

Investigation of double-carbapenem efficiency in experimental sepsis of colistin-resistant *Klebsiella pneumoniae*

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ABSTRACT

OBJECTIVE: *Klebsiella pneumoniae*, a Gram-negative pathogen, especially which produces carbapenemase, is seen as a major threat to public health due to rapid plasmid-mediated spread of resistance and limited therapeutic options available for treatment. Although colistin has been recognized as a “last resort” antimicrobial for multidrug-resistant *K. pneumoniae* infections, these isolates have developed resistance to colistin as a result of its intensive use. The aim of this study was to evaluate the efficacy of double-carbapenem treatment of colistin-resistant *K. pneumoniae* experimental sepsis in mice.

METHODS: In the study, 8–10-week-old Balb-c mice were divided as control groups (positive and negative) and treatment groups (colistin, ertapenem+meropenem, and ertapenem+meropenem+colistin). Sepsis was developed in mice by an intraperitoneal injection of colistin resistant *K. pneumoniae*. Antibiotics were given intraperitoneally 3 h after bacterial inoculation. Mice in each subgroup were sacrificed with overdose anesthetic at the end of 24–48 h and cultures were made from the heart, lung, liver, and spleen. Furthermore, homogenates of lung and liver were used to detect the number of colony-forming units per gram. Bacterial clearance was evaluated in lung and liver at different time points.

RESULTS: When the quantitative bacterial loads in the lung and liver tissues are evaluated, no statistically significant difference was observed between different antibiotic treatments ($p>0.05$). All three treatment options were not effective, especially in 24 h. Only the decrease in bacterial load at the 48th h of the group treated with ertapenem + meropenem + colistin was found significant ($p<0.05$) compared to the 24 h.

CONCLUSION: In the light of these data, it was understood that double-carbapenem application was not sufficient in the treatment of experimental sepsis in mice with colistin-resistant *K. pneumoniae*. Furthermore, ertapenem + meropenem + colistin combined therapy was not found to be superior to colistin monotherapy or double-carbapenem therapy.

Keywords: Colistin resistance; experimental sepsis; *Klebsiella pneumoniae*.

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In recent times, causing hospital infections Gram-negative pathogens with several antibiotic resistances have become important health-care problem worldwide. *Klebsiella pneumoniae*, a member of *Enterobacteriaceae* family, is rod shaped with a prominent capsule, Gram negative, lactose fermenting bacillus. Multiple drug-resistant *Klebsiella* strains are increasing day by day and there are limited number of effective antimicrobial agents such as polymyxins and tigecycline in the treatment of Gram-negative pathogens [1–3]. All strains of *K. pneumoniae* are ampicillin resistant and also nosocomial isolates can be multidrug resistant due to the presence of the acquired plasmids. Mortality and treatment failure rates are becoming higher in bacteremia developing with the existence of extended-spectrum beta-lactamase (ESBL) encoding plasmid-bearing species [4–6]. On top of all this, carbapenem-resistant *K. pneumoniae* (CRKP) infections are becoming more common today with the development of different resistance mechanisms such as change in outer membrane permeability, efflux upregulation of the pumping system, or the production of beta-lactamases that hydrolyze carbapenems [7–9].

K. pneumoniae, especially which produces carbapenemase, is seen as a major threat to public health due to rapid plasmid-mediated spread of resistance and limited therapeutic options available for treatment. Colistin treatment in CRKP infections is preferred as a last resort. However, especially in recent years with the frequent use of colistin; unfortunately, colistin-resistant strains of *K. pneumoniae* have emerged. Therefore, the search for new antibiotics in the treatment of CRKP infections or the use of antibiotics (such as fosfomycin, tigecycline, ceftazidime/avibactam, meropenem/vaborbactam, meropenem, and gentamicin) in different combination has been on the agenda in terms of developing a new solution. One of the treatment approaches in CRKP infections preferred in recent years is the combination of carbapenems with colistin [10–12]. In this study, it was aimed to investigate the efficacy of both double-carbapenem usage (meropenem and ertapenem) and also double-carbapenem combination with colistin in experimental sepsis of colistin-resistant *K. pneumoniae*.

