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# A novel β-lactam-aminoglycoside combination in veterinary medicine: The co-use of ceftiofur and gentamicin to combat resistant *Escherichia coli*

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**ABSTRACT:** The focus of this study was to evaluate the efficacy of ceftiofur+gentamicin combination to increase the success of antimicrobial inhibition against resistant *Escherichia coli (E.coli)* strains isolated from animals. Interaction between drugs was determined using checkerboard method and the fractional inhibitory concentration index was interpreted as synergism, antagonism and indifference. The combination was defined as bactericidal or bacteriostatic based on the minimum bactericidal test results. Mutant prevention concentration test was used to evaluate the resistance tendency suppression potential of the combination. The synergistic effect was detected for all *E. coli* strains by the checkerboard method; even the strains that were resistant to the individual compounds in the combination. Based on the results of minimum bactericidal concentration test, the combination exhibited bactericidal effect against all *E. coli* strains. In addition, the individual mutant prevention concentrations of ceftiofur and gentamicin decreased up to 125-fold by using the combination for the inhibition of resistant *E. coli* strains. The results indicated that killing potential of co-use of the compounds is much stronger than their individual use. The combination achieved to decrease the mutant prevention concentrations and this can reduce the risk of emergence of single mutations during treatment done with suggested doses.

***Keywords*:** Ceftiofur, gentamicin, resistant *Escherichia coli*, antimicrobial combination

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## INTRODUCTION

As a member of the intestinal microbiota, *Escherichia coli* (*E. coli*) is responsible for many intestinal or extraintestinal opportunistic infections in humans and animals (Hopkins et al., 2005; Ingerson Mahar and Reid, 2011). Due to the lack of effective therapeutic options in the veterinary field, the treatment of infections caused by resistant *E. coli* is a major concern. Resistance gene-based dose optimization is one pragmatic approach to combat resistant bacteria. However, resistance gene variability is a limiting factor affecting the success of treatment (Cengiz et al., 2013). Antimicrobial combinations should be considered for the treatment of infections caused by various resistance gene-containing strains. Combination therapy involves the co-use of two or more compounds with synergistic interactions and increases the treatment potential of the infection provided that the combination does not lead to increased toxicity (Sun et al., 2016; Tamma et al., 2012). The synergistic activity of β-lactam plus aminoglycoside has been widely investigated against many infectious agents (Tamma et al., 2012). β-lactams inhibit peptidoglycan biosynthesis, disrupting bacterial cell wall synthesis and increasing the influx of aminoglycoside to block ribosomal protein synthesis (Kohanski et al., 2010). Ceftiofur (CEF) is a semi-synthetic member of third generation cephalosporins and is resistant to several β-lactamases (Meegan, 2013). As an aminoglycoside, gentamicin (GEN) inhibits protein synthesis by binding to the bacterial 30S ribosomal subunit. Based on the results of recent studies, β-lactam+aminoglycoside remains an effective therapeutic option for the treatment of critical clinical cases (Theelen, 2019). The aim of this study was to determine the efficacy and resistance prevention potential of CEF+GEN as a novel β-lactam+aminoglycoside combination. Therefore, the interaction between CEF and GEN was determined, and the bactericidal characteristics of the combination and its resistance tendency toward *E. coli* strains under the combination pressure were evaluated.

## MATERIAL AND METHODS

For isolation of *E. coli*, samples collected from cattle were directly spread onto Eosine Methylen Blue Agar-Levine and MacConkey Agar, and incubated under aerobic conditions. Candidate *E. coli* colonies were identified by API 20 E, and results were evaluated by API-Web system. Broth microdilution testing was performed to determine the minimum inhibitory concentrations (MICs) of the antimicrobials according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2016). Antimicrobials were obtained as an analytical standard powder (Fluka). MICs were defined as the minimum concentration of antibiotic that inhibited growth of the organism. PCR and qRT-PCR were used to characterize the presence and expression levels of molecular mechanisms of the resistance as described previously (Sahinturk et al., 2016). Briefly, *gyrA*, *parC* and *oqxB* genes were PCR amplified using specific primers and PCR products of *gyrA* and *parC* were sequenced. qRT-PCR was used to determine expression level of *marA*, *acrB*, *soxS* and *ompF* genes. *E. coli* AG100 was used as a control strain. Overexpression was defined as a 1.5-fold increase in the genes.

