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The *in vitro* activity of danofloxacin plus ceftiofur combination: implications for antimicrobial efficacy and resistance prevention

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Abstract

Due to the high prevalence of multi-drug resistant bacteria, combination therapy is an efficient choice for treatment of infections caused by highly resistant strains. In this study, the efficacy of ceftiofur plus danofloxacin combination was investigated against resistant *Escherichia coli*. The interaction between the two drugs was determined by checkerboard tests and time-kill assays. The combination was defined as bactericidal or bacteriostatic based on the minimum bactericidal concentration test results. Mutant prevention concentration test was used to evaluate the resistance tendency suppression potential of the combination. The combination had a synergistic effect against 83.00% of the isolates as verified by the checkerboard and time-kill assays. The combination was defined as bactericidal against all *E. coli* strains, since minimum bactericidal concentration: minimum inhibitory concentration ratios were below four thresholds and also markedly reduced mutant prevention concentration values of ceftiofur up to 4000-fold compared to its single use. Ceftiofur plus danofloxacin combination inhibited growth of *E. coli* strains which were resistant to ceftiofur or newer generation of fluoroquinolones. Our results suggest that ceftiofur plus danofloxacin combination has a bactericidal characteristic and can be an important alternative for the treatment of infections caused by resistant *E. coli*.

Introduction

Escherichia coli is frequently responsible for several common bacterial infections in human and animals. The prevalence of multi-drug resistant (MDR) *E. coli* strains is increasing rapidly due to the spread of mobile genetic elements and, therefore, single drug clinical treatment strategies may not be effective against resistant bacteria.[1,2] Based on the scientific reports and experiences from clinical treatments, combination therapy may be more effective to inhibit MDR bacteria.[1] For instance, synergistic activity of β-lactam-aminoglycoside combinations against Gram-negative bacteria has been best established and similar synergism has been shown for β-lactams (penicillins and cephalosporins) and fluoroquinolone (FQ) combinations.[1] *In vitro* synergism rate between β-lactams and FQs against Gram-negative organisms has ranged from 17.00% to 82.00%.[2] However, there is no reported drug interaction between β-lactams and FQs in veterinary field.

Ceftiofur (CEF) is a third-generation cephalosporin antibiotic and used for the treatment of bacterial infections of respiratory tract in cattle and swine. The CEF exerts its anti-bacterial action by inhibition of bacterial cell wall synthesis.[3] In *Salmonella* and *E. coli* isolates from animals, resistance to third-generation cephalosporins was uncommon.[4] Danofloxacin (DAN) is a synthetic second-generation FQ with a broad-spectrum anti-bacterial activity and used in the treatment of respiratory disease in chickens, cattle and pigs. The DAN acts through inhibition of bacterial DNA-gyrase, an enzyme which is also referred as topoisomerase II and is responsible for maintaining the DNA topography of.[5] In *E. coli*, high resistance rates (> 60.00%) were observed for second-generation FQs including DAN.[6]

The objective of this study was to investigate the activity of CEF plus DAN combination against MDR *E. coli* from animals. The pharmacodynamic variables such as

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minimum bactericidal concentration (MBC) and mutant prevention concentration (MPC) were determined to demonstrate the therapeutic efficacy of the combination as suggested by European Medicine Agency Guideline.[7] Before the determination of pharmacodynamics variables, fractional inhibitory concentration index (FICI) was used to evaluate the interaction between CEF and DAN.

Materials and Methods

Bacterial strains. For isolation of *E. coli* strains, samples collected from cattle were directly spread onto eosin methylene blue agar-Levine (Becton Dickinson, Sparks, USA) and MacConkey agar (Becton Dickinson), and incubated under aerobic conditions. Candidate *E. coli* colonies were identified by API 20 E (BioMerieux Inc., Hazelwood, USA), and results were evaluated by API-Web system. The susceptibility profiles of the selected six *E. coli* isolates are shown in Table 1.

Fractional inhibitory concentration index. The FICIs of the CEF (Sigma-Aldrich, Taufkirchen, Deutschland) plus DAN (Sigma-Aldrich) combination were determined using the checkerboard method.[8] Dilutions ranging from 1/32x minimum inhibitory concentration (MIC) to 4x MIC were tested for each antimicrobial agent. The FICI was interpreted as follows: FICI ≤ 0.50 = synergy, FICI > 4.00 = antagonism and FICI > 0.50 – 4.00 = indifference/additive. The FIC index/indices were calculated as follows:

FIC_A = MIC drug A in combination / MIC drug A alone

FIC_B = MIC drug B in combination / MIC drug B alone

$FICI / \Sigma FIC = FIC_A + FIC_B$.

