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RESEARCH ARTICLE

The *in vitro* Antibacterial Activity of Florfenicol in Combination with Amoxicillin or Cefuroxime against Pathogenic Bacteria of Animal Origin

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ABSTRACT

This study evaluated the *in vitro* activity of florfenicol (F) in combination with amoxicillin (AM) or cefuroxime (CRM) against pathogenic bacteria of animal origin, including *E. coli*, *S. aureus, S. cholerasuis* and *P. mirabilis*. The MIC of AM ranged from 16 to 256 µg/ml. The MBC of AM (64 µg/ml) was four-fold higher than its MIC value (16 µg/ml) for *E. coli*, and similar to the MIC for the other three species. The MIC of F ranged from 8 to 16 µg/ml. The MBC values of F for *E. coli*, *S. aureus,* and *S. cholerasuis* were eight-fold higher than the respective MIC values, and 32-fold higher than the MIC of *P. mirabilis.* The MIC of CRM ranged from 8 to 128 µg/ml. The MBC of CRM was the highest (≥ 256 µg/ml), except for *E. coli*. The F/AM combination resulted in synergism (FIC index ≤ 0.5) for *E. coli*, *S. aureus,* and *P. mirabilis* and in-difference (FIC index >1) for *S. cholerasuis.* For F/CRM combination, synergism (*E. coli* and *S. cholerasuis*) and in-difference (*S. aureus* and *P. mirabilis*) were observed. Killing rate study showed a 1.5 - > 3 log 10 cfu/ml reduction of *E. coli* with F/AM compared to AM or F alone. The highest activity of the combinations was observed when F comprised at least 50% of the combination. Further studies using many bacterial isolates and various proportion of each drug would reveal the potential of a combination product containing F and AM/CRM for use in veterinary practice.

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INTRODUCTION

The use of antimicrobial compounds in veterinary practice improves animal health and production. However, their use in animals, particularly for growth enhancement, has come under much scrutiny, as it has been shown to contribute to the increased prevalence of antibiotic-resistant bacteria of human significance (Mathew *et al*., 2007).

Antibacterial drug resistance is a growing concern worldwide, with some pathogenic bacteria exhibiting resistance to virtually all available drugs (Lister, 2006). In addition to the benefits of higher efficacy or safety profiles than the individual drugs, combination therapy with two or more antimicrobial agents is considered to be a potentially effective means of minimizing the emergence rate of bacterial resistance (Eliopoulos and Moellering, 1991). In this regard, a number of antibacterial drug combinations, including amoxicillin/ clavulinic acid, ampicillin/sulbactam, trimethoprim/ sulfonamide, trimetoprim/sulfadimethoxine, and florfenicol/tylosin have been used in veterinary area (Escudero *et al*., 1996; Fernández-Varón *et al*., 2005; Kim *et al*., 2008).

As part of our long-term research that focuses on developing safe & effective combination products for use in veterinary area, we have evaluated a number of antibacterial drug combinations (Kim *et al*., 1997; Kim *et al*., 2008). Here in, we report the *in vitro* antibacterial activity of florfenicol/ amoxicillin and florfenicol/ cefuroxime combinations against pathogenic bacteria of animal origin.

MATERIALS AND METHODS

Bacterial strains

Field isolates of *Escherichia coli, Staphylococcus aureus* (*S. aureus*), *Salmonella cholerasuis* (*S.*

Pak Vet J, 2011, 31(2): 141-144.

cholerasuis) and *Proteus mirabilis* (*P. mirabilis*) from animals and two quality control organisms (*E. coli* ATCC 25922 and *S. aureus* ATCC 29213) were used in this study.

