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IN VITRO SYNERGY AND POSTANTIBIOTIC EFFECT OF COLISTIN COMBINATIONS WITH MEROPENEM AND VANCOMYCIN AGAINST *ENTEROBACTERIACEAE* WITH MULTIPLE CARBAPENEM RESISTANCE MECHANISMS

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ABSTRACT

Aim: The aim of the study was to determine *in vitro* synergy and postantibiotic effect of colistin alone and combined with meropenem or vancomycin against *Enterobacteriaceae* producing multiple carbapenemases; combinations of two metallo-β-lactamases (MBL) or MBL with OXA-48. Colistin-resistant strain positive for OXA-48 was also included in the study.

Methods: The antibiotic susceptibility was tested by broth microdilution method. Synergy was tested by chequerboard, time-kill and 2-well method. PAE was determined by viable counting.

Results:The chequerboard analysis revealed synergy for colistin combination with meropenem in all isolates with FICI values ranging from 0.12 to 0.24. FICI values for combinations with vancomycin were below 0.5 indicating synergy in two out of four isolates. *K. pneumoniae* 609815 positive for OXA-48 and colistin resistant showed the most pronounced and consistent synergy effect with meropenem in both chequerboard and time-kill method. Synergy effect in time-kill curves, was observed for *K pneumoniae* 145846 with two MBLs and colistin resistant *K. pneumoniae* 609815 positive for OXA-48, with both combinations including meropenem and vancomycin. Colistin alone exhibited short postantibiotic effect (PAE) against all tested isolates. Meropenem markedly prolonged the PAE in two isolates in contrast to vancomycin which did not demonstrate significant effect on the duration of PAE

Conclusions: The synergy effect and the duration of PAE was strain and antibiotic dependent but not related to the resistance gene content.

Key words: carbapenem resistance, *Klebsiella pneumoniae*, colistin, VIM-1, NDM,-1 OXA-48

Carbapenem-resistance in *Enterobacteriaceae* has increased globally during the past decade, particularly in *Klebsiella pneumoniae*, and is typically associated with carbapenemase

production as the most important resistance mechanism [1]. Therapeutic options for infections caused by carbapenem-resistant *Enterobacteriaceae* are limited and colistin is often the only remaining treatment option. Colistin (also known as polymyxin E) is a multi-component polypeptide antibiotic discovered in the 1950s. However, colistin resistance emerged recently in *Enterobacteriaceae* [2]. Persistent suppression of bacterial growth after short antimicrobial exposure is called the postantibiotic effect (PAE) [3]. Both carbapenems and colistin were found to produce PAE in Gram-negative bacteria as individual drugs but there are only few reports on PAE induced by combination of colistin with carbapenems [4-6]. Clinical studies demonstrated that combination antibiotic therapy is associated with better outcome than monotherapy for the treatment of severe infections with these strains, even if the isolated strains are resistant *in vitro* to the individual drugs [7]. Improved clinical outcomes have been reported for the combinations of colistin and a carbapenem, tigecycline, fosfomycin and vancomycin [7]. Recently, isolates with multiple carbapenemases, including combinations of VIM-1, NDM-1 and OXA-48, or KPC with broad spectrum β-lactamase were found in *Enterobacteriaceae* in Zagreb [8-9]. Moreover, colistin resistance was reported in a OXA-48 producing *Klebsiella pneumoniae* from Zagreb (unpublished results) and *Enterobacter aerogenes* from Pula [10]. Such isolates pose a serious therapeutic problem in our hospitals, particularly in *Enterobacter* spp which is prone to develop adaptive resistance to colistin due to upregulation of efflux pumps or loss of outer membrane porins. There are many reports on colistin synergy with other antibiotics against KPC and MBL positive *Enterobactericae* [11.12,13]. However, there are no reports on the synergy testing and PAE induced by colistin combination with other antibiotics, in the isolates with multiple carbapenemases, or those positive for OXA-48 and colistin resistant.

