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RESEARCH ARTICLE

The *in vitro* Antibacterial Activity of Enrofloxacin-Trimethoprim Combination against Five Bacterial species

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ABSTRACT

The aim of the current study was to investigate the combination effect of enrofloxacin and trimethoprim by their inhibitory and bactericidal activities against five bacterial species (*E. coli*, *P. hemolytica*, *S. aureus*, *S. cholerasuis*) and a field isolate *S. typhimurium*. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), fractional inhibitory concentration (FIC) and time killing rate were performed using these isolates. Both antibiotics has shown similar MIC ranging from equal to 3 fold dilutions difference for each of the bacteria tested except for *E. coli* where enrofloxacin has shown better activity with more than ten fold dilutions less than trimethoprim. The fractional inhibitory concentration index from the results of checkerboard for enrofloxacin and trimethoprim showed a synergistic effect for *P. hemolytica* and *S. typhimurium* (field isolate), while no difference was observed for the remaining tested bacteria. In the combination of the two antibiotics with different ratios, compared to the MICs of the two antibiotics tested alone, the concentration of the two antibiotics in the combination has shown a 2-8 fold reduction against all bacteria tested. Furthermore, as the concentrations of enrofloxacin increase and trimethoprim decrease the minimum inhibitory concentrations for *E. coli*, *P. hemolytica* and *S. aureus* has shown a decrease. The other two bacteria didn't show any change. Although all the combined ratios had similar MIC and MBC values compared to MIC and MBC tested alone, the concentration of each antibiotic in the combined ratios was lower by more than ten-fold compared to the MIC and MBC alone for both antibiotics. The time kill rate study for the antibiotics alone or in combination against *E. coli* and *S. aureus* had revealed higher inhibitions of bacterial growth with a difference of 2-4 log cfu/ml bacteria by the combination antibiotics after 12 hrs of incubation than tested alone. In summary, combination therapy with these two antibiotics may serve additive to synergistic effect and broad spectrum activity against the tested bacteria.

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INTRODUCTION

Enrofloxacin (ENR), the third-generation fluoroquinolone, is effective in treatment of a wide range of bacteria in animals. Moreover, it is effective against microorganisms that are resistant to other antibiotics such as aminoglycosides, tetracyclines, macrolides and β-lactam (Shim *et al*., 2003; Shoorijeh *et al*., 2012). Trimethoprim (TMP) is a commonly used antibacterial substance against gram-positive and negative bacteria. It blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase (Hsu *et al*., 1998; Tu *et al*., 1988). Although both ENR and TMP are suitable to treat both gram-positive and negative bacteria, there is still increasing concern over the pathogen resistance originated from both animals and human for both antibiotics (Gottlieb *et al*., 2008; Lykkerberg *et al*., 2007; Reinhardt *et al*., 2002).

The number of antibiotic-resistant bacteria is increasing around the world due to use of antibiotic, (Credito *et al*., 2009). Combined antibiotics of amoxicillin/clavinic, ampicillin/sulbactam, trimetoprim/ sulfadimethoxine,, trimetoprim/sulfonamide, florfenicol/tylosin have been used in veterinary area (Escudero *et al.,* 1996; Fernández-Varón *et al.,* 2005; Kim *et al.,* 2008).

Combination of ampicillin-aminoglycoside on group B streptococci and glycopeptides and vancomycin on *S. aureus* showed synergistic effects (Aeshlimann *et al*., 2000; Mandal *et al*., 2003). Also, synergism of trimethoprim and ciprofloxacin *in vitro* has been reported (Huovien *et al.*, 1992). However, to use drugs in combination information about their combined efficacy is needed. Therefore, this study was aimed to evaluate combination inhibitory and bactericidal activities of enrofloxacin and trimethoprim against five bacterial species.

MATERIALS AND METHODS

Antibiotics and Bacteria: Standard antibiotics powder of enrofloxacin (ENR) and trimethoprim (TMP) were obtained from Zhejiang Gaabang Pharmaceutical Co., Ltd and Shouguang Fukang Pharmaceutical Co., Ltd China respectively. *S. typhimurium* was isolated from Gyeougsangbuk-do livestock research institute (Korea). Standard bacterial strain *P. hemolytica* (ATCC 55518), *S.cholerasuis* (ATCC 7001), *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) were obtained from the Korean Culture Center of Microorganisms (Seoul, Korea).

Determination of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MICs of enrofloxacin and trimethoprim alone or in combination for five bacterial species were determined by broth micro dilution method according to NCCLS (National Committee on Clinical Laboratory Standards, USA) guidelines (NCCLS, 2003). Briefly, all tested organisms were cultured on tryptic soya agar plates from beads previously stored at -70˚C and incubated overnight at 37°C. After 24 h, pure colonies of 4 to 5 in number were transferred to 5ml sterile MHB (Mueller Hinton Broth) and incubated over night at 37ºC. Serial two-fold dilutions of the antimicrobial agents were prepared in Mueller-Hinton Broth (MHB) in 96-well plates. The standard inocula were prepared by direct suspension in MHB and adjusted with sterile saline until the turbidity matched a 0.5 McFarland standard from the overnight culture. Drug-containing wells were inoculated with the diluted bacterial suspension that gave a final concentration of ~105 cfu/ml. The 96 well microtiter plate was sealed by paraffin and incubated at 37˚C for 24 h. The MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. 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. From and above MIC, 100 µl samples was taken and dropped on to Mueller Hinton Agar (MHA, BD, USA) plates and then incubated at 37˚C for 18-24 hrs. A concentration at which there was a reduction in colony counts by 99.9% from the original inoculum size was considered to represent the MBC.