Time-kill experiments. Time-kill experiments were performed as described previously.[9] Synergy was defined as a ≥ 2.00 $\log_{10}$ decrease in the colony count at 6 or 24 hr with the combination treatment compared to the initial inoculum. The drug combination was considered to be antagonistic if there was a ≥ 2.00 $\log_{10}$ increase in CFU mL^{-1}, and a < 2.00 $\log_{10}$ change in CFU mL^{-1} was interpreted as no interaction.

Minimum bactericidal concentration. The MBCs were determined on six representative isolates as previously described.[10] The MBC was defined as the lowest concentration showing ≥ 99.90% killing compared to the initial inoculum. The CEF plus DAN combination was defined as bactericidal and bacteriostatic for MBC: MIC ratios 1 - 4 and ≥ 8, respectively.[11]

Mutant prevention concentration. The MPC of the CEF plus DAN was determined based on the method of Blondeau *et al*.[12] The *E. coli* isolates were incubated overnight at 37.00 ˚C in 100 mL of Mueller-Hinton broth (MHB; Becton Dickinson), after which the cultures were centrifuged at 9,000 rpm for 15 min. The supernatant was discarded and pellet was re-suspended in 3.00 mL of MHB to achieve > 10^{10} CFU mL^{-1}. A 100-µL aliquot of this culture was used to inoculate plate count agar plates containing a 1x FIC-64x FIC range of the CEF plus DAN combination.

The plates were incubated at 37.00 ˚C for 72 hr and examined every 24 hr for growth of *E. coli.* The MPC was determined as a concentration that allowed no growth of bacteria at the end of the 72-hr incubation. Each experiment was conducted in duplicate.

Table 1. Phenotypic and molecular characterization of *E. coli* strains, checkerboard and time-kill data with the interpretations.

Isolate ID***	Resistance mechanism							Checkerboard			Time-kill			
	QRDR*		PMQR**	MDR				DAN/CEF (µg mL^{-1})	FICI	Interpretation	Log reduction		Interpretation	
	gyrA	*parC*	*oqxB*	*marA*	*acrB*	*soxS*	*ompF*	MIC			6 hr	24 hr	6 hr	24hr
E175				↓↓	↓	↓↓	↑	0.002/0.512	0.54	SYN	3.04	5.24	SYN	SYN
E222	Ser83Leu	Ser80Ile		↓↓	↓↓	↑↑	↑	0.512/0.512	0.50	SYN	2.11	2.50	SYN	SYN
E245	Ser83Leu, Asp87Glu			↑	↑↑	↑↑	↑	0.512/2	0.38	SYN	0.46	0.59	ADD	ADD
E246	Ser83Leu			↑↑	↑↑	↑↑↑	↓↓↓	0.256/0.256	0.38	SYN	0.46	0.40	ADD	ADD
E269				↓↓	↓	↑↑	↑	0.032/0.512	0.54	SYN	2.09	4.68	SYN	SYN
E306	Ser83Thr		+	↓↓	↓↓	↑	↑	1/0.064	1.01	ADD	3.23	5.78	SYN	SYN

DAN: Danofloxacin, CEF: ceftiofur MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration, SYN: Synergism, ADD: Additive.

MDR: Multidrug resistance; compared to AG100; ↑: 1–5 fold increased; ↑ ↑: 5–10 fold increased; ↓: 1–5 fold decreased; ↓↓: 5–10 fold decreased; ↓↓↓: ≥ 10 fold decreased.

* Quinolone resistance determining region; **Plasmid-mediated quinolone resistance; *** The antimicrobial resistance profiles of the *E. coli* strains: E175: Sulfamethoxazole; E222: Nalidixic acid, ciprofloxacin, sulfamethoxazole, trimethoprim, tetracycline, oxytetracycline, and chloramphenicol; E245: Nalidixic acid, ciprofloxacin, orbifloxacin, gatifloxacin, ampicillin, ceftiofur, tetracycline, oxytetracycline erythromycin and chloramphenicol; E246: Nalidixic acid, gatifloxacin, ampicillin, trimethoprim, gentamicin, tetracycline, oxytetracycline, chloramphenicol and colistin; E269: Nalidixic acid, , sulfamethoxazole, trimethoprim, tetracycline, oxytetracycline and colistin; E306: Nalidixic acid, ciprofloxacin, orbifloxacin, ampicillin, trimethoprim, tetracycline, oxytetracycline, erythromycin and chloramphenicol.

Results

Synergy test results of the combination are given in Table 1. The FICI values for *E. coli* isolates ranged from 0.38 to 1.01. The combination synergistically acted against five of the *E. coli* isolates. Indifference was detected against one *E. coli* isolate, E306. Antagonism was not observed for any of *E. coli* isolates by the checkerboard method.