MATERIALS AND METHODS

Challenge Microorganism

The microorganism used in this study was isolated from the blood culture of a patient hospitalized in the Neurosurgery Intensive Care Unit of Erciyes Univer-

Highlight key points

- Experimental sepsis was developed in 8–10 weeks old Balb-c mice by an ip injection of colistin resistant *K. pneumoniae*.
- Double carbapenem therapy formed by using ertapenem and meropenem which were combined with colistin.
- Ertapenem+meropenem+colistin group had the lowest bacterial counts at the 48th h among all therapy groups.

sity Medical Faculty Hospital and this *K. pneumoniae* isolate was obtained from Assoc. Prof. Dr. Aycan Gundogdu, Erciyes University Medical Faculty Department of Medical Microbiology. This isolate has been identified as colistin minimum inhibitory concentration (MIC)=64, imipenem MIC=4, meropenem MIC=4, and ESBL positive.

Animals

In this study, 8–10-weeks-old Balb/c female mice weighing 20–25 g were used. These mice obtained from Erciyes University Experimental Research and Application Centre and housed there throughout the experiment. Fifty mice were included in the study, which were housed by their groups in cages and they were allowed to reach water and food as *ad libitum*. Mice environment were illuminated for 12 h day and night and the temperature of the room was adjusted to 24°C (20–25°C).

In Vivo Study

In this study, a total of 50 mice were used. These animals were divided into control (negative and positive) and treatment groups (colistin, ertapenem+meropenem, and ertapenem+meropenem+colistin). Furthermore, each group had 10 mice comprising 5 mice in subgroups for each time point of 24–48 h. With an excluding of negative control group, a dose of bacterial suspension ($0.2 \text{ ml } 12 \times 10^8 \text{ cfu/ml}$) was injected to mice intraperitoneally. Antibiotic treatments were started 3 h after the administration of bacterial inoculum doses to treatment groups. Colistimethate sodium 5 mg/kg/day in two equal doses, ertapenem 20 mg/kg/day single dose, and meropenem 100 mg/kg/day in three equal doses were administered intraperitoneally to the mice. Drug dosages were administered according to the previous experimental studies [13–15]. All mice in the subgroups were sacrificed at 24–48 h with an overdose of anesthetic (150 mg/kg ketamine hydrochloride, Pfizer, Turkey). Samples were taken from the lung, liver, spleen, and heart and cultured

TABLE 1. Bacterial counts (CFU/gr) of lung and liver tissues

	Bacterial counts (mean±SEM)			
	Lung (Log ₁₀ CFU/g)		Liver (Log ₁₀ CFU/g)	
	24 h (n=5)	48 h (n=5)	24 h (n=5)	48 h (n=5)
Positive control group (n=10)	4.935±0.371	4.932±0.766	5.714±0.295	5.478±1.412
Colistin group (n=10)	5.017±1.225	4.344±0.723	5.326±0.982	5.348±1.075
Ertapenem+ meropenem group (n=10)	4.786±0.461	4.070±1.269	5.073 ±0.559	4.362 ±0.671
Ertapenem + meropenem + colistin group (n=10)	4.683±0.586	3.871±1.072	^a 4.948 ±0.438	^a 4.176±0.603

a: Significant difference between the 24th and 48th h (p<0.05); SEM: Standard error of mean.

on trypticase soy agar (Merck, Germany) to check both for the growth of bacteria and development of sepsis. Sepsis was defined as detection of bacterial growth at least two organs. Lungs and livers of mice were homogenized in saline sterile field and after 10-fold serial dilutions, cultivated into tryptic soy agar (TSA, Merck, Germany) for quantitative culture. Bacterial loads in lungs and livers were calculated as cfu/gr at 24–48 h.

Statistical Analysis

Results were analyzed using Statistical Package for the Social Sciences (SPSS) 15.0 (SPSS Inc., Chicago, Ill., USA). Data were presented as the mean±standard error of the mean. Logarithmic transformations were used when constructing statistical models to describe the relationship between two measurements. One-way analysis of variance was used for data with a normal distribution pattern. All pairwise multiple comparison procedures were performed by the Student-Newman-Keuls method. P<0.05 was considered statistically significant.