The aim of the study was to determine *in vitro* synergy and postantibiotic effect of colistin alone and combined with meropenem or vancomycin against *Enterobacteriacea*e producing multiple carbapenemases or carbapenemase combined with colistin resistance.

The study was performed on the collection of previously characterized strains from Croatia [8-9]. The study collection included: *Enterobacter cloacae* 209377 positive for VIM-1, NDM-1, OXA-48, TEM-1 and CTX-M-15, *Klebsiella pneumoniae* 145846 positive for NDM-1 VIM-1, TEM-1, and SHV-11, *Klebsiella pneumoniae* 38985 positive KPC-2, TEM-1 and SHV-11, and *Klebsiella pneumoniae* 609815 positive for OXA-48, SHV-11 and colistin

Minimum inhibitory concentrations (MICs) of colistin, vancomycin and meropenem were determined by the broth microdilution method according to the CLSI guidelines [14].

The synergy of colistin with meropenem or vancomycin was tested by chequerboard method yielding the fractional inhibitory concentration index (FICI), 2-well method and time-kill method (TK) as described previously [15]. Antibiotic concentrations used during TK experiments represented mean steady-state concentrations of non-protein bound drug in human body fluids: 4 mg/L for colistin, 10 mg/L for meropenem and 9 mg/L for vancomycin. Synergy in TK was defined as $\geq$ 2 log10 decrease in colony count at 24 h with the antimicrobial combination compared to the most active single agent. Bactericidal effect was defined as $\geq$ 3 log10 decrease in the colony count after 24 h compared with the starting inoculum. PAE was determined by a standard viable counting method after exposure to colistin alone and combined with vancomycin or meropenem in the same concentrations as for TK.

All but one strain (609815, OXA-48) were susceptible to colistin with MIC values ranging from 0.5 to 2 mg/L. The strains exhibited variable MICs of meropenem with values ranging from 8 to >128 mg/L. The chequerboard analysis revealed synergy for colistin combination with meropenem in all tested isolates, with FICI values ranging from 0.12 to 0.24. FICI values

for combinations with vancomycin were 0.5 indicating synergy in two isolates (*K. pneumoniae* 14846 and *K. pneumoniae* 609815- colistin resistant) and above 0.5 indicating no synergy in the remaining two isolates (Table 1). 2- method revealed the growth of all strains at 0.25 x MIC of colistin alone, but absence of growth when the strains were exposed to 2 x MIC of colistin alone (Table 1). No growth was observed when the strains were exposed to 0.25 x MIC and 2 x MIC of colistin combined with both meropenem and vancomycin confirming synergy in all tested strains (Table 1).

Colistin, alone and in any combination with other antibiotics was associated with 3 log decrease in CFU/ml during the first hour of the experiments in two (*K. pneumoniae* 145 846 positive for NDM-1 and VIM-1 and *E. cloacae* 209377 positive for VIM-1, NDM-1 and OXA-48) out of four strains (Fig. 1). However, considerable regrowth occurred for colistin alone and in combination, but the addition of meropenem or vancomycin postponed the regrowth for 1 to 3 h, as shown in Fig. 1. Nevertheless, regrowth occurred in most cases after 24h, in spite of the additional antibiotic. Bactericidal effects after 24 h were found for the combinations with both vancomycin and meropenem, against *K. pneumoniae* strain 145846 positive for NDM-1 and VIM-1 positive for two MBLs and *E. cloacae* 209377 positive for VIM-1, NDM-1 and OXA-48 positive for two MBLs and OXA-48, with CFU reduction of 3 to 4 log10 compared to the starting inoculum. Synergy effect defined as $\geq$ 2 log10 decrease with the antibiotic combination compared to colistin alone, was observed for *K pneumoniae* 145846 and colistin resistant *K. pneumoniae* 609815 positive for OXA-48, with both combinations including meropenem (3-4 log10 decrease compared to colistin alone) and vancomycin (2-4 log10 decrease compared to colistin alone) as shown in Table 1 and Fig 1. All tested strains exhibited very short to moderate PAEs induced by colistin alone ranging from 0.9 h (*K. pneumoniae* 60815) to 2.6 (*E. cloacae* 209377) as shown in Table 2 and Fig. 1. The duration of PAE was only slightly prolonged by vancomycin in four tested strains and

