AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

DOI:
10.1556/030.2022.01785
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RESEARCH ARTICLE

Time kill-assays of antibiotic combinations for multidrug resistant clinical isolates of OXA-48 carbapenemase producing *Klebsiella pneumoniae*

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Received: May 21, 2022 • Accepted: June 2, 2022

ABSTRACT

Treatment of infections caused by OXA-48 carbapenemase producing multidrug-resistant isolates often necessitates combination therapy. *In vitro* effect of different antibiotic combinations against multidrug-resistant (MDR) *Klebsiella pneumoniae* isolates were evaluated in this study.

Meropenem-tobramycin (MER+TOB), meropenem-ciprofloxacin (MER+CIP), colistin-meropenem (COL+MER), colistin-ciprofloxacin (COL+CIP) and colistin-tobramycin (COL+TOB) combinations were tested by time kill-assays. Each antibiotic alone and in combination at their Cmax values were tested against 4 clinical *K. pneumoniae* isolates at 1, 2, 4, 6, 8, 12 and 24 h. Effect of colistin and its associations were also assessed at 30 min. Bactericidal activity was defined as $\geq 3\log_{10}$ CFU mL^{-1} decrease compared with initial inoculum. Synergy was defined as $\geq 2\log_{10}$CFU mL^{-1} decrease by the combination compared with the most active single agent. Presence of $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{KPC} and $bla_{\text{CTX-M-1}}$ genes was screened by PCR using specific primers.

The $bla_{\text{OXA-48}}$ gene was identified together with $bla_{\text{CTXM-1}}$ group gene in all isolates. COL+MER demonstrated to be synergistic and bactericidal. MER+TOB showed synergistic and bactericidal effect on two strains although, regrowth was seen on other two strains at 24 h. MER+CIP exhibited indifferent effect on the strains.

Combination therapy could be a potential alternative to treat MDR *K. pneumoniae* infections. This combination might prevent resistance development and secondary effects of colistin monotherapy. MER+TOB and MER+CIP might have an isolate-dependent effect, that may not always result in synergism.

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KEYWORDS

OXA-48 carbapenemase, MDR *Klebsiella pneumoniae*, time kill-assay, combination therapy

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INTRODUCTION

Klebsiella pneumoniae is an emerging nosocomial pathogen which belongs to ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) group pathogens and causes a wide range of clinical diseases such as urinary tract infections, pneumonia and blood stream infections [1–3]. CRKP (Cabapenem resistant *K. pneumoniae* strains have dramatically risen worldwide over the last decades [1]. Polymyxins (colistin), aminoglycosides, tigecycline, and ceftazidime/avibactam are currently the treatment options for the CRKP strains [4]. It is noteworthy that, colistin is the last treatment choise due to its toxic effects. Ceftazidime/avibactam is more reliable than colistin, but it does not inhibit metallo-β-lactamase enzymes [imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactamase (NDM)] [5].

OXA-48 producing *K. pneumoniae* strains are usually multidrug-resistant (MDR) due to $bla_{\text{OXA-48-like}}$ genes and these strains can coharbor extended-specrum beta-lactamases (ESBLs) ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}), AmpC enzymes as well as fluoroquinolone and aminoglycoside resistance genes. Combination therapy against MDR isolates will be life-saving until finding different new beneficial strategies or developing new drugs to use [6]. *In vitro* effect of different antibiotic combinations against MDR *K. pneumoniae* isolates were evaluated in this study.

MATERIAL AND METHODS

Strain collection and antimicrobial susceptibility tests

A collection of four MDR and *bla*OXA-48 carbapenemase-producing *K. pneumoniae* clinical isolates (Kpn1, Kpn2, Kpn3, Kpn4) were studied due to their different resistance patterns. The isolates were obtained from clinical samples (Kpn1, Kpn2, Kpn3; blood samples and Kpn4; urine sample) of hospitalized patients. In this study, antibiotics which are the most preferred by clinicians in the practice and currently available in Turkey, were studied [7]. Antibiotic MICs were determined using standard broth microdilution method. Susceptibility results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) clinical breakpoint guidelines [8].

Synergy tests

Meropenem-tobramycin (MER+TOB), meropenem-ciprofloxacin (MER+CIP), colistin-meropenem (COL+MER), colistin-ciprofloxacin (COL+CIP) and colistin-tobramycin (COL+TOB) combinations were tested by time kill-assays, each antibiotic alone and in combination at their Cmax values were tested against the four clinical *K. pneumoniae* isolates at 1, 2, 4, 6, 8, 12 and 24 h. Effect of colistin and its association were also assessed at 30 min. Bactericidal activity was defined as $\geq 3\log_{10}$ CFU mL^{-1} decrease compared to initial inoculum. Synergy was defined as $\geq 2\log_{10}$ CFU mL^{-1} decrease by the combination compared to the most active single agent [9].

Genotypic detection of resistance gene

Presence of $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{KPC} and $bla_{\text{CTX-M-1}}$ genes were screened by PCR using specific primers [10].