The *in vitro* activity of the combination against *E. coli* isolates, based on the time-kill method, is shown in Figure 1 and Table 1. At both incubation time points (6- and 24-hr), the combination therapy resulted in a ≥ 2.00 $\log_{10}$ reduction in viable counts against four of the *E. coli* isolates and the synergism rate was recorded as 66.00%. Indifference (< 2.00 $\log_{10}$) was observed for the other two *E. coli* isolates. Regrowth was not observed for any of *E. coli* isolates after 24-hr incubation.

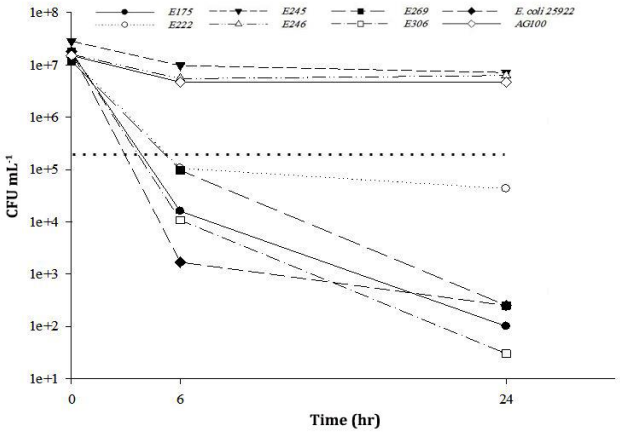

Fig. 1. Time-kill curve of ceftiofur plus danofloxacin combination against multi-drug resistant *E. coli* isolates and control strain.

The MBCs showed 1000-fold decrease for CEF when combined with DAN. In one *E. coli* isolate (E175), MBC value of DAN increased from 0.06 µg mL^{-1} to 2.00 µg mL^{-1}. For the rest of *E. coli* isolates, at least 2-fold reduction was determined for DAN. The MBC:MIC ratios of the combination were one for three *E. coli* isolates (E246, E269 and E306), two for one *E. coli* isolate (E245) and four for two *E. coli* isolates (E175 and E222), and the combination showed bactericidal effect against all studied *E. coli* isolates (Table 2).

The most remarkable reduction was noted for MPC values of CEF in the combination compared to its single use (Table 2). The MPC values of CEF in the combination ranged from 0.12 µg mL^{-1} to 4.00 µg mL^{-1}. In MPC tests, the combination could reduce the MPC of CEF up to 4000-fold. Similar to MBC test results, an increase of MPC was observed for the same *E. coli* isolate, E175. At least 2-fold decrease was determined in the MPC values of DAN in other *E. coli* isolates (Table 2). Mutant prevention index (MPI) values of the combination ranged from 4.00 to 128. The MPI values of the combination were lower than CEF for five *E. coli* isolates and DAN for three *E. coli* isolates.

Discussion

Recent studies have shown that β-lactam plus FQ combination has *in vitro* and *in vivo* synergy against extended-spectrum β-lactamase-producing *E. coli* and *Pseudomonas aeruginosa* isolates.[13,14] In addition, the combinations of these two groups reduce the likelihood of resistance in Gram-negative bacilli compared to monotherapy.[13,15] Al-Hasan *et al*. have stated that β-lactam plus FQ combination therapy for bacteremia caused by Gram-negative bacilli appears more promising.[16] The results of our study showed that in checkerboard test CEF plus DAN combination was more effective against MDR *E. coli* isolates due to synergistic interaction between the compounds. Synergistic interaction between CEF and DAN against MDR *E. coli* isolates was lower in time-kill assays (66.00%). The data provided from checkerboard tests and time-kill assays can be different due to the protocols followed.[17,18] Different results can be recorded for compounds from the same antimicrobial class even if the same synergy test is applied.[15,19,20] Drago *et al*.[19] have shown that cephalosporin (ceftriaxone and cefotaxime) plus FQ (levofloxacin and moxifloxacin) combination has synergistic effect against resistant *Streptococcus pneumoniae* and this combination was suggested as an alternative to the previous combinations. They also found that levofloxacin and moxifloxacin showed a different rate of synergy with parenteral cephalosporins. Since the mechanism underlying the activity of antibiotics in combination should be the same for

Table 2. Pharmacodynamic profile of ceftiofur plus danofloxacin combination.