ranged from 1.16 (*K. pneumoniae* 38985) to 2.8 h (*E. cloacae* 209377). Addition of meropenem prolonged significantly the duration of PAE (>2h) in two tested strains (*E. cloacae* 209377 and *K. pneumoniae* 38985) as shown in Table 2 and Fig. 1. The PAE was consistently the longest with *E. cloacae* 209377 harbouring VIM-, NDM-1, and OXA-48 (Table 2). The addition of meropenem to colistin resulted in synergistic activity in chequerboard method and 2-well method with all tested strains. This is probably due to increased permeability leading to higher concentrations of meropenem in periplasmic space with the combination. However, the synergy in TK was reported only for *K. pneumoniae* 145846 and for colistin resistant *K. pneumoniae* 609815. The addition of vancomycin produced synergy in TK method with the *K. pneumoniae* 145846 with two MBLs and colistin resistant *K. pneumoniae* 609815 which was confirmed by chequerboard method. 2- well method found synergy with all tested strains and antibiotic combinations. The discrepancy between 2-well and TK can be explained by the fact that 2-well method detects bacteriostatic activity while TK method determined bactericidal activity. The discrepancies between chequerboard and 2-well method can be explained by the fact that 2-well detects the growth or absence of growth with only two different antibiotic concentrations (0.25 x MIC and 2 x MIC) whereas chequerboard tests the range of 12 antibiotic dilutions. The differences could also be due to the specific characteristics of the strains which are not influenced by the resistance gene content. Moreover, colistin and meropenem, in combination, in physiologically attainable concentrations, showed an evident and potent *in vitro* PAE activity in half of the tested strains.. Vancomycin did not significantly prolong the duration of PAE induced by colistin. The longest PAE was noticed for *E. cloacae* 209377 positive for two MBLs, OXA-48 and ESBL. The *E. cloacae* strain possessed three carbapenemases and it is possible that the acquisition of the resistance genes was on expense of reduced fitness and prolonged response to antibiotic exposure. The shortest PAE was detected for colistin

resistant *K. pneumoniae* which could be explained by ineffectiveness, of colistin, probably due to modification of the outer membrane lipopolysaccharide, although the mechanism of the colistin resistance was not clarified in this study. Our study demonstrated that the PAE was strain and antibiotic dependent, but was not influenced by the type of carbapenemase. Biological differences between the strains might have influenced the response to the antibiotics and the duration of PAE. The bibliographical data point out to the fact that bacterial isolates belonging to the same or different species demonstrate huge differences in the duration of PAE [3-5]. Interestingly, the colistin resistant and OXA-48 positive strain showed the most pronounced synergy effect regardless of the testing method, but the addition of meropenem or vancomycin did not prolong significantly the duration of PAE. The possible explanation for the synergy effect is that colistin which causes an electrostatic interaction with lipid A which consequently discrupts the outer membrane of Gram-negative bacteria allows the large molecules such as vancomycin to pass through the lipid layer and reach the receptor at the cell wall and enables meropenem to achieve higher concentrations in the periplasmic space. Prevention of heteroresistance to colistin could also be one explanation for the synergy, but the population analysis was not done in this study. Unexpectedly, the phenomenon is more pronounced in colistin resistant compared to colistin susceptible strains. The KPC-positive organism (38985) exhibited no synergy in TK method neither with vancomyin nor with meropenem. This could be explained by increased fitness of this individual strain in the presence of antibiotics in comparison with other strains. Moreover, KPC carbapenemase exerts very high hydrolysis of carbapenem substrates which could, in part, explain the lack of synergy with meropenem.