Chemicals

Pure powders of florfenicol (F), amoxicillin (AM) and cefuroxime (CRM) were obtained from Daesung microbiological labs (Kyunggi-Do, Korea). Stock solutions and working solutions were prepared according to the instruction of the manufacturer. For combination studies, F and AM or CRM were combined at 3:1, 1:1 and 1:3 (w/w) ratios of F and AM or CRM, respectively.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of florfenicol, amoxicillin and cefuroxime, alone or in combination, was determined against the field isolates and quality control organisms by a standard broth microdilution method (Anonymous, 2006). Briefly, Serial two-fold dilution of the antimicrobial agents was prepared in Muller-Hinton Broth (MHB) in 96-well plates. Cultures were grown overnight at 37°C from beads previously stored at -70°C. The standard inocula were prepared by direct suspension in MHB and adjusted with sterile saline until the turbidity matched a 0.5 McFarland standard. Drug-containing and control wells were inoculated with the diluted bacterial suspension that gave a final concentration of ~ 10^5 cfu/ml. The MIC was determined as the lowest concentration of each drug at which no visible growth was observed by visual examination of the plates from below after 24 h incubation at 37°C.

To determine the MBC, 100 µl samples from wells with higher than or equal to the MIC were subcultured on Trypticase soy agar plates and incubated overnight at 37°C. A reduction in colony counts by 99.9% from the original inoculum size was considered to represent the MBC.

Determination of fractional inhibitory concentration (FIC)

The FIC of F/AM or F/CRM combination was determined by a standard checkerboard method (Eliopoulos and Moellering, 1991). The FIC index was calculated according to the equation:

FIC index = $FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$

where, MIC_A and MIC_B are the MIC of drug A and B alone, and C_A and C_B are the concentrations of each drug in the combination in wells corresponding to the MICs. The FIC interpretative criteria were as follows: synergism (FIC index ≤ 0.5), additive/ indifference (0.5 < FIC index < 2), and antagonism (FIC index ≥ 2).

Killing rate analysis of F/AM combinations

To obtain further evidence on the effects of antibacterial drug combinations, we performed a time-kill analysis using different ratios of F and AM and a field strain of *E. coli.* Time-kill studies were performed in MHB with starting inocula of 10^5 cfu/ml. Aliquots (100 µl) were withdrawn from culture tubes containing F, AM, F/AM combination, or drug-free control tubes before and at 1, 3, 6, 9, 12, and 24 h after incubation at 37°C and subjected to serial 10-fold dilutions in saline. One hundred microliters of the suspensions were then dropped onto quadrants of Trypticase soy agar (Becton, Dickinson and Co., Sparks, MD). Once dry, the plates were incubated at 37°C for 24 h to determine viable counts as cfu/ml. A previously described interpretative criteria was used to describe the type of antibacterial interactions (Eliopoulos and Moellering, 1991). Synergy was defined as a ≥ 2 $\log_{10}$ cfu/ml reduction after 24 h incubation with the combined drugs, in comparison with the most active drug alone. Indifference/additive interaction was defined as a < 2 $\log_{10}$ cfu/ml reduction after 24 h incubation with the combined drugs, in comparison with the most active drug alone. Antagonism was defined as a ≥ 2 $\log_{10}$ cfu/ml increase after 24 h incubation with the combined drugs, compared to the level of killing by the most active drug alone.

RESULTS

The *in vitro* antibacterial activities, in terms of MIC, MBC and FIC, of AM, F, and CRM alone or in combination are presented in Table 1. The MIC of AM for the four tested strains ranged from 16 to 256 µg/ml. The MBC of AM (64 µg/ml) was four-fold higher than its MIC value (16 µg/ml) for *E. coli*, while no differences were observed between the respective MIC and MBC values for *S. aureus, S. cholerasuis* and *P. mirabilis* isolates. The MIC of F for the four tested strains ranged from 8 to 16 µg/ml. The MBC values of F for *E. coli*, *S. aureus,* and *S. cholerasuis* were eight-fold higher than the respective MIC values, while the highest MBC/MIC ratio of 32 was recorded for *P. mirabilis* isolate. The MIC of CRM ranged from 8 to 128µg/ml depending on the tested organism. However, the MBC of CRM was the highest (≥ 256 µg/ml) compared to that of AM and F, for all isolates except *E. coli*.