RESULTS

Antibiotic MICs (mg L^{-1}) of the four *K. pneumoniae* isolates are given in Table 1. The $bla_{\text{OXA-48}}$ gene was identified together with $bla_{\text{CTXM-1}}$ group gene in all isolates. Time kill assay curves are showed in Fig. 1.

Kpn1

MER, TOB alone exhibited bacteriostatic profile, MER+TOB resulted in rapidly bactericidal effect, synergistic effect (at 2h, 6h respectively) with complete inhibition growth at 24h. On the contrary, MER+CIP was indifferent. For colistin and its combinations bactericidal effect was reached within first 30 min (Fig. 1).

Kpn2

MER presented bacteriostatic profile (1-log10 CFU mL^{-1} inoculum reduction at 6h) while COL, CIP, and TOB could not reduce inoculum at any time. Conversely, COL+MER was bactericidal, and synergistic at 4 h. Although regrowth was observed at 12 h, synergistic effect was maintained. MER+TOB exhibited bacteriostatic, and synergistic pattern at 8 h but this effect was lost at 24 h. MER+ CIP, COL+TOB, COL+CIP were indifferent (Fig. 1).

Kpn3

TOB and CIP could not reduce the initial inoculum. MER exhibited bactericidal effect that was lost with regrowth at 24 h. MER+TOB presented synergy and bactericidal effect at 24 h and impeded regrowth. MER+CIP was indifferent. Although this isolate was susceptible to colistin, this antibiotic alone was bacteriostatic as they were COL+TOB and COL+CIP. Conversely COL+MER showed potent synergy, bactericidal action within first hour and no growth was observed from second hour (Fig. 1).

Kpn4

TOB and CIP did not reduce inoculum, MER exhibited a bacteriostatic profile (2.5 log-CFU mL^{-1} inoculum reduction at 4 h) and regrowth after 8 h. However, MER+CIP presented a bactericidal pattern at 4 h and synergistic effect at 6 h. Regrowth was observed at 24 h. MER+TOB was bactericidal (at 4 h) although not

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Table 1. Antibiotic MICs (mg L^{-1}) of four *K. pneumoniae* isolates

Antibiotic	Kpn1 Blood culture	Kpn2 Blood culture	Kpn3 Blood culture	Kpn4 Urine culture
Amoxicilin/Clavulanate	>256 R	>256 R	>256 R	>256 R
Piperacillin/Tazobactam	>256 R	>256 R	192 R	>256 R
Cefotaxime	>32 R	>32 R	>32 R	>32 R
Cefotaxime/Cefotaxime+CLAV.	>16/>1	>16/>1	>16/>1	>16/>1
Ceftazidime	24 R	>256 R	32 R	>256 R
Ceftazidime/Ceftazidime+CLAV.	>32/<0.64	>32/>4	>32/>4	>32/>4
Cefoperazone/Sulbactam	32	128	128	>256
Meropenem	1.5 I	24 R	>32 R	24 R
Ertapenem	>256 R	>256 R	>256 R	>256 R
Imipenem	2 I	4 R	6 R	12 R
Ciprofloxacin	>32 R	>32 R	>32 R	16 R
Ofloxacin*	>32 R	>32 R	>32 R	12 R
Tobramycin	12 I	16 R	32 R	>1024 R
Amikacin	6 S	6 S	12 S	>256 R
Gentamicin	0.38 S	64 R	64 R	>256 R
Tigecycline*	8 R	0.75 S	1.5 R	0.36 S
Colistin*	0.19 S	3 R	0.38 S	0.5 S