The limitation of the study is relatively small number of tested strains and the fact that results were strain dependent and method dependent. Our preliminary results on small number of tested strains demonstrated that meropenem has a potential of exerting synergy with colistin

and prolonging PAE but further study including larger number of isolates are needed to confirm these results.

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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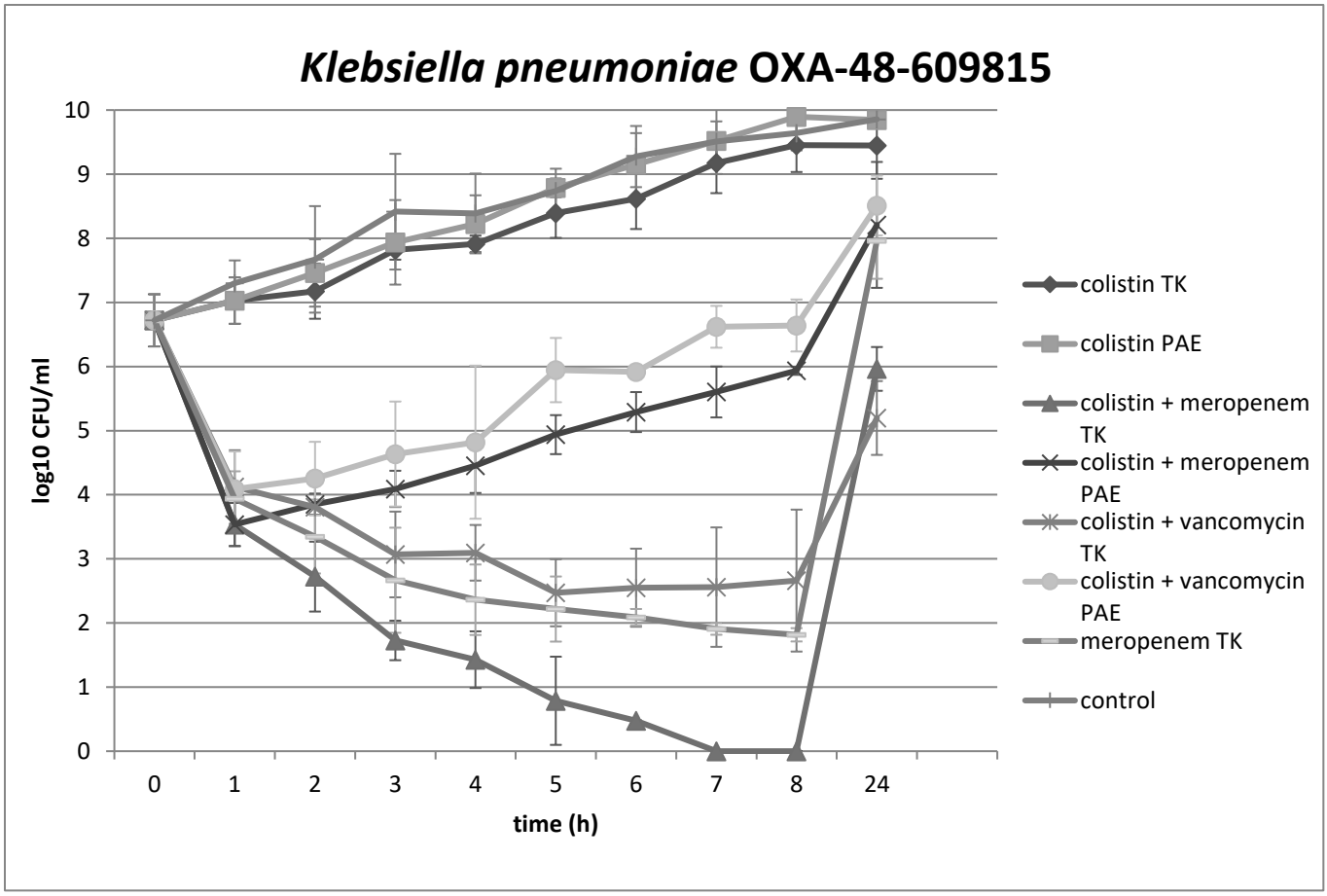
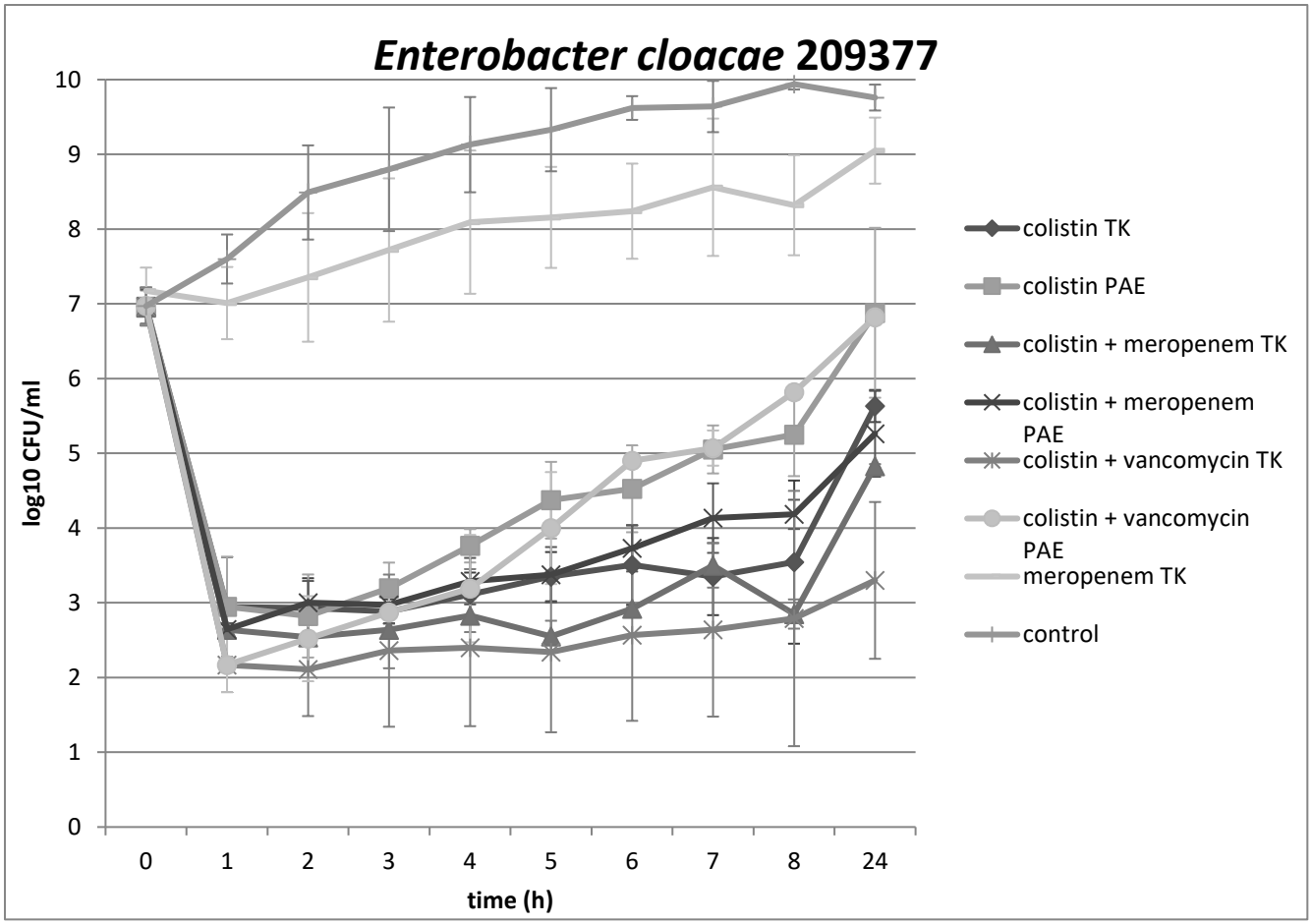
FIGURE LEGEND

