Original Article

**In vitro** efficacy of meropenem in combination with colistin, ampicillin-sulbactam against multidrug-resistant blaNDM-1-positive *acinetobacter baumannii* strains

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**Abstract:** Multidrug-resistant *Acinetobacter baumannii* strains increase the mortality rate of patient after surgery. Evidences have showed that combined antibiotic treatments have presented more efficient than single antibiotic treatment for the treatment of *acinetobacter baumannii* strains. The purpose of this study was to analyze **in vitro** efficacy of synergistic effects of Meropenem (MEP), Colistin (COT), and Ampicillin-sulbactam (AMPS) on multidrug-resistant acinetobacter baumannii strains with blaNDM-1. The *baumannii* strains with blaNDM-1 had high antimicrobial activity (≤32 mg/L). Notable changes were noted in the minimum inhibitory concentration (MIC) in blaNDM-1-positive *baumannii* strains before and after performing the drug resistant test. We showed that synergistic antibiotic therapy markedly suppressed blaNDM-1 growth. The mRNA detection demonstrated that resistance genes (*rmtA*, *OXA-24*, *TEM-1*, and *IMP*) were decreased after synergistic antibiotic therapy. Significant increasing was observed in the comparative adeB mRNA levels in blaNDM-1-positive *baumannii* strains after synergistic antibiotic therapy compared with control (78.66±14.48% vs. 10.08±26.35%; P=0.001). In conclusion, findings in the current study suggest that synergistic effects of MEP, COT, and AMPS are potential therapeutic schedule against multidrug-resistant acinetobacter blaNDM-1-positive *baumannii* strains **in vitro**.

**Keywords:** *Acinetobacter baumannii*, meropenem, colistin, ampicillin-sulbactam, blaNDM-1

**Introduction**

*Acinetobacter baumannii* is an aerobic gram-negative coccobacillus, which is an opportunistic pathogen and is one of the most prevalent pathogenic bacterium for patients in hospital [1]. Clinical observations have indicated that *Acinetobacter baumannii* is emerged as a common nosocomial pathogen that can lead to severe infections including septicemia, pneumonia, meningitis, urinary tract infections, and infections stemming from wounds [1, 2]. In recent years, the emergence of multidrug-resistant *Acinetobacter baumannii* has become a critical problem and increased the mortality rate for limiting therapeutic options for infectious diseases caused by multidrug-resistant *Acinetobacter baumannii* [3]. Although carbapenems antibiotics therapy is efficient for *Acinetobacter baumannii* infections, the emergence of multidrug-resistant *Acinetobacter baumannii* strains resistance to various antimicrobial treatments has been increasingly reported [4, 5].

Recently, Biglari et al have reported the intra-species genotypic diversity among *P. aeruginosa* and *Acinetobacter baumannii*, which is associated with multidrug-resistant acinetobacter *Acinetobacter baumannii* [6]. Study also has reported that the various genes are potential targets in the treatment of multidrug-resistant acinetobacter *Acinetobacter baumannii* strains [7]. Ning et al have indicated that combination regimen of Meropenem, cefoperazone-sulbactam and minocycline is an efficient strategy for the treatment of extensive burns with pan-drug resistant *Acinetobacter baumannii* infection [8]. In addition, Colistin, co-treatments of rifampicin, and meropenem have been administered as single agents in a model of pneumonia caused by a carbapenem-resistant
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*Acinetobacter baumannii* [9]. Synergistic interactions between colistin and meropenem against drug-resistant and pandrug-resistant *Acinetobacter baumannii* were identified in patients who infected multidrug-resistant *Acinetobacter baumannii* strains [10]. However, the therapeutic schedules of combined antibiotic treatments for ampicillin-sulbactam against multidrug-resistant *Acinetobacter baumannii* strains need to be developed to improve the therapeutic effects.

In this study, we evaluated the synergistic effects of Meropenem (MEP), Colistin (COT), and ampicillin-sulbactam (AMPS) on multidrug-resistant blaNDM-1-positive *Acinetobacter baumannii* strains. We reported that synergistic antibiotic therapy markedly suppressed growth of blaNDM-1 strains and down-regulated resistance genes (*rmtA*, *OXA-24*, *TEM-1*, and *IMP*) in blaNDM-1-positive *Acinetobacter baumannii*.