Isolate ID	Pharmacodynamic parameters														
	MICs (µg mL^{-1})			MBCs (µg mL^{-1})			MBC: MIC			MPCs (µg mL^{-1})			MPIs		
	DAN	CEF	DAN+CEF	DAN	CEF	DAN+CEF	DAN	CEF	DAN+CEF	DAN	CEF	DAN+CEF	DAN	CEF	DAN+CEF
E175	0.064	1.00	0.002/0.512	0.064	8.00	2.00/0.008	1.00	8.00	4.00	0.512	128	64.00/0.256	8.00	128	128
E222	2.00	2.00	0.512/0.512	8.00	8.00	2.00/2.00	4.00	4.00	4.00	8.00	128	4.00/4.00	4.00	64.00	8.00
E245	2.00	16.00	0.512/2.00	8.00	64.00	4.00/1.00	4.00	4.00	2.00	32.00	256	8.00/2.00	16.00	16.00	4.00
E246	1.00	2.00	0.256/0.256	4.00	2.00	0.256/0.256	4.00	1.00	1.00	32.00	128	1.00/1.00	32.00	64.00	4.00
E269	1.00	1.00	0.032/0.512	4.00	1.00	0.032/0.512	4.00	1.00	1.00	16.00	32.00	0.128/2.00	16.00	32.00	4.00
E306	1.00	4.00	1.00/0.064	4.00	4.00	0.512/0.032	4.00	1.00	1.00	4.00	512	2.00/0.128	4.00	128	4.00

MIC: Minimum inhibitory concentration, DAN: Danofloxacin, CEF: Ceftiofur, MBC: Minimum bactericidal concentration, MPC: Mutant prevention concentration, MPI: Mutant prevention index

levofloxacin and moxifloxacin, the observed difference could be attributable to the difference in intrinsic activity of two molecules.[19] The MIC is commonly used to compare *in vitro* intrinsic activity of antibiotics.[21]

The *in vitro* antimicrobial activity of drugs is usually assessed by determining the MIC and MBC after overnight aerobic incubation of a standard and inoculum size of bacteria in low protein liquid medium at pH 7.20. For bactericidal drugs, the MBC is usually the same as the MIC or generally not more than four-fold greater. The MBC of bacteriostatic drugs can be many-fold greater than the MICs.[22] The results of this study showed that MBC/MIC ratios of CEF plus DAN combination for all MDR *E. coli* isolates ranged from 1 to 4 and were equal or below four-fold threshold. Therefore, the CEF plus DAN combination can be classified as bactericidal. The bactericidal effect of CEF plus DAN combination was also seen at MICs. For instance, MBCs of CEF plus DAN combination were equal to the MICs for three of six MDR *E. coli* isolates. Moreover, MBCs of CEF plus DAN were below the resistance clinical breakpoints (R > 8 for CEF and R > 2 for DAN). The MBC and MIC provide no information on time course of the antimicrobial effect at fluctuating drug concentrations and are determined using standard bacterial inoculum (10^5 CFU mL^{-1}). The MPC is the concentration of a drug preventing the growth of single-step mutant present in a large bacterial population and has potential to allow testing antimicrobial efficacy in bacterial densities at infection site (10^{8-10} CFU g^{-1} of tissue or pus).[19,23,24] Antimicrobial combinations with a low MPC/MIC ratio (MPI) have high killing activity against resistant pathogens. In addition, combination therapy can prevent emergence of resistant bacteria when MPCs are below the clinical breakpoints.[25] The results of this study showed that MICs of CEF resistant *E. coli* strains with double mutations in *gyrA* can be reduced up to eight times when CEF is combined with DAN. The MPC of CEF in the combination was also lower than the MIC of CEF alone. In contrast, the highest MPI value of CEF plus DAN was observed for the most susceptible isolate, *E. coli* E175, which has no quinolone resistance-determining region mutations. There is no explanation or data available for the enhanced MBC and MPC values of *E. coli* E175. These data indicated that MPCs were not always accurately predicted from the MIC values. The difference between MPC and susceptibility data is probably due to the used inoculum densities.[26] The MPC measurement is fundamentally different from standardized susceptibility measurements because it utilizes an inoculum accounting for the first-step resistant cells presence.[26] The low MPI values observed for five of six MDR *E. coli* isolates indicated that CEF plus DAN has an important potential to reduce the tendency of MDR *E. coli* isolates to become highly resistant or untreatable pathogens.

Due to the synergistic interaction, CEF plus DAN combination should be considered for the inhibition of MDR *E. coli* isolates from animals, even for *E. coli* isolates resistant to CEF or newer generation of FQ such as orbifloxacin and gatifloxacin. The combination has a bactericidal characteristic and this makes the combination an important alternative for the treatment of infections caused by MDR *E. coli* in vitally important tissues and organs. Further studies should be designed to show the *in vivo* activity of the combination in order to determine the effect of pharmacokinetic variability and host defence system on the efficacy of CEF plus DAN combination.

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Conflict of interest

None declared.

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