Fig. 1. Time kill curves and postantibiotic effect of colistin alone and combined with meropenem or vancomycine against strains: *K. pneumoniae* 609815 positive for OXA-48, SHV-11 and colistin resistant, *E. cloacae* 209377 positive for VIM-1, NDM-1, OXA-48, TEM-1 and CTX-M-15, *K. pneumoniae* 145846 positive for NDM-1 VIM-1, TEM-1, and SHV-11 and *K. pneumoniae* 38985 positive KPC-2, TEM-1 and SHV-11- Mean value of three experiments is shown.


Klebsiella pneumoniae OXA-48-609815
log10 CFU/ml
time (h)
0
1
2
3
4
5
6
7
8
24
0
1
2
3
4
5
6
7
8
9
10
colistin TK
colistin PAE
colistin + meropenem TK
colistin + meropenem PAE
colistin + vancomycin TK
colistin + vancomycin PAE
meropenem TK
control


Enterobacter cloacae 209377
log10 CFU/ml
time (h)
0
1
2
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4
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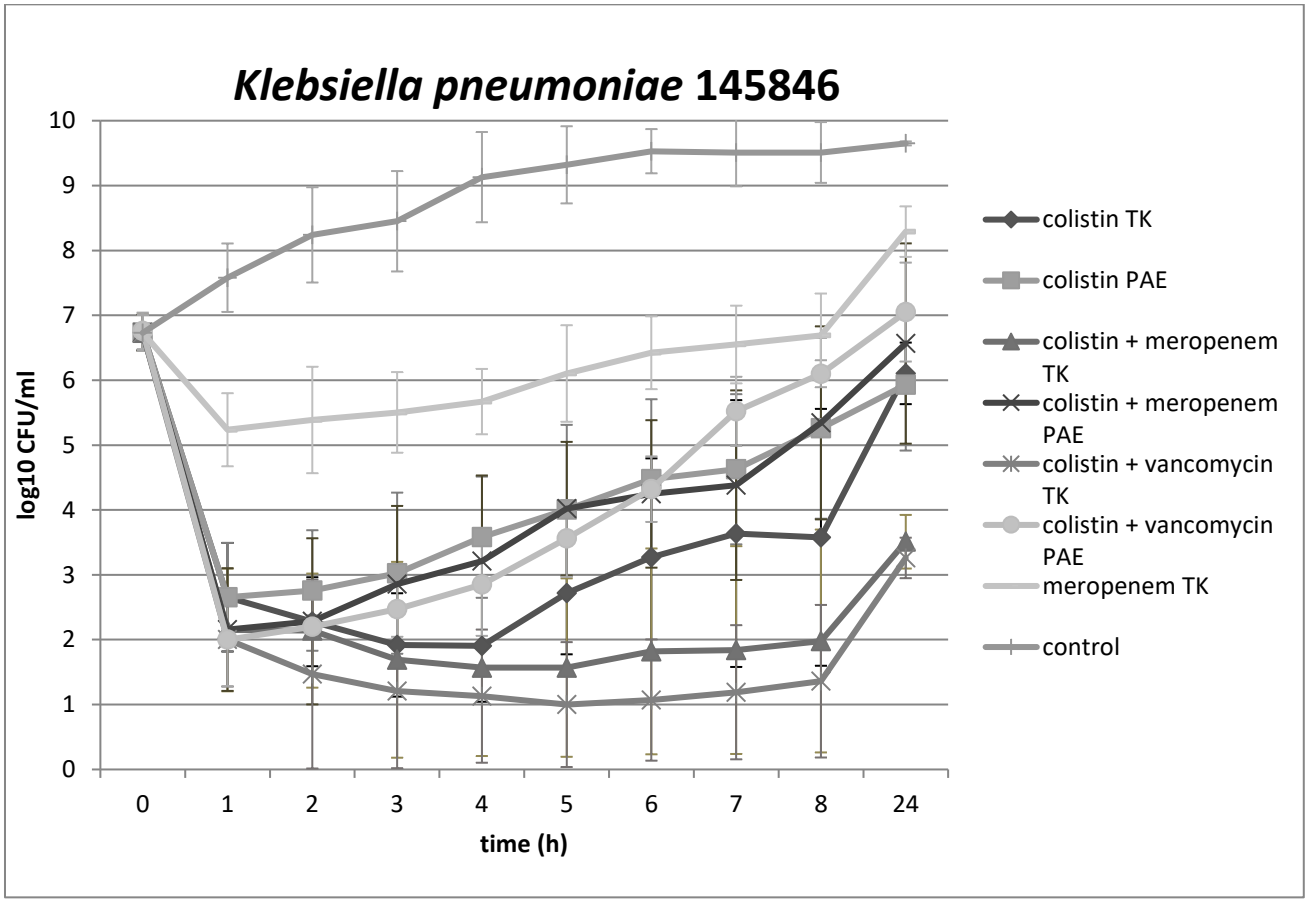
6
7
8
9
10
0
1
2
3
4
5
6
7
8
24
colistin TK
colistin PAE
colistin + meropenem TK
colistin + meropenem PAE
colistin + vancomycin TK
colistin + vancomycin PAE
meropenem TK
control