As shown in Table 1, The FIC indices for F/AM combination were < 0.5 for *E. coli*, *S. aureus,* and *P. mirabilis* isolates, indicating synergistic interaction between the two drugs. However, F/AM combination showed an indifferent interaction (FIC index >1) for *S. cholerasuis.* For F/CRM combination, synergistic interaction for *E. coli* and *S. cholerasuis* and indifference for *S. aureus* and *P. mirabilis* were observed. No antagonism was obtained for both F/AM and F/CRM combinations against all tested strains.

Table 2 shows the *in vitro* antibacterial activity of F/AM and F/CRM combined at three different proportions, including 75% F: 25% AM/CRM, 50% F: 50% AM/CRM, and 25% F: 75% AM/CRM. Combination between AM and F resulted in 1- to 32-fold reductions in the MIC values of AM depending on the tested bacterial species. However, the MIC of F when combined with AM showed only a 2-fold changes compared to F used alone, which is not considered significant with susceptibility assays (Lister, 2006). A 75% AM: 25% F ratio had generally the lowest activity (the highest MIC of the combination). Similarly, F/CRM combinations resulted in bacterial species - dependent

Pak Vet J, 2011, 31(2): 141-144.

Table 1: *In vitro* antibacterial activity of amoxicillin or cefuroxime in combination with florfenicol

Antibiotics		*E.coli*		*S. aureus*		*S. cholerasuis*		*P. mirabilis*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MICs	AM/F (alone)	16/8	64/64	256/16	256/128	64/8	64/64	16/8	16/256
(μg/mL)	AM/F (combination)	1/0.5		8/0.5		1/8		1/0.25	
	FIC index	0.13		0.06		1.01		0.09	
MICs	CRM/F (alone)	16/8	32/64	8/16	≥256/128	128/8	≥256/64	8/4	≥256/256
(μg/mL)	CRM/F (combination)	0.03/4		8/0.5		8/0.25		8/0.25	
	FIC index	0.50		1.03		0.09		1.06	

AM: Amoxicillin, CRM: cefuroxime, F: florfenicol

Table 2: Minimum inhibitory concentrations of florfenicol/ amoxicillin and florfenicol/cefuroxime combined at different proportions.

Drugs	MIC (μg/ml)			
	E. coli	*S. aureus*	*S. cholerasuis*	*P. mirabilis*
AM	8	256	256	16
F	8	16	4	8
AM75% +F25%	4	32	8	8
AM50% + F50%	8	16	8	4
AM25% + F75%	8	16	8	4
CRM	32	1	64	8
F	8	16	4	4
CRM75% +F25%	16	2	16	16
CRM50% +F50%	8	2	8	8
CRM25% + F75%	8	2	8	8

AM: amoxicillin, CRM: cefuroxime, F: florfenicol

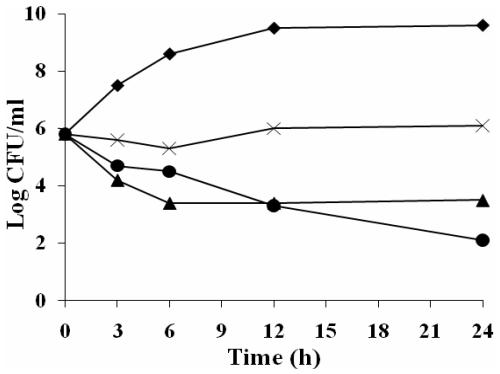

Fig. 1: Killing rate of amoxicillin (AM), florfenicol (F) or their combination against *E.coli*. -◆-: Control, -●-: AF, -▲-: AM, -×-: F.

reduction in MICs of both drugs. CRM 75% F: 25% ratio had the lowest activity, while 50% F: 50% CRM and 75% F: 25% CRM had comparable or 2 to 8-fold higher activity than F or CRM alone.