RESULTS

Bacterial loads in lung and liver for all mice are presented in Table 1. When the quantitative bacterial loads in the lung tissues are evaluated, the highest bacterial growth in the 24th h belongs to the colistin group. Bacterial loads of lungs in the positive control group were higher than the ertapenem+ meropenem and ertapenem + meropenem + colistin groups. However, when the differences between the groups were analyzed statistically, no significant difference was found (p=0.893). At the 48th h, although the bacterial load of lung in the positive

control group was higher than all the treatment groups, this difference did not statistically significant (p=0.336), in spite of it was found to be quite high compared to the group of ertapenem + meropenem + colistin treatment.

When the quantitative bacterial loads in the liver tissue are examined, it was observed that the highest bacterial growth at the 24th h was in the positive control group while there were no significant differences between the groups in terms of statistical significance (p=0.175). At the 48th h, as in all other quantitative bacterial load calculations; in the ertapenem + meropenem + colistin group had the lowest bacterial counts but there was also no significant difference between the groups (p=0.123). When the bacterial loads of the organs at the 24th and 48th h were compared, only a statistically significant difference was found between ertapenem + meropenem + colistin groups' 24th h and 48th h bacterial loads (p=0.049).

DISCUSSION

Sepsis is defined as life-threatening organ dysfunction resulting from the reaction of the host to infection and it is an important public health problem mostly affects older adults [16]. Especially, Gram-negative pathogens cause infections that require serious health care in adults and children too. In recent years, changes in antibiotic susceptibility profiles of Gram-negative microorganisms have been observed. In parallel with this situation, the prevalence of infection is gradually increasing due to *K. pneumoniae* producing ESBL [17]. *K. pneumoniae*, the main pathogen responsible for local infections such as cystitis and pneumonia, and common infections that can result in severe sepsis and death, remains the most common factor of hospital- and community-acquired

infections [18]. This pathogen, bringing high maintenance costs with it, requires a long-term antimicrobial treatment process, which may also include parenteral antibiotic therapy. The treatment options in *K. pneumoniae* infections have been limited by the presence of plasmid-mediated resistance in *K. pneumoniae* species and also the presence of antibiotic resistance starting with ESBLs and AmpC lactamases to the development of colistin resistance. This situation brought new treatment options to the agenda [19].

Souli et al. [20] worked with 27 patients from two institutions in Athens, Greece, between 2012 and 2015. They used the combination of double carbapenem as a rescue therapy for patients infected with *K. pneumoniae* carbapenemase-2 producing *K. pneumoniae* and cannot be treated otherwise. While 22.2% of clinical isolates were sensitive to gentamicin, 18.5% to colistin or fosfomycin, and 7.4% to tigecycline, none were found sensitive to meropenem (all isolates, MIC-2 mg/l) or ertapenem. All patients had double-carbapenem combination for a 10-day period and successful results were obtained in 77.8% of patients [20]. In contrast to this, in this experimental sepsis study; the combined antibiotic, we have applied to the colistin-resistant *K. pneumoniae* performed on 50 mice, did not have a superiority to the traditional ones. Dual carbapenem combined therapy has recently been considered as a possible therapeutic strategy, since current treatment options against CRKP are limited due to high resistance to different antibiotic groups, including polymyxins [20]. In a study by Oliva et al. [21], double-carbapenem treatment consisting of ertapenem + meropenem (high dose) has been evaluated in a group of patients with CRKP infection. A total of 15 patients were included in the study and reported that 80% of patients showed good response to the double-carbapenem treatment regimen. For 11 of the 14 strains tested by the checkerboard method (78.6%) and synergism have been detected. In the study of time kill, in which ertapenem and meropenem were evaluated, there was no significant difference ($p > 0.05$) as a result of use alone, in the test using 1 MIC meropenem + 1 MIC ertapenem, a significant decrease was shown in \log_{10} CFU/ml compared to other combinations of use [21]. Incompatible with the results of the study; in this experimental sepsis study, it was found that the use of double carbapenem does not provide an advantage over other treatments. It is thought that this different result has emerged because the isolate used in the study was resistant to both colistin and meropenem. In another study conducted by Oliva et al.

