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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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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 C_{max} 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*_{OXA-48}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{KPC} and *bla*_{CTX-M-1} genes was screened by PCR using specific primers.

The *bla*_{OXA-48} gene was identified together with *bla*_{CTX-M-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



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 (Carbapenem 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 choice 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*_{OXA-48-like} genes and these strains can harbor extended-spectrum beta-lactamases (ESBLs) (*bla*_{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 C_{max} 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*_{OXA-48}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{KPC} and *bla*_{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*_{OXA-48} gene was identified together with *bla*_{CTX-M-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



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
Amoxicillin/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

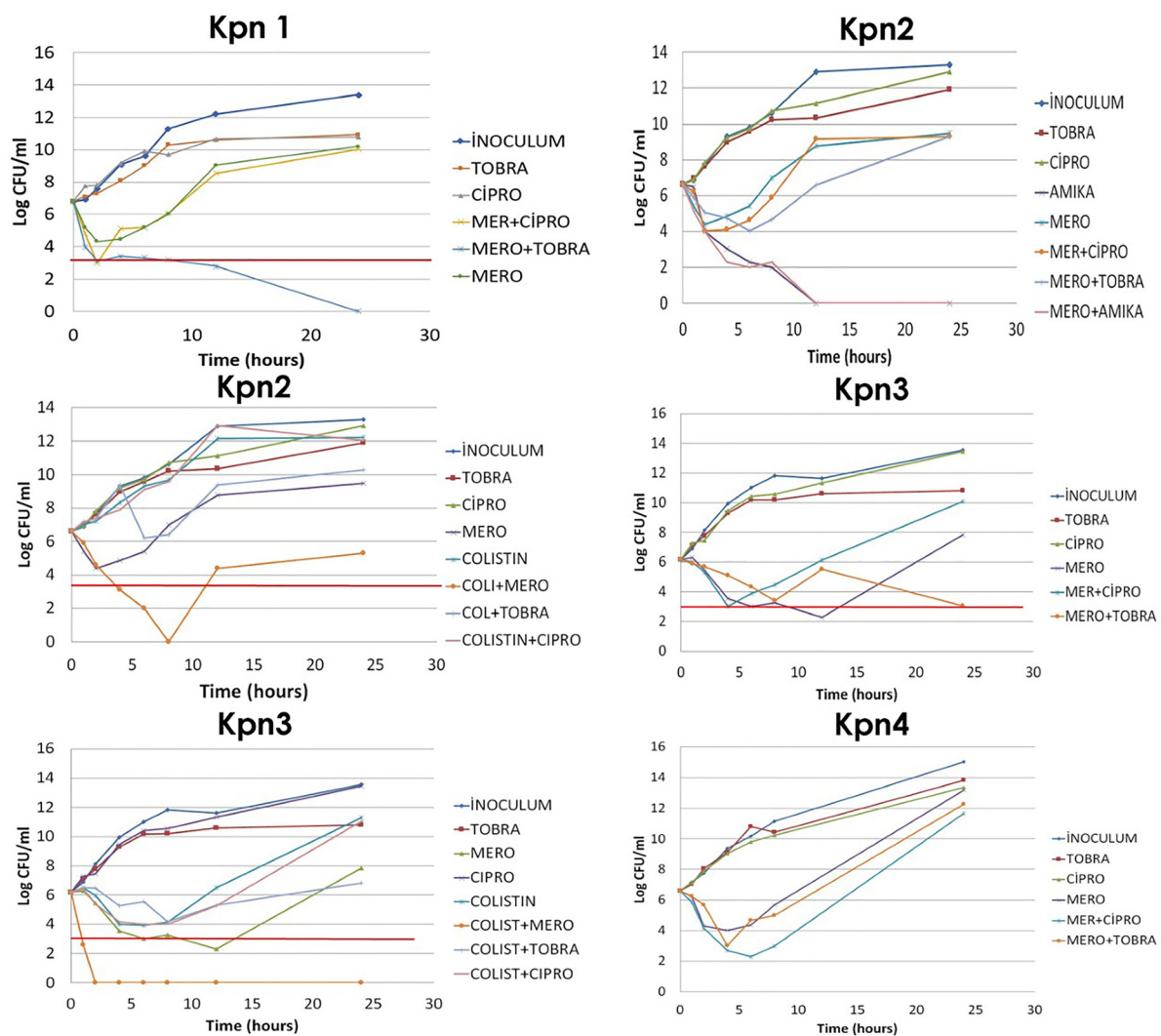


Fig. 1. Time kill curves of antibiotics (meropenem, tobramycin, ciprofloxacin, colistin) alone and in combination against OXA-48 carbapenemase-producing *K. pneumoniae* strains



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 chosen 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 against 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 to be superior compared to monotherapy [14]. Even if there are so many clinical studies that investigate antibiotic combinations against 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 against 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 approach to control infections caused by MDR *K. pneumoniae* strains [21, 22].

This study is limited to four OXA-48 producing *Klebsiella pneumoniae* 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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