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NOTE

Assessment of synergistic interactions of danofloxacin and orbifloxacin against quinolone-resistant *Escherichia coli* isolated from animals by the checkerboard and time-kill methods

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In recent years, detection of fluoroquinolone (FQ) resistance determinants in *Escherichia coli* (*E. coli*) isolated from animals indicated that this is an important public health issue and can create a high risk for the treatment of infectious diseases at the recommended available dosage regimens.[1] In Gram-negative bacteria, resistance to FQs primarily occurs from gene mutations in the quinolone resistance-determining region (QRDR) of the genes encoding the drug target enzymes (DNA gyrase and topoisomerase IV).[2] In addition, the pentapeptide repeat proteins (QnrA, QnrB and QnrS) increase MIC of FQ for *E. coli* (0.125–16 $\mu g\,ml^{-1}$) by protecting DNA gyrase from inhibition.[3,4]

FQs have been approved for use in the treatment of infectious diseases around the world. Danofloxacin, a member of second-generation FQs, is a synthetic FQ with broad-spectrum antibacterial activity. It is used in the treatment of respiratory disease in chickens, cattle and pigs. Orbifloxacin is a member of third-generation FQs and developed for use in companion animal medicine. In canine practice, orbifloxacin is indicated for the treatment of various infections, including urinary, skin and otitis infections.

There are a few reported drug interactions among FQs that have veterinary significance.[5–7] Interactions between main compounds (enrofloxacin and ibafloxacin) and their metabolites (ciprofloxacin and 8-hydroxy-ibafloxacin) against *E. coli*, *Staphylococcus* spp. and *Pseudomonas aeruginosa* (*P. aeruginosa*) were investigated in these studies. In addition, Pankey and Ashcraft[8] showed that there was a synergistic interaction between ciprofloxacin and gatifloxacin against *P. aeruginosa*. The objective of this work was to identify if a synergistic interaction between danofloxacin and orbifloxacin against FQ-resistant *E. coli* isolates from animals occurs. The drug combination studies were carried out using the checkerboard and time-kill methods.

Seven *E. coli* isolates carrying *gyrA* mutations or *qnr* genes from the Laboratory of Molecular Pharmacology were selected for the checkerboard and time-kill studies. The *gyrA* mutant *E. coli* isolates were obtained from three healthy fowl (E224, E245, E246), the *qnr*-containing *E. coli* isolates were from one healthy cow (E101), one cow (E103) and one sheep (E248) with gastroenteritis, and one healthy dog (E300).

Broth microdilution testing was performed to determine the MICs of the compounds according to the guidelines of the Clinical Laboratory Standards Institute.[9] *E. coli* ATCC25922 was used as control for antimicrobial susceptibility testing.

QRDR and plasmid-mediated quinolone resistance (PMQR) genes were amplified using specific primers[10,11] and PCR products of *gyrA* were sequenced by Macrogen Inc. (Korea). The DNA sequences of *gyrA* were analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Presence of the *qnrA* and *qnrS* genes was determined by PCR amplification, as described previously by Robicsek *et al.*[12] and Cengiz *et al.*[1] The primers used are as follows: *gyrA*, 5′-ACGTACTAGGCAATGACTGG-3′ (forward) and 5′-AGAAGTCGCCGTCGATAGAAC-3′ (reverse); *qnrA*, 5′-ATTTCTCACGCCAGGATTTG-3′ (forward) and 5′-GATCGGCAAAGGTTAGGTCA-3′ (reverse); and *qnrS*, 5′-ACGACATTCGTCAACTGCAA-3′ (forward) and 5′-TAAATTGGCACCCTGTAGGC-3′ (reverse).

Fractional inhibitory concentration index/indices (FIC index/indices) of danofloxacin and orbifloxacin were determined using checkerboard method.[13] Danofloxacin concentrations ranged from 0.064 to 256 $\mu g\,ml^{-1}$ and orbifloxacin concentrations ranged from 0.128 to 512 $\mu g\,ml^{-1}$. 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

FIC index/ΣFIC = $FIC_A + FIC_B$

The FIC index was interpreted as follows: synergy = FIC index $\leqslant$ 0.5; indifference = 0.5 < FIC index $\leqslant$ 4; antagonism = FIC index > 4.

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Table 1 Resistance mechanisms and MICs of *E. coli* isolates, checkerboard and time-kill data with the interpretations

	Resistance mechanism		MIC (μg ml^{-1})		Checkerboard		Time-kill				
								Log reduction		Interpretation	
Isolate ID	*gyrA*	*qnr*	DAN	ORB	ΣFIC	Interpretation	DAN/ORB (μg ml^{-1})	6 h	24 h	6 h	24 h
E101	—	*qnrA1*	128	256	**0.24**	SYN	16/32	**2.42**	−1.21	SYN	IND
E103	—	*qnrS1*	32	128	1	IND	16/64	**2.01**	**3.15**	SYN	SYN
E224	S83L, D87N	—	32	64	1	IND	16/32	**2.99**	**2.92**	SYN	SYN
E245	S83L, D87E	—	2	8	**0.5**	SYN	0.5/2	**3.27**	**3.3**	SYN	SYN
E246	S83L	—	1	2	**0.37**	SYN	0.25/0.25	**2.54**	−1.69	SYN	IND
E248	—	*qnrS1*	1	128	**0.09**	SYN	0.03/4	**3.55**	−1.97	SYN	IND
E300	—	*qnrS1*	2	4	**0.31**	SYN	0.5/0.25	**3.74**	−1.1	SYN	IND

Abbreviations: DAN, danofloxacin; ORB, orbifloxacin.
Bold values indicate significance.