Fractional Inhibitory Concentration (FIC): Antibiotic combinations were tested by the checkerboard titration method using 96-well micro-titer plates. The fractional inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the following equation: $\sum FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$ where MIC_A and MIC_B are the MIC of drug A and B alone, respectively and C_A and C_B are the concentrations of the drugs in combination, respectively. Drug-drug interaction was considered synergistic if the FIC index ≤ 0.05, indifference if the FIC index was >0.5 and ≤4 and antagonistic if FIC index is > 4.

Time-kill rate: The time-kill analysis study was performed with *E. coli* and *S. aureus.* Drug concentrations of 0.5 x, 1 x and 2 x MIC in 10 ml MBH (Mueller Hinton broth) were prepared in glass culture tubes. Aliquots of exponentially growing cultures (5 x 10^8 colony forming units /ml) were inoculated in to the prepared antimicrobial agents containing broth. Before and at 1, 3, 6, 9, 12, and 24 h after incubation at 37ºC, 50 µl bacterial suspension from the different MIC concentrations was taken and subjected to 10-fold serial dilutions in saline. And, 100µl of the suspension was plated onto agar plates to obtain viable colonies. The control experiment consisted of plating cultures of $5X10^5$ CFU/ml without antibiotics. Synergy was defined as ≥ 2 log_{10}CFU/ml reductions after 24 h of incubation with the combined drug, in comparison with the most active drug alone; antagonism was defined as ≥ 2 log_{10}CFU/ml increases after 24 h of incubation with the combined drug, compared to the level of killing of the most active drug alone.

RESULTS

Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration: The results of the MICs and MBCs of ENR and TMP are summarized in Table 1. Both antibiotics has shown similar MIC ranging from equal to 3 fold dilutions difference for each of the bacteria tested except for *E. coli* where ENR has shown better activity with more than 10 fold dilutions less than TMP. However, for all tested bacteria, higher bactericidal activity was observed by ENR with less than 4 folds dilution than TMR except for *P. hemolytica* which showed equal MBC.

Fractional Inhibitory Concentration (FIC): The FIC index from the results of checkerboard for ENR and TMP showed a synergistic effect for *P. hemolytica* and *S. typhimurium* (field isolate), indifference for the remaining tested bacteria (Table 2).

MIC and MBC of Enrofloxacin-Trimethoprim Combination at different ratio: The MICs of combined antibiotic results at three different ratios (1:3, 3:7 and 2: 3) are summarized in Table 3. As the concentrations of ENR increase and TMP decrease the MICs for *E. coli, P. hemolytica* and *S. aureus* for the combination has shown a

decrease. The other two bacteria didn't show any change. Compared to the MICs of the two antibiotic tested alone, the concentration of the two antibiotic in the combination has shown a 2-8 fold reduction in the five bacteria tested. The MBC has shown similar activity with no or less than two fold dilution difference among the different ratio of the antibiotic combination for the tested bacteria.

Time-Kill Study: After 9-12 h of incubation at 0.5 x MIC of ENR, TMP and ET37 an exponential re-growth of the bacterial species was observed (Fig 1A). On the other hand at 1 x and 2 x MIC the re-growth was not observed for the combined ET37 antibiotic (Fig. 1 B and C) showing a synergistic activity between the two drugs.

In the time kill study 2-4 fold differences in log CFU/ml were observed against *E. coli*, and *S. aurous* at 1x and 2x MIC after 12 h and 24 h of incubation. *S. aurous* had shown more susceptibility than *E. coli* for all antibiotics tested.

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of trimethoprim and enrofloxacin

Organism	Concentration (µg/ml)			
	Enrofloxacin		Trimethoprim	
	MIC	MBC	MIC	MBC
E. coli (ATCC 25922)	0.015625	0.0156	0.5	8
P. hemolytica (ATCC 55518)	0.25	0.5	0.0625	0.5
S. aureus (ATCC 29213)	0.25	0.5	1	16
S. cholerasuis (ATCC 7001)	0.0625	0.125	0.0625	4
S. typhimurium (field isolated.)	16	32	64	128

Table 2: *In vitro* interaction between enrofloxacin and trimethoprim against test bacteria

Organism	Enrofloxacin (µg/ml)	Trimethoprim (µg/ml)	Enrofloxacin/ Trimethoprim (µg/ml)	FIC index
E. coli (ATCC 25922)	0.015	1.00	0.02/0.02	1.35
P. hemolytica (ATCC 55518)	0.25	0.06	0.06/0.01	0.41
S. aureus (ATCC 29213)	0.25	2.00	0.13/0.5	0.77
S. cholerasuis (ATCC 7001)	0.015	0.50	0.01/0.25	1.17
S. typhimurium (field isolates)	32.00	128.00	16/1	0.50