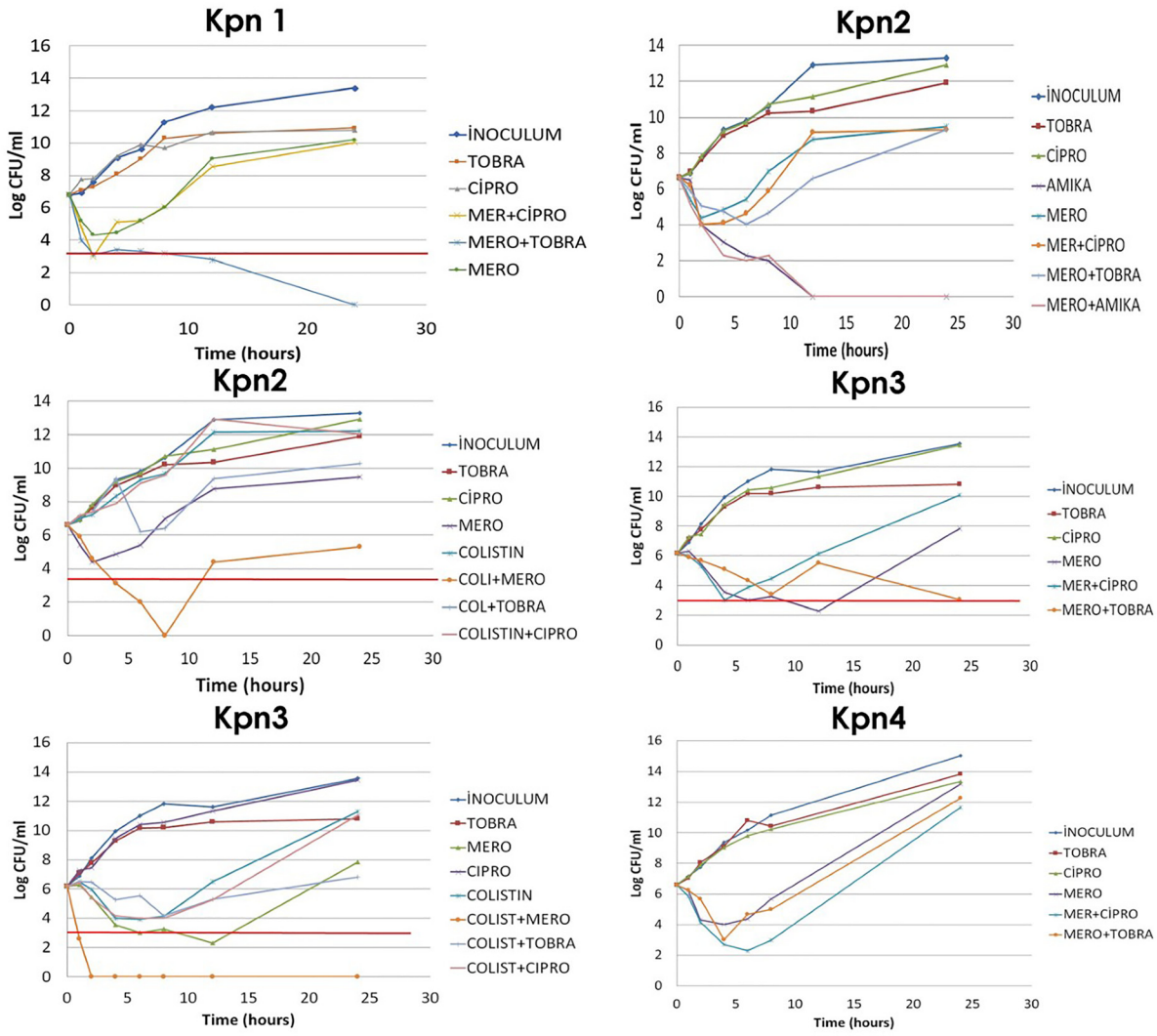


Fig. 1. Time kill curves of antibiotics (meropenem, tobramycin, ciprofloxacin, colistin) alone and in combination against OXA-48 carbapenemase-producing *K. pneumoniae* strains

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synergistic and regrowth began at 6 h. For colistin and its associations bactericidal effect was reached within first 30 min (Fig. 1).

DISCUSSION

Combination regimens are choosen by clinicians for MDR *Enterobacterales* although studies are mostly limited to *in vitro* synergy tests [11]. Despite the fact that time–kill synergy assays provide data on bactericidal activity over time and more accurately reflect in vivo synergy, these assays are labour intensive and not practical for routine clinical laboratories and for a large screen [12].

Combination therapy containing a high-dose carbapenem has been found clinically effective aganist carbapenem resistant *K. pneumoniae* strains [13]. In contrast, in an Indian study that analyzed the effectiveness of mono and combination therapy strategies against CRGNB (Carbapenem Resistant Gram Negative Bacteria) non-bacteremic infections, combination therapy was not found be superior compared to monotherapy [14]. Even if there are so many clinical studies that investigate antibiotic combinations aganist KPC producing *K. pneumoniae*, there are limited studies with OXA-48 producing *K. pneumoniae* [13, 15].

Polymyxin and carbapenem combinations have been previously evaluated against *K. pneumoniae carbapenemase* (KPC)-producing strains and synergistic effects have been reported in several studies [16, 17]. Furthermore, most of the *in vitro* synergy studies have been performed with VIM-producing isolates. In a time kill study, a 24 h synergy of meropenem and colistin has been previously observed against a VIM-producing strain [18, 19].

In this study, synergistic effects were observed with COL+MER combinations againsts OXA-48 producing *K. pneumoniae* strains in accordance with our previous time kill assay study that was conducted with eight OXA-48 producing *K. pneumoniae* strains [6].

Novel approaches are urgently needed to treat these infections. RNA-based antimicrobial strategies are currently under investigation. But most studies have been limited to reference strains, such as *E. coli* MG1655 [20]. However, phage therapy is a new aproach to control infections caused by MDR *K. pneumoniae* strains [21, 22].

This study is limited to four OXA-48 producing *Klebsiella pneumonae* strains, more large scale studies are needed to strengthen the potential benefits of antibiotic combinations.

CONCLUSIONS

COL+MER has demonstrated to be synergistic and bactericidal. It could be a potential alternative to treat MDR *K. pneumoniae* infections [23]. This combination might prevent resistance development and secondary effects of colistin monotherapy. MER+TOB and MER+CIP might have an isolate-dependent effect, that may not always result in synergism.

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