Time-kill experiments were slightly modified from the method described by Petersen *et al.*[14] A liquid overnight bacterial culture of the *gyrA* mutant and *qnr*-containing *E. coli* isolates was diluted with Mueller–Hinton Broth (Becton, Dickinson and Company, Sparks, MD, USA) and drug stock solutions to achieve an initial inoculum of $\sim 10^6$ c.f.u. ml^{-1}. Each 10 ml culture was incubated at 37 °C, and samples were withdrawn for the determination of bacterial counts at 0, 6 and 24 h. Colony counts were determined by plating 100 μl of each diluted sample onto Plate Count Agar (Becton, Dickinson and Company) with an automated spiral plater (WASP; Don Whitley Scientific Ltd., Shipley, UK) and then counting using an colony counter (UVITEC Cambridge, Cambridge, UK). Synergy was defined as a $\geqslant 2$ $\log_{10}$ decrease in colony count at 6 or 24 h with the combination compared with the initial inoculum. The drug combination was considered to be antagonist if there was a $\geqslant 2$ $\log_{10}$ increase in c.f.u. ml^{-1} and indifference was the interpretation of a <2 $\log_{10}$ change in c.f.u. ml^{-1}.

The MICs of danofloxacin and orbifloxacin for *E. coli* ATCC25922 were 0.032 and 1 μg ml^{-1}, respectively. Microbiological activity (MIC_{90}) of danofloxacin and orbifloxacin to *E. coli* isolated from animals was reported as 0.015–0.25 and 0.5 μg ml^{-1}, respectively.[15] *E. coli* isolates presented an alteration in *gyrA* (E224, E245, E246: Ser-83 → Leu; E224: Asp-87 → Asn, E245: Asp-87 → Glu) and the *qnr* genes detected were *qnrA1* (E101) and *qnrS1* (E103, E248, E300). The amino-acid substitutions in *gyrA* were at the most frequently identified site (codon 83).[2] The MICs of the compounds and FIC values of the combination for *gyrA* mutant and *qnr*-containing *E. coli* isolates are shown in Table 1. FIC index of the combination for resistant *E. coli* isolates ranged from 0.09 to 1. The incidence of synergy and additivity/indifference was 71% and 29%, respectively. Antagonism was not detected for any of *E. coli* isolates by checkerboard method.

By using the time-kill method, the *in vitro* activity of the combination against *gyrA* mutant and *qnr*-containing *E. coli* isolates are shown in Table 1. At 6-h incubation, the combination resulted $\geqslant 2$ $\log_{10}$ reduction in viable counts against all *E. coli* isolates and it showed synergic activity. At 24-h incubation, this was also achieved for E103, E224 and E245 isolates. However, regrowth was observed for four of seven *E. coli* isolates after 24 h incubation.

There are increasing numbers of antibiotic-resistant infections, especially by Gram-negative bacteria, which are innately multi-drug resistant.[16] In addition, Gram-negative bacteria such as *E. coli* are increasingly resistant to the few effective agents available for treatment via the acquisition of transmissible elements, and when isolated from animals have multiple and different mechanisms of antibiotic resistance.[1,17] Therefore, to restore the efficacy of licensed veterinary FQs against resistant Gram-negative bacteria has become important.

The two methods used most commonly to assess antimicrobial interactions *in vitro* are the checkerboard and time-kill assays.[7] In this study, these methods were used to assess synergy of danofloxacin + orbifloxacin combination against seven clinical isolates of *E. coli*. Synergy using the checkerboard method was detected for five of seven *E. coli* isolates with 0.09–0.5 ΣFIC. Pankey and Ashcraft[8] used Etest synergy method and found a similar interaction between ciprofloxacin and gatifloxacin against *P. aeruginosa* with 19% incidence. Enrofloxacin is unique in that it is partially metabolized to ciprofloxacin and both active drugs circulate in treated animals.[5] Lautzenhiser *et al.*[7] showed that for staphylococcal and *E. coli* isolates, FIC indices of enrofloxacin + ciprofloxacin combination were between 0.5 and 4.0, indicating that the combination acted additively *in vitro*. The combination of ibafloxacin plus its major active metabolite 8-hydroxy-ibafloxacin had synergistic action in two *E. coli* isolates and additive effects in *E. coli* ATCC25922.[6] The synergistic activity of the active metabolite contributes additionally to the antimicrobial activity of the parent compound.[5–7] In this study, by the time-kill method, synergy was mainly shown after 6 h of exposure for all isolates accompanied by regrowth after 24 h for four of them. These results showed that for *E. coli* synergy incidence detected by the time-kill method is higher than checkerboard method. Elipoulos and Moellering[13] indicated that in contrast to the checkerboard technique, which typically provides only inhibitory data, the killing-curve technique measures the microbicidal activity of the combination being tested. For this reason, it is presumably more relevant for clinical situations in which bactericidal effect.[13]

Cengiz *et al.*[18] showed that the genetic mechanisms of FQ resistance were determinative for the bactericidal activity of enrofloxacin alone against *E. coli*. The results of this study clearly indicated that danofloxacin (second-generation FQs) and orbifloxacin (third-generation FQs) can exert synergistic activity against some strains FQ-resistant *E. coli* isolates, and this combination could be considered for augmenting of their efficacy *in vivo*.

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