Klebsiella pneumoniae 145846
log10 CFU/ml
time (h)
0
1
2
3
4
5
6
7
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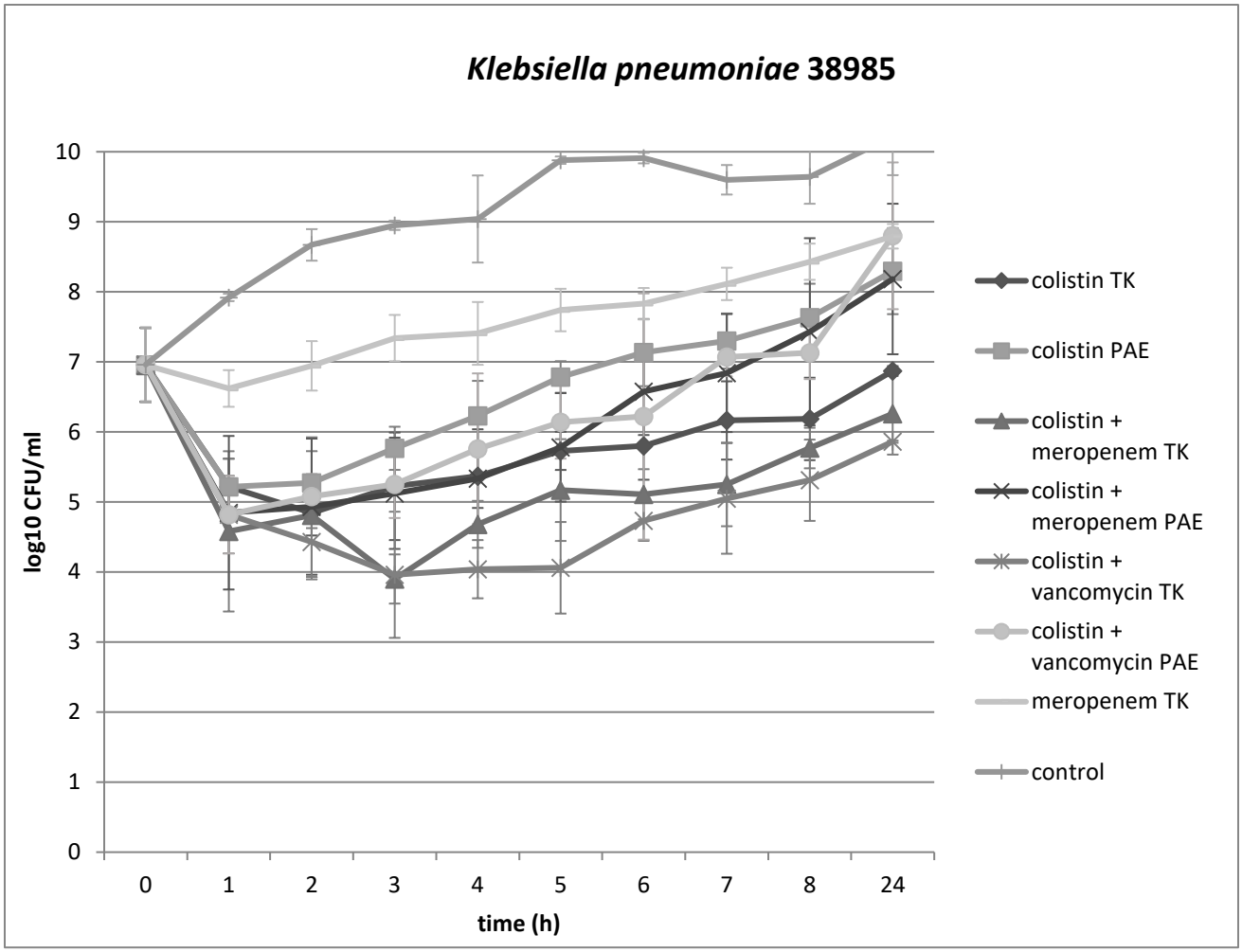
9
10
0
1
2
3
4
5
6
7
8
24
colistin TK
colistin PAE
colistin + meropenem TK
colistin + meropenem PAE
colistin + vancomycin TK
colistin + vancomycin PAE
meropenem TK
control


