kpn8 and Ecl6) were selected to determine the bactericidal effect of the combination, and a more rapid



**Figure 2** Time-kill curves of four representative *Enterobacteriaceae* isolates. (A) Isolate Kpn1; (B) isolate Kpn6; (C) isolate Kpn8; (D) isolate Ecl6. Each isolate was tested by five technical replicates and four biological replicates.

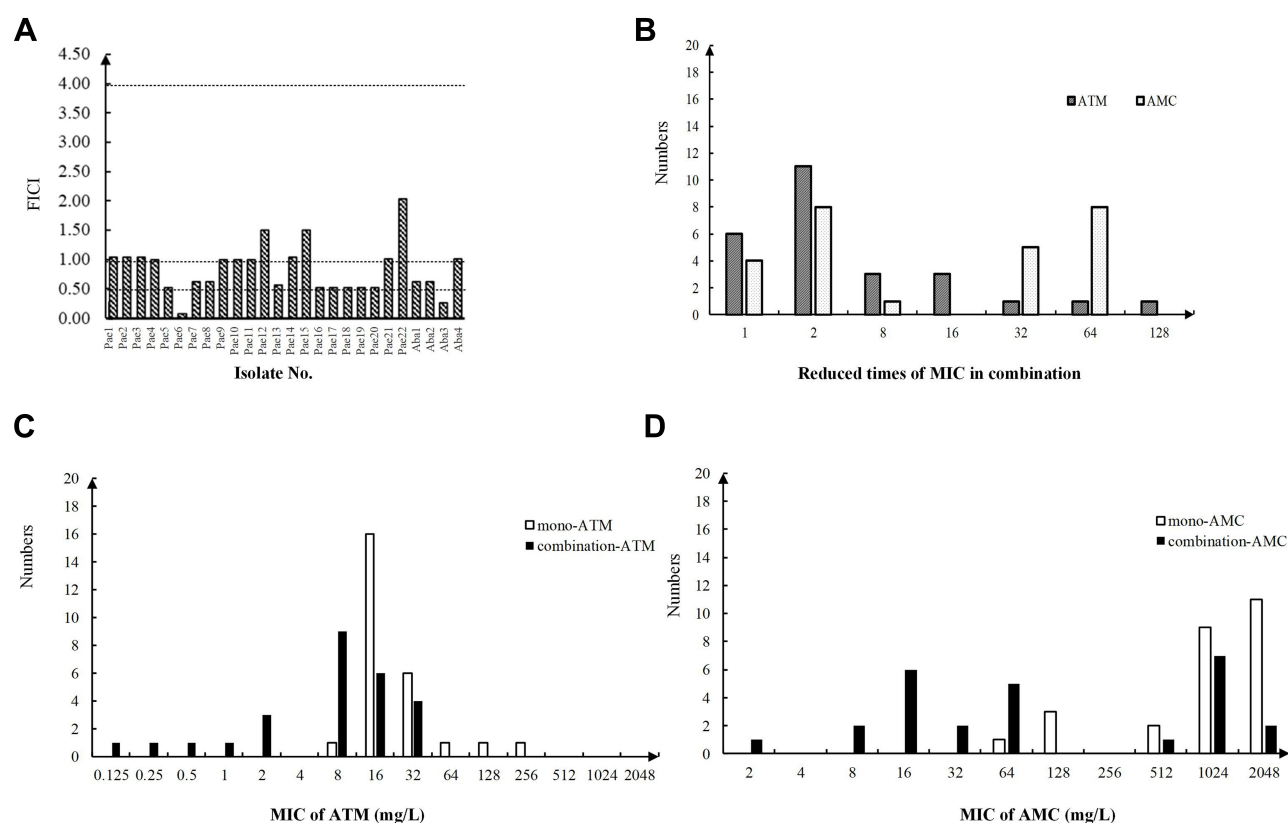
bactericidal effect was observed in the three combination groups than in the control group and mono-ATM group; this effect appeared in the first hour and led to a 5- to 7-fold reduction in Log<sub>10</sub> (CFU/mL) at the 6 hour time-point in the time-kill assay (Figure 2). In summary, the results confirmed that the combination of ATM and AMC was efficient against *Enterobacteriaceae* isolates that coproduce MBL and ESBLs.<sup>8,11</sup>

*P. aeruginosa* and *A. baumannii* were the two species of Pseudomonadales isolates that harboured MBL genes in our research, and both of these species were thought to have complex and different mechanisms of resistance to AMC, such as  $\beta$ -lactamases and efflux pumps.<sup>27,28</sup> In this study, *bla*<sub>OXA-10</sub> and/or *bla*<sub>PER</sub> were the main ESBL genes harboured by the 13 *P. aeruginosa* isolates. The effects of the combined treatment of partial synergy and indifference were commonly observed against *P. aeruginosa* with ratios of 40.9% (9 isolates) and 36.4% (8 isolates), respectively. In addition, among four *A. baumannii* isolates, the combined treatment caused a synergistic effect against only one isolate, a partial synergistic effect against two isolates and an indifference effect against one isolate (Supplemental Table 2, Figure 3A). Compared to that of mono-antibiotic against Pseudomonadales isolates, the MICs of the ATM and AMC combination presented lower values in most cases, with the MIC decreasing to over two

dilution times (Figure 3B–D). The susceptibilities to ATM or AMC in combination were restored in 7 *P. aeruginosa* isolates (7/22, 38.2%) and 3 *A. baumannii* isolates (3/4, 75%) (Supplemental Table 2). Since the antimicrobial resistance due to multidrug efflux pumps was a major problem in Pseudomonadales isolates, the pump inhibitors might have a strong potential in application.<sup>29,30</sup> In addition, the new molecules extracted from nature also showed power in antimicrobial activity including *P. aeruginosa*.<sup>31–35</sup>

In general, we provide evidence that the combination of ATM and AMC presents encouraging effects against clinical MBL-GNOs and might be a promising option to prevent the progression of drug resistance and to protect the use of ATM in the clinic. However, our results have some limitations that need to be considered. First, the conclusion of our research is based on an in vitro model, while the situation in vivo would be very different, since ATM and AMC have different PK/PD parameters. Thus, more evidence to determine the appropriate ratio of ATM and AMC and more clinical experiences are still needed in the future. Second, AMC is weaker than ceftazidime/avibactam and meropenem/vaborbactam in terms of bactericidal effects both in vivo and in vitro. Therefore, patients who are suffering from severe infections, such as bloodstream infection, would obtain more benefits with those new agents.





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