Fig. 1 shows the interaction F and AM against *E. coli* by killing rate analysis. The combination of F and AM at 1 : 1 ratio resulted in > 3 log 10 cfu/ml reduction compared to the killing rate of F alone, and ~ 1.5 log 10 cfu/ml reduction compared to the killing rate of AM alone, indicating an interaction ranging from additive to synergism.

DISCUSSION

The *in vitro* antibacterial activity of a bacteriostatic agent (florfenicol) and two bacteriacidal agents (amoxicillin and cefuroxime) was examined in this study. A small MBC/ MIC ratio (< 4 to 6) is usually expected for bactericidal agents, while the MBC of bactericidal drugs are many-fold higher than their MIC (Dowling, 1996; Levison, 2004). Accordingly, a small MBC/MIC ratio of 1-2 for AM, and a higher ratio of 2-32 for F were obtained in this study. However, CRM had a small MBC/MIC ratio of 2 only for *E. coli*, whereas a higher ratio was required with other species of bacteria, probably indicating species-dependent bactericidal activity of this agent.

It was generally believed that combination of two bactericidal drugs results in synergism, while combination of bactericidal and bacteriostatic agent often has antagonism (Daschneri, 1976). As opposed to this notion, however, several studies have shown synergistic or additive interactions between many bacteriostatic and bactericidal agents. For example, synergistic or additive interactions between the bactericidal penicillin/ cephalothin and bacteriostatic rolitetracycline have been reported (Daschneri, 1976). Consistently, combination of F with AM/CRM in the present study has resulted in synergistic/additive interactions depending on the tested species of bacteria. As shown in Table 1 and 2, combination of F and AM/CRM has resulted in many-fold reductions in the MIC of AM/CRM than the individual drugs, and the best activity was obtained when the combination consisted of at least 50% of F. In view of the increasing rate of resistance to older antibacterial drugs like AM in several veterinary pathogens (Kim *et al*., 2007: Russi *et al*., 2008), combination therapy with the relatively newer agents like F may represent a greater potential in terms of minimizing both treatment failure and emergence of resistant bacteria.

Currently, different techniques, including time-kill, checkerboard, and E test are used to evaluate the interaction of antibacterial agents *in vitro*, with each method having its own advantages and disadvantages (White *et al*., 1996). Combination of F and AM has resulted in a synergistic interaction against *E. coli* with the checkerboard method with an FIC index of 0.13 (Table 1). Using the time kill analysis, however, a < 2 $\log_{10}$ cfu/ml reduction was observed for F/AM combination compared to the most active single agent (AM) alone, while the combination led to a > 3 $\log_{10}$ cfu/ml reduction compared to the killing profile of F alone. The discrepancy between the two methods may have resulted from the inherent differences in the endpoints determined by the two techniques i.e. the checkerboard method is merely a measure of the inhibitory activity, whereas the time-kill method of synergy testing assesses bactericidal activity (White *et al*., 1996). However, these findings should be validated in further studies using different bacterial species and various concentrations of both drugs.

It was beyond the scope of this investigation to evaluate the mechanisms of interaction between the combined drugs. Florfenicol inhibits microbial protein synthesis by binding to the 50 S subunit of the 70 S ribosome and impairing peptidyl transferase activity (Dowling, 2006). Amoxicillin and CRM are β-lactams that impair the development of bacterial cell walls by interfering with transpeptidase enzymes responsible for the formation of the cross-links between peptidoglycan strands (Daschner, 1976). Several studies have demonstrated synergistic interactions between β-lactams and other antibacterial agents, such as aminoglycosides, in which the inhibition of cell wall synthesis by β-lactams significantly enhanced the uptake of other drugs (Weiss and Lapointe, 1995; Güzel and Gerçeker, 2008). Similar phenomenon might happen in our study in which synergism of the compounds might arise as a result of increased cellular uptake of F due to AM/CRM-induced inhibition of bacterial cell wall synthesis. However, this hypothesis warrants further confirmatory research.

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