**Materials and methods**

*Antimicrobial susceptibility testing*

The antimicrobial disk diffusion susceptibility test was use to analyze the antimicrobial susceptibility of *Acinetobacter baumannii* strains using MEP (30 μg), COT (30 μg), AMPS (30 μg) or Co-treatment (MEP+COT+AMPS, 30 μg) at 35°C for 48 h. The results were presented as “resistant” according to the Clinical and Laboratory Standards Institute (CLSI) guidelines described in previous report [11].

*Acinetobacter baumannii strains growth*

Table 1. Sequences of primers were used in this study

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence</th>
<th>Reverse</th>
<th>Forward</th>
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<tbody>
<tr>
<td>rmtA</td>
<td>5’-TCTATTTCTACTACACCAGCCCG-3’</td>
<td>5’-TTAAAGTTTGGTGTGGCTGAT-3’</td>
<td></td>
</tr>
<tr>
<td>OXA-24</td>
<td>5’-AGGATATTGGTGAAGTCG-3’</td>
<td>5’-GTTTATAGCTGGGCACGAA-3’</td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>5’-ACATTCTCACCTTGAGTTT-3’</td>
<td>5’-CCATTGCAAATCGCTGCAT-3’</td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>5’-CACCATTCTGCCAGGAGCA-3’</td>
<td>5’-TCCTGCTGTGTCCCTTGT-3’</td>
<td></td>
</tr>
<tr>
<td>abeS</td>
<td>5’-TTGGTGTCAGGCACGGATATT-3’</td>
<td>5’-ACCAATGCAGCCAGCTAAGT-3’</td>
<td></td>
</tr>
<tr>
<td>rpoB</td>
<td>5’-CGGAGTCAACGAGTTGTC-3’</td>
<td>5’-ACGCTTCTCCATGGTG-3’</td>
<td></td>
</tr>
</tbody>
</table>

AMPS, 30 μg or Co-treatment, 30 μg were added in each well of a 6-well round bottom microtitre plate. Subsequently, 100 μl of bacterial suspension grown were inoculated into each well at 37°C for 48 h. The growth of *Acinetobacter baumannii* strains were estimated based on optical density at 620 nm in each well. Each experiment was performed in duplicates in three independent and presented as the median values.

*Real-time reverse transcription PCR (RT-PCR)*

Bacteria were grown in Luria-Bertani (LB) broth until mid-log phase. Total RNA was isolated from Bacteria cells (1.0 g) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) DNase-treated RNA templates were prepared using the RNeasy Kit (Qiagen, Hilden, Germany). The cDNA was synthesized using random primer hexamers. All forward and reverse primers were synthesized by Invitrogen (Table 1, Thermo Fisher Scientific, Inc.). RT-PCRs were performed using a 7300 thermocycler (Applied Biosystems) with a SYBR green PCR master mix system (TaKaRa, Tokyo, Japan). The PCR procedure included 95°C for 10 s, followed by 45 cycles of 95°C for 15 s and 57.2°C for 30 s. Relative mRNA expression changes were calculated by the 2^ΔΔCq method (19). The results were expressed as the n-fold compared to the rpoB gene control.

*Knockdown of blaNDM-1*

*Acinetobacter baumannii* strains ACMH-6200, ACMH-6201 cells were grown in Luria-Bertani (LB) and harvested in mid-log phase. Cells were transfected the following siRNAs to silence blaNDM-1 target gene using CRISPR-Cas9 system according to the manufacturer’s instructions [13].

*Statistical analysis*

Statistical analysis was completed using SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA) with the assistance of Microsoft (Microsoft Corporation, Redmond, WA, USA). Data are presented as the mean ± standard deviation of triplicate experiments. Differences
among multiple groups were determined using the Student’s t test and P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of the antimicrobial susceptibility of Acinetobacter baumannii strains with blaNDM-1

The antimicrobial susceptibility of Acinetobacter baumannii strains with blaNDM-1 was performed. As shown in Figure 1A, blaNDM was positive in Acinetobacter baumannii strains ACMH-6200 and ACMH-6201 compared to ATCC-7978. We demonstrated that the overall prevalence of resistance ACMH-6200 was: MEP 22.4%, COT 32.8%, and AMPS 18.6% (Figure 1B). The overall prevalence of resistance ACMH-6201 was: MEP 25.2%, COT 34.6%, and AMPS 22.6% (Figure 1C). We showed that ATCC-7978 demonstrated lower prevalence of resistance for MEP 4.2%, COT 8.8%, and AMPS 6.8% (Figure 1D). These results indicate that Acinetobacter baumannii strain with blaNDM-1 has stronger drug resistance than blaNDM-1-negative Acinetobacter baumannii strain (Table S1).