Klebsiella pneumoniae 38985
log10 CFU/ml
time (h)
0
1
2
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6
7
8
24
colistin TK
colistin PAE
colistin + meropenem TK
colistin + meropenem PAE
colistin + vancomycin TK
colistin + vancomycin PAE
meropenem TK
control

Table 1. Synergistic effect of colistin combined with either vancomycin or meropenem with three different methods

Strain	Chequerboard Colistin+ vancomycin (FICI)	Chequerboard Colistin + meropenem (FICI)	Time-kill (colistin +vancomycin) ≥2log10 reduction	Time-kill (colistin +meropenem) ≥2log10 reduction	Two well method Colistin+meropenem	Two well method Colistin +vancomycin
Enterobacter cloacae 209377	- (1,003)	+ (0.12)	-	-	Synergy	Synergy
Klebsiella pneumoniae - 145846	+ (0.5)	+ (0.12)	+	+	Synergy	Synergy
Klebsiella pneumoniae 38985	- (1,003)	+ (0.24)	-	-	Synergy	Synergy
Klebsiella pneumoniae 609815	+ (0.5)	+ (0.12)	+	+	Synergy	Synergy

Table 2 Duration of postantibiotic effect of colistin alone and combined with vancomycin or meropenem (mean value and standard deviation are shown)

Colistin alone			
E.cloacae 209377	*K.pneumoniae* 145846	*K.pneumoniae* 38985	*K.pneumoniae* 609815
Mean: 2.6 SD: 0.86	Mean: 1.26 SD: 1.15	Mean: 1.06 SD: 0.41	Mean: 0.9 SD: 0.26
Colistin + vancomycin			
E.cloacae 209377	*K.pneumoniae* 145846	*K.pneumoniae* 38985	*K.pneumoniae* 609815
Mean: 2.8 SD: 0.65	Mean: 1.38 SD: 0.68	Mean: 1.16 SD: 0.35	Mean: 1.34 SD: 0.23
Colistin+meropenem			
E.cloacae 209377	*K.pneumoniae* 145846	*K.pneumoniae* 38985	*K.pneumoniae* 609815
Mean: 6.1 SD: 1.87	Mean: 1.43 SD: 0.65	Mean: 3.16 SD: 1.75	Mean: 1.36 SD: 0.55