Six *E. coli* isolates with various resistance determinants and profiles were used in this study (Table 1). *E. coli* E245 was resistant to both CEF and GEN, and *E. coli* E246 was resistant to GEN only. The 4/6 of *E. coli* strains were susceptible to CEF and GEN. In this study, the efficacy of the combination was tested against *E. coli* strains resistant to compounds in the combination and the resistance preventive potential of the combination was evaluated against all *E. coli* strains. The interaction between drugs was determined by the checkerboard test. The fractional inhibitory concentration indexes (FICIs) provided from the checkerboard test were interpreted as follows: FICI≤ 0.5 = synergy; FICI > 4.0 = antagonism; and FICI > 0.5-4 = indifference (Table 1) (Elipoulos and Moellering, 1996). The minimum bactericidal concentration (MBC) and mutant prevention concentration (MPC) of the combination were determined as previously described (Blondeau et al., 2001; Hansen and Blondeau, 2005). The MBC was defined as the lowest concentration showing ≥ 99.9 % death compared with the initial inoculum. The combination was defined as bactericidal and bacteriostatic for MBC:minimum inhibitory concentration (MIC) ratios of 1-4 and ≥ 8, respectively (Table 2) (Maaland et al., 2015). The MPC was determined as the concentration that allowed no growth of bacteria at the end of the 72-h incubation period (Table 2). The inoculum densities used for MIC-MBC and MPC determination were $10^7$ cfu/ml (equivalent to 0.5 MacFarland turbidity) and $10^{10}$ cfu/ml, respectively.

## RESULTS

The FICI values of the combination therapy for *E. coli* strains are shown in Table 1. The FICIs of the combination ranged from 0.1 to 0.5. A synergistic ef-

fect was detected for all *E. coli* strains by the checkerboard method. Based on the MBC:MIC ratios, the combination exhibited bactericidal effect against all *E. coli* strains (Table 2). The MBC:MIC ratio was two for *oqxB* containing-*E. coli* E306 and one for the rest of *E. coli* strains. The individual MPCs ranged from 32 μg/ml to 512 μg/ml for CEF and from 16 μg/ml to 4096 μg/ml for GEN (Table 2). The MPCs of the combination ranged from 0.256 μg/ml to 64 μg/ml. The individual MPCs of CEF and GEN decreased by up to 128-fold using the combination for the inhibition of resistant *E. coli* strains. The MPC:MIC ratio of the combination ranged from 2 to 32 for *E. coli* strains. The highest MPC:MIC ratio was detected for the most susceptible isolate, *E. coli* E175.

**Table 1.** Resistance profiles and mechanisms of *E. coli* and checkerboard data with the interpretations

| | | Resistance mechanism | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | QRDR[a] | | PMQR[b] | MDR[c] | | | |
| Isolate ID | Resistance profile[d] | gyrA | parC | oqxB | marA | acrB | soxS | ompF |
| E175 | SMX | | | | ↓↓ | ↓ | ↓↓ | ↑ |
| E222 | NAL, CIP, SMX, TMP, TET, OTC, CHL | Ser83Leu | Ser80Ile | | ↓↓ | ↓↓ | ↑↑ | ↑ |
| E245 | NAL, CIP, ORB, GAT, AMP, CEF, GEN, TET, OTC, ERY, CHL | Ser83Leu Asp87Glu | | | ↑ | ↑↑ | ↑↑ | ↑ |
| E246 | NAL, GAT, AMP, TMP, GEN, TET, OTC, CHL, CST | Ser83Leu | | | ↑↑ | ↑↑ | ↑↑↑ | ↓↓↓ |
| E269 | NAL, SMX, TMP, TET, OTC, CST | | | | ↓↓ | ↓ | ↑↑ | ↑ |
| E306 | NAL, CIP, ORB, AMP, TMP, TET, OTC, ERY, CHL | Ser83Thr | | + | ↓↓ | ↓↓ | ↑ | ↑ |

[a]: quinolone resistance determining region
[b]: plasmid-mediated quinolone resistance
[c]: compared to AG100; ↑: 1–5 fold increased; ↑ ↑: 5–10 fold increased; ↓: 1–5 fold decreased; ↓↓: 5–10 fold decreased; ↓↓↓: ≥ 10 fold decreased.
[d]: SMX: sulfamethoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, SMX: sulfamethoxazole, TMP: trimethoprim, TET: tetracycline, OTC: oxytetracycline, CHL: chloramphenicol, ORB: orbifloxacin, GAT: gatifloxacin, AMP: ampicillin, CEF: ceftiofur, ERY: erythromycin, CST: colistin

**Table 2.** Pharmacodynamic profile of ceftiofur+gentamicin combination

| Isolate ID | Pharmacodynamic parameters | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MICs (μg/ml) | | FICI | | MBCs (μg/ml) | | | MBC:MIC | | | MPCs (μg/ml) | | | MPC:MIC | | |
| | CEF S≤8, R>8 | GEN S≤2, R>4 | Conc. (μg/ml) | Intp.[a] | CEF | GEN | CEF +GEN | CEF | GEN | CEF +GEN | CEF | GEN | CEF +GEN | CEF | GEN | CEF +GEN |
| E175 | 1 | 4 | 0,128/1 | 0,3 | 8 | 4 | 0.128/1 | 8 | 1 | 1 | 128 | 128 | 4/32 | 128 | 32 | 32 |
| E222 | 2 | 4 | 0,128/1 | 0,3 | 8 | 4 | 0.128/1 | 4 | 1 | 1 | 128 | 128 | 2/16 | 64 | 32 | 16 |
| E245 | 16 | 128 | 1/16 | 0,1 | 64 | 256 | 1/16 | 4 | 2 | 1 | 256 | 4096 | 4/64 | 16 | 32 | 4 |
| E246 | 2 | 64 | 0,512/8 | 0.3 | 2 | 64 | 0.512/8 | 1 | 1 | 1 | 128 | 512 | 1/16 | 64 | 8 | 2 |
| E269 | 1 | 4 | 0,256/1 | 0,5 | 1 | 4 | 0.256/1 | 1 | 1 | 1 | 32 | 32 | 2/8 | 32 | 8 | 8 |
| E306 | 4 | 2 | 0,512/0,064 | 0,1 | 4 | 2 | 1/0.128 | 1 | 1 | 2 | 512 | 16 | 2/0.256 | 128 | 8 | 4 |