Synergistic inhibitory effects of MEP, COT, and AMPS for baumannii strains with blaNDM-1 growth

The inhibitory effects of MEP, COT, and AMPS for Acinetobacter baumannii strains growth were investigated in vitro (Figure S1). We showed that synergistic inhibitory effects of MEP, COT, and AMPS were greater than single agent for ACMH-6200 and ACMH-6201 growth (Figure 2A, 2B). These results suggest that synergistic treatment of MEP, COT, and AMPS is a potential strategy for the treatment of blaNDM-1-positive Acinetobacter baumannii strains.
Effects of MEP, COT, and AMPS for changes of resistance genes

To identify the role of MEP, COT, and AMPS for Acinetobacter baumannii strains growth, resistance genes of Acinetobacter baumannii strain were analyzed. We found that resistance genes rmtA, OXA-24, TEM-1, and IMP were decreased after synergistic antibiotic therapy in ACMH-6200 and ACMH-6201 (Figure 3A, 3B, **P<0.01). We also showed that adeB mRNA expression levels were increased in ACMH-6200 and ACMH-6201 (Figure 3C, 3D, **P<0.01). Results also demonstrated that genes expression of rmtA, OXA-24, TEM-1, IMP and adeB were down-regulated in single antibiotic therapy (MEP, COT, or AMPS) compared to control in both ACMH-6200 and ACMH-6201 (**P<0.01). These results indicate that synergistic antibiotic therapy can down-regulate resistance genes in Acinetobacter baumannii strain in vitro (Table S2).

Effects of MEP, COT, and AMPS on blaNDM-1 and β-lactamases production

Previous study has identified that β-lactamases involves in the drug resistance of Acinetobacter baumannii strain. Here, we investigated the effects of synergistic antibiotic therapy on
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blaNDM-1 and β-lactamases production. We showed that synergistic antibiotic therapy decreased blaNDM-1 and β-lactamases mRNA production in ACMH-6200 and ACMH-6201 (Figure 4A, 4B, **P<0.01). Results showed that single antibiotic therapy (MEP, COT, or AMPS) significantly decreased blaNDM-1 and β-lactamases mRNA expression compared to control in ACMH-6200 and ACMH-6201. These results suggest that synergistic antibiotic therapy (MEP, COT, and AMPS) can down-regulate blaNDM-1 and β-lactamases production in Acinetobacter baumannii strain in vitro (Table S3).

Effect of blaNDM-1 on the growth and fitness of baumannii strain

To determine whether blaNDM-1 knockdown can inhibit the Acinetobacter baumannii strains growth were analyzed for the blaNDM-1-knockdown (SiR-blaNDM-1) ACMH-6200 and ACMH-6201 strains and the wild-type strains. As shown in Figure 5A, 5B, blaNDM-1 knockdown increased the inhibitory effects of synergistic antibiotic therapy (MEP, COT, and AMPS) for ACMH-6200 and ACMH-6201 strains (*P<0.05, **P<0.01). These results indicate that blaNDM-1 may be a potential target for multidrug-resistant blaNDM-1-positive Acinetobacter baumannii strains.

Discussion

Multi-drug resistant Acinetobacter baumannii can cause outbreaks for infectious patients in hospital [14]. Evidences have indicated that blaNDM-1-positive Acinetobacter baumannii strains had higher multi-drug resistant than blaNDM-1-negative Acinetobacter baumannii [15, 16]. In recent years, ampicillin-sulbactam therapy significantly decreased the risk of death caused by Acinetobacter baumannii [16]. In this study, we further investigated the inhibitory effects of synergistic antibiotic therapy (MEP, COT, and AMPS) for blaNDM-1-positive Acinetobacter baumannii strains. We reported that synergistic antibiotic therapy of MEP, COT,
and AMPS significantly inhibited blaNDM-1-positive *Acinetobacter baumannii* strains growth via decreasing blaNDM-1, β-lactamases production and drug resistance genes expression.

Currently, multi-drug resistant *Acinetobacter baumannii* has been regarded as an opportunistic pathogen, especially to immunocompromised patients who were hospitalized in intensive care units (ICU) [17]. Previous study has demonstrated the *in vitro* and *in vivo* analysis of antimicrobial agents alone and in combination against multi-drug resistant *Acinetobacter baumannii* [18]. In this study, we demonstrated that synergistic antibiotic therapy of MEP, COT, and AMPS can markedly inhibit growth of ACMH-6200 and ACMH-6201 *Acinetobacter baumannii* strains.