Table 3: Minimum inhibitory concentration and minimum bactericidal concentration of trimethoprim and enrofloxacin combination at different ratio

Organism	Concentration (µg/ml)					
	E25+T75		E30+T70		E40+T60	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (ATCC 25922)	0.0625	0.0625	0.03125	0.03125	0.03125	0.0625
P. hemolytica (ATCC 55518)	0.5	1	0.25	0.25	0.125	0.25
S. aureus (ATCC 29213)	0.5	0.5	0.25	0.5	0.25	0.5
S. cholerasuis (ATCC 7001)	0.03125	0.0625	0.03125	0.03125	0.03125	0.03125
S. typhimurium (field isolated.)	32	≥64	32	≥64	32	≥64

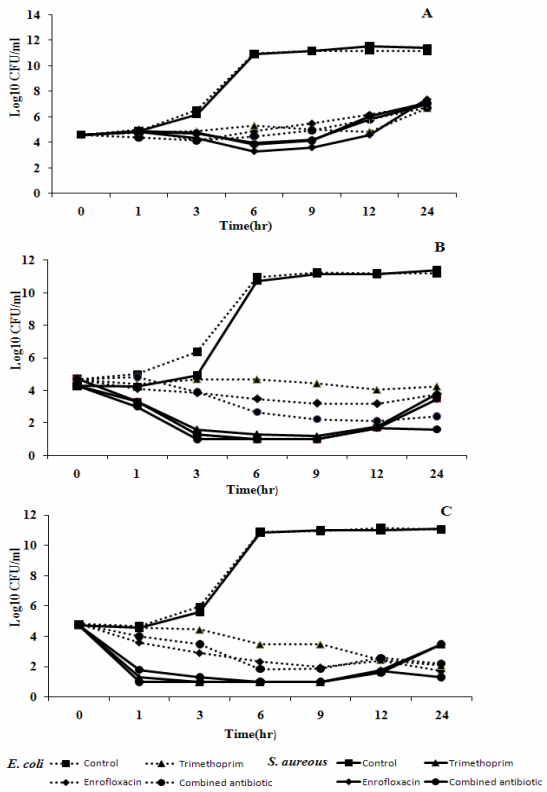

Fig. 1: Time-kill analysis of *E. coli* and *S. aureus* in the presence of the antibiotics at 1/2 x MIC (A), 1xMIC (B), 2xMIC(C).

DISCUSSION

After determining the MICs and MBCs of ENR and TMP (Table 1) using five bacteria, we assessed the importance of the bacterial-species and concentration on the activities of these antibiotics. Under our standard conditions (about 10^5 CFU/ml) and on a molar basis, the MIC of ENR was equal to that of TMP for *S. cholerasuis* while for *S. typhimurium*, *S. aureus* and *P. hemolytica* showed a similar MIC 2-3 fold dilution less for ENR. E. coli show an exceptionally very high susceptibility to ENR with more than 15 fold dilution lesser than TMP under the same conditions. At the same time the MBCs for ENR showed less than 3 fold dilutions for all the bacteria tested except for *P. hemolytica* which showed equal MBC for both antibiotics. These results suggest better efficacy of the ENR than that of the TMPs when given alone in the tested bacteria. Furthermore the MICs of ENR and TMP observed in the current study alone were similar compared to the report by Lee and Lee (2007) for *S. typhimarium* and *E. coli* by ENR. This also coincides with the expectation from the perspectives of mechanism of action in that the antibacterial activity of ENR is bacteriostatic agent and TMP bactericidal.

The Checkerboard method used to analyze the combined effect of the two bactericidal and bacteriostatic antibiotics, also revealed synergistic for two isolates and indifferent interaction for the remaining tested bacteria. The synergistic effect observed only for the two bacteria tested was less than expected. This might be due to the

susceptibility of all the bacteria isolates used in this study for both antibiotics.

To further analyze the combined effect of the two antibiotics, the combined antibiotics in different ratios were assessed for their MIC and MBC activities. Although all the combined ratios had similar MIC and MBC values compared to MIC and MBC tested alone, the concentration of each antibiotic in the combined ratios was lower compared to the MIC and MBCs alone. The time kill rate study for the antibiotics alone or in combination against *E. coli* and *S. aurous* has shown no synergistic or antagonistic. The combined drugs could decrease the growth after 12 h compared with individual drugs and this showed that the combined (ET37) had better inhibition effect on mutant growth.

Although we didn't check the mutant prevention concentration (MPC) (Gebru *et al*., 2011) in the current study, the exponential re-growth after 12 h in both antibiotics tested alone and inhibition by the combination suggests, combined drug could inhibit the growth of mutants and lower the chance of developing drug resistance. Using combined drugs is an alternative to prevent drug resistance. Combination therapy with these two drugs studied may have served additive to synergistic effect and broad spectrum against the tested bacteria. However, further study with resistant bacterial strains is recommended.

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