[a]: Synergistic interaction

## DISCUSSION

β-lactam-aminoglycoside combinations are primarily preferred to expand the spectrum of action and have synergistic effects. These combinations have been used as an initial therapeutic option since 1984 (Rafei, 2018). All aminoglycosides can cause varying degrees of ototoxicity and nephrotoxicity. Nephrotoxicity is the most common adverse effect of aminoglycoside treatment (Prescott et al., 2013). β-lactam-aminoglycoside combinations are also considered a less toxic therapeutic option in addition to having potential synergistic and expanded spectrum activities. In veterinary medicine, aminoglycosides are most often combined with penicillin (EMA, 2017). Cephalosporins are also synergistic with aminoglycosides for the treatment of neutropenic human patients with infections caused by resistant strains (Prescott et al., 2013; Rafei, 2018). Therefore, the focus of this study was to compare the efficacy of the individual use of the antimicrobials with synergistically acting CEF+GEN combination against *E. coli* strains with resistance to many antimicrobials. The results of this study showed that CEF+GEN effectively inhibited *E. coli* strains with varying susceptibility profiles and different resistance determinants. Multidrug resistance (MDR) is a potential limiting factor in the treatment of infectious bacteria. FICI data showed that the CEF+-GEN combination could more effectively inhibit *E. coli* strains resistant to compounds in the combination and those resistant to many other antimicrobials from different groups. The use of a second antimicrobial can reduce the risks for patients infected with MDR organisms and will provide adequate coverage for potential pathogens causing an infection (Tamma et al., 2012). In clinical trials, the efficacy of β-lactam-aminoglycoside combinations has also been shown. For example, the combination of amikacin and ampicillin was found to be suitable for the treatment of foals with sepsis (Theelen, 2019). Similarly, the CEF+GEN combination can also be used to increase the clinical success of treatments for infections caused by *E. coli* strain even when it is resistant to CEF and/or GEN. Noel et al. (2018) showed that addition of amikacin to ceftalazone/tazobactam bacterial clearance were increased and emergence of resistance to ceftalazone/tazobactam was prevented. Tschudin-Sutter et al. (2018) indicated that combination therapy with β-lactam+aminoglycoside might improve mortality of *Pseudomonas aeruginosa* causing blood stream infection. Based on the results of previous studies, CEF+GEN combination can be preferred without risk of emergence of resistance to inhibit various resistance determinant-carrying *E. coli* strains instead of monotherapy with CEF or GEN alone. The results of this study showed that the MICs of the CEF+GEN combination were equal to their MBCs. Based on the MBC:MIC ratio, the combination was defined as bactericidal against all *E. coli* strains. The co-use of CEF and GEN caused a decrease in their individual MBCs by up to 64-fold. The MBCs of CEF and GEN were below the clinical breakpoint of CEF (S≤8 μg/ml, R>8 μg/ml) for all *E. coli* strains and below that of GEN (S≤2 μg/ml, R>4 μg/ml) for four *E. coli* strains. The genetic mechanism of resistance can be determinative for the bactericidal activity of antimicrobials (Cengiz et al., 2013). Therefore, sustaining of bactericidal activity of each compound in the combination is crucial by decreasing their individual MBCs. The other benefit of the CEF+GEN combination was a change in the individual MPCs of CEF and GEN. The MPCs decreased for all *E. coli* strains, and the MPC:MIC ratio decreased for four strains. The distance between

MPC and MIC is determinative for the emergence of resistant sub-populations during antimicrobial therapy (Hansen and Blondeau, 2005). The lowering MPC:MIC ratio may reduce the risk of emergence of resistance or evolving of resistant strains to highly resistant strains. As a clinical perspective, this improvement may increase the success of antimicrobial therapy applied by CEF+GEN combination.

## CONCLUSION

In conclusion, the combination decreased the MPCs and narrowed the range between the MIC and MPC. This improvement can reduce the risk of the emergence of single mutations during treatment with currently approved doses. In addition, the bactericidal effects of the compounds sustained at concentrations below the clinical breakpoints of the individual compounds by their use in a combination. This result indicates that the killing potential of the co-use of the compounds is much stronger than their individual use.

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## CONFLICT OF INTEREST STATEMENT

None declared by the authors.

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