Extended-spectrum β-lactamase (ESBL) produced by antibiotic-resistant bacteria is a potential mechanism to underlie the phenomenon of the evolution of antibiotic-resistant bacteria [19, 20]. Report has revealed that the involvement of β-lactamase enhanced drug resistance of *Acinetobacter baumannii* strains [21]. In this study, we found that β-lactamase is up-regulated in blaNDM-1-positive *Acinetobacter baumannii* strains. This finding indicates that β-lactamase involves in the multi-drug resistant *Acinetobacter baumannii*.

The blaNDM-1 gene is associated with the potential for the spread of between *P. aeruginosa* and *Acinetobacter baumannii* in clinical and environmental settings [22]. Experimental evidence has found that intra- and inter-species transfer of a plasmid harbouring blaNDM-1 gene in *Acinetobacter baumannii* increased the antibiotic resistance [23]. In this study, we reported that blaNDM-1-positive *Acinetobacter baumannii* strains had stronger antibiotic resistance than blaNDM-1-negative *Acinetobacter baumannii* strains. We indicated that blaNDM-1 knockdown decreased antibiotic resistance of *Acinetobacter baumannii*, which suggest that blaNDM-1 may be a potential target for multi-drug-resistant blaNDM-1-positive *Acinetobacter baumannii* strains.

Antibiotic resistance of *Acinetobacter baumannii* strains involve in various resistance genes [24-26]. Studies have showed that OXA-24 and rmtA are novel class β-lactamase with carbapenemase activity in an *Acinetobacter baum-

In conclusion, this study reported that blaNDM-1-positive *Acinetobacter baumannii* strains presented strong antibiotic resistance. Findings in the current study indicate that synergistic effects of MEP, COT, and AMPS are potential therapeutic strategy against multidrug-resistant blaNDM-1-positive *Acinetobacter baumannii* strains. Notably, we also indicate that blaNDM-1 is a potential target for the treatment of patients infected with *Acinetobacter baumannii* strains.

**Disclosure of conflict of interest**

None.

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**References**


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Table S1. blaNDM gene expression in *Acinetobacter baumannii* strains ACMH-6200, ACMH-6201 and ATCC-7978

<table>
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<tr>
<th><em>Acinetobacter baumannii</em> strains</th>
<th>blaNDM mRNA level</th>
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<tbody>
<tr>
<td>ACMH-6200</td>
<td>5.80 7.50 6.20</td>
</tr>
<tr>
<td>ACMH-6201</td>
<td>6.20 7.60 7.20</td>
</tr>
<tr>
<td>ATCC-7978</td>
<td>0.50 0.23 0.32</td>
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Table S2. Genes levels *rmtA*, *OXA-24*, *TEM-1*, *IMP* and *adeB*

<table>
<thead>
<tr>
<th>Gene</th>
<th>ACMH-6200</th>
<th>ACMH-6201</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MEP</td>
</tr>
<tr>
<td>rmtA</td>
<td>7.82</td>
<td>8.43</td>
</tr>
<tr>
<td>OXA-24</td>
<td>10.54</td>
<td>8.26</td>
</tr>
<tr>
<td>TEM-1</td>
<td>10.45</td>
<td>8.38</td>
</tr>
<tr>
<td>IMP</td>
<td>9.98</td>
<td>7.36</td>
</tr>
<tr>
<td>adeB</td>
<td>1.48</td>
<td>1.05</td>
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<tr>
<td>ACMH-6200</td>
<td>1.38</td>
<td>0.86</td>
</tr>
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Figure S1. Effects of synergistic antibiotic therapy on the growth of ACMH-6200 and ACMH-6201.
The efficacy of meropenem, colistin, ampicillin-sulbactam against blaNDM-1

**Table S3. Changes of resistance genes β-lactamases and blaNDM-1**

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<tr>
<th>Gene</th>
<th>Control</th>
<th>MEP</th>
<th>AMPS</th>
<th>COT</th>
<th>Co-treatment</th>
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<td>ACMH-6200</td>
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<tr>
<td>β-lactamases</td>
<td>5.34</td>
<td>6.69</td>
<td>5.70</td>
<td>5.72</td>
<td>3.53</td>
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<td>6.12</td>
<td>5.50</td>
<td>3.42</td>
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<td>β-lactamases</td>
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<td>4.00</td>
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<td>1.88</td>
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<td>4.13</td>
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