[22], in 2017, activity of different antibiotic combinations with and without colistin (colistin + meropenem/doripenem, colistin + tigecycline, colistin + rifampicin, gentamicin + tigecycline, colistin + rifampicin, gentamicin + tigecycline + meropenem + meropenin + tropecycline + antimicrobial combination) in 39 CRKP strains has been evaluated *in vitro*. Besides, triple combinations of colistin + meropenem + tigecycline also have been tried. In addition to this, double-carbapenem application was tested by conducting time kill studies for meropenem + ertapenem. Gentamicin-based combinations showed high levels of synergy, while meropenem + ertapenem exhibited synergistic effect in just 12/39 strains. Combined treatments using $2 \times \text{MIC}$ meropenem were found more bactericidal and synergistic than combined use of $1 \times \text{MIC}$ meropenem [22]. In this *in vivo* study, when lung and liver bacterial loads were evaluated in the 24th and 48th h groups; although bacterial loads in treatment groups decreased especially at the 48th h, there was no statistically significant difference between the positive control group and the treatment groups. At the 48th h, only bacterial burden in liver was decreased statistically significantly in the ertapenem + meropenem + colistin group ($p < 0.05$). Carbapenem resistance due to OXA-48 enzymes in *K. pneumoniae* increases, especially in the Middle East and European regions, and there are limited treatment options. Evren et al. [23] evaluated the *in vitro* synergistic activity of fosfomycin against *K. pneumoniae* strains producing OXA-48 in combination with imipenem, meropenem, colistin, and tigecycline. Twelve different carbapenem-resistant OXA-48 producing *K. pneumoniae* isolates were included in that study and the synergistic activity of imipenem, meropenem, colistin and tigecycline, and fosfomycin was evaluated using the checkerboard method. It has been found that the combination of fosfomycin shows synergistic effect with imipenem, meropenem, and tigecycline at 42%, 33%, and 33%, respectively. The combination of fosfomycin and colistin has completely antagonistic effect against all strains. It was also reported that no statistically significant difference was found between imipenem, meropenem, and tigecycline with fosfomycin *in vitro* synergistic activities ($p > 0.05$) [23]. In this present study, similar to the study mentioned, ertapenem + meropenem + colistin combination showed a considerable synergistic effect in reducing the bacterial load in the liver tissue at the 48th h compared to the ertapenem + meropenem combination. However, no statistically significant difference was found between different treatment options ($p > 0.05$). Colistin

is the last and sometimes the only therapeutic option for CRKP infections due to the increasing resistance and limited choice of antibiotics. Unfortunately, combined antibiotic therapy came to the fore-after pathogens also developed resistance in colistin monotherapy [24]. Su et al. [25] tested the efficacy of colistin, trimethoprim-sulfamethoxazole, and colistin-trimethoprim-sulfamethoxazole *in vitro* in 36 CRKP clinical isolates. The combination of trimethoprim-sulfamethoxazole and colistin showed strong synergistic and bactericidal activity compared to colistin and trimethoprim-sulfamethoxazole as treatment options [25]. As in this study, the combined antibiotic treatment of ertapenem-meropenem-colistin that we have applied has only been effective in reducing the bacterial liver load at 48 h ($p < 0.05$).

In this experimental sepsis study with colistin-resistant *K. pneumoniae*, the effectiveness of double-carbapenem administration and its combination with colistin was investigated. According to the data, it was observed that the combined treatments of both ertapenem + meropenem and ertapenem + meropenem + colistin reduce the bacterial load at the 24th h compared to colistin monotherapy and this reduction continued until the 48th h, but no bacterial eradication occurred in any group during this period. It was determined that double-carbapenem administration was not effective enough in the treatment of colistin-resistant *K. pneumoniae* experimental sepsis. At the same time, ertapenem + meropenem + colistin combined therapy was not found to be superior to other options of pharmacological treatments (colistin and ertapenem + meropenem). Therefore, if combined therapy is desired in clinical practice, it is thought that it would be more appropriate to apply different combinations of antibiotics in colistin-resistant strains instead of combined therapy with colistin + ertapenem + meropenem. Furthermore, additional experimental animal studies were thought to be useful to determine the therapeutic value of combined antibiotic therapy that can be used in colistin-resistant *K. pneumoniae* infections. Furthermore, new discovery of antibiotics is not expected nowadays and innovative therapeutic options such as cell-based treatments (mesenchymal stem cells, exosomes, etc.) and bacteriophages therapy or monoclonal antibodies can be considered in the treatment of these kinds of difficult infections [26, 27].

Ethics Committee Approval: The Erciyes University Animal Experiments Local Ethics Committee granted approval for this study (date: 11.01.17, number: 17/005).

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