

Comparative *in vitro* killing activities of enrofloxacin when used alone and in combination with doxycycline against *E. coli* isolates from dogs and cats

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Abstract

Enrofloxacin (ENR) exhibits bactericidal activity while doxycycline (DOX), a drug of choice for *Ehrlichia canis* (*E. canis*), demonstrates bacteriostatic effect. When Gram-negative bacteria and *E. canis* co-infection occurs, using enrofloxacin concurrently with doxycycline is inevitable. The objectives of this study were to compare *in vitro* killing activities and killing times of enrofloxacin when used alone with those of its combination with doxycycline against ten *Escherichia coli* (*E. coli*) isolates from dogs and cats using time-kill curves. The combination treatment was administered either simultaneously (ENR+DOX at $t=0$ hr) or separately (DOX at $t=0$ followed by ENR at $t=12$ hr). The best killing activity was found with enrofloxacin alone, with a log reduction of 3.96 ± 0.49 (97.96-100.00% kill) within 2 hr of drug administration, followed by the simultaneous combination with a log reduction of 3.78 ± 0.37 (98.73-100.00% kill) within 6 hr, and the separated combination with a log reduction of 3.51 ± 0.47 (99.19-100.00% kill) within 12 hr. Comparing time to 3 log reduction (T3K), the separated combination killed *E. coli* significantly slower than enrofloxacin alone (8.04 ± 0.94 vs 2.47 ± 0.40 hr; $p < 0.05$), which was consistent with time to elimination (TE) (14.97 ± 1.35 vs 5.83 ± 0.58 hr; $p < 0.05$). This study establishes that the killing activities of enrofloxacin were reduced and delayed when used in combination with doxycycline, especially with 12-hour doxycycline pre-treatment, suggesting the antagonistic interaction between enrofloxacin and doxycycline when concurrently used against *E. coli* from dogs and cats.

Keywords: doxycycline, drug interaction, *E. coli*, enrofloxacin, *in vitro* killing

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Introduction

Fluoroquinolones are one of the most commonly used antibacterial drugs in general veterinary practice (Escher et al., 2011). This group of drugs acts by inhibiting DNA replication, leading to bacterial cell death (bactericidal activity). As a veterinary approved fluoroquinolone, enrofloxacin (ENR) has often been used due to its broad spectrum of activity against Gram-negative and some Gram-positive bacteria (Wayne et al., 2011). Another commonly chosen group of antibacterial drugs is tetracyclines, particularly doxycycline (DOX). This group of antibacterials thwarts bacterial protein synthesis, keeping bacterial cells in the stationary phase, resulting in the inhibition of bacterial growth (bacteriostatic activity) (MacDougall and Chambers, 2011). Despite its bacteriostatic activity against Gram-positive and some of Gram-negative bacteria, doxycycline is often prescribed as the drug of choice for *Ehrlichia canis* (*E. canis*) treatment (Wayne et al., 2011).

However, in the case of Gram-negative bacteria like *Escherichia coli* (*E. coli*) and *E. canis* co-infection, using enrofloxacin in combination with doxycycline is necessary. Therefore, interaction between these two drugs must be concerned. Regarding the general principle of antibacterial combination, bactericidal agents like aminoglycosides, beta-lactams and fluoroquinolones should not be used together with bacteriostatic agents such as macrolides, phenicols and tetracyclines (Ocampo et al., 2014). This is because such combination will generally result in antagonism. One of the reasons behind this antagonistic interaction is that the killing mechanisms of the former group depend on the active bacterial growth which can be inhibited by the latter (Boothe, 2012).

Based on the evidence that antagonism prevails among bactericidal-bacteriostatic combinations, many quinolones such as nalidixic acid, norfloxacin and ciprofloxacin exhibited less bactericidal activities when combined with bacteriostatic agents such as chloramphenicol, erythromycin, tetracycline and trimethoprim (Ocampo et al., 2014). However, some fluoroquinolones such as ciprofloxacin and enrofloxacin remained highly bactericidal against stationary-phased bacteria, except for moxifloxacin (Sulochana et al., 2009; Podos et al., 2012).

Nevertheless, no study regarding interaction between enrofloxacin and doxycycline has been published. To shed light on antibacterial interaction, our study aimed to compare *in vitro* killing activities and times of enrofloxacin alone to those of its combination with doxycycline against a common pathogen, *E. coli*, isolated from dogs and cats.

Materials and Methods

***E. coli* isolates:** *E. coli* isolates used in this study were clinical isolates from dogs and cats submitted to the Veterinary Diagnostic Laboratory, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, between 2012 and 2013, for bacterial identification and antibiograms. Based on

their susceptibility determined by VITEK2 system (bioMérieux, North Carolina, USA), a total of 10 *E. coli* isolates susceptible to both enrofloxacin and doxycycline were selected and stored at -80°C in storage media composed of tryptic soy broth (TSB) 70% and glycerol 30% until further analyses.

Susceptibility of *E. coli* to enrofloxacin and doxycycline: Susceptibility to enrofloxacin and doxycycline was determined in all *E. coli* isolates using MIC strips (Liofilchem®, Roseto degli Abruzzi, Italy) according to the manufacturer's instructions, with concentrations ranging from 0.002 to 32 µg/ml for enrofloxacin, and 0.016 to 256 µg/ml for doxycycline. Inhibition zones of the tested isolates were analyzed based on susceptible MIC breakpoints (≤ 0.5 µg/ml for enrofloxacin, and ≤ 4 µg/ml for doxycycline), along with *E. coli* ATCC® 25922 as quality control according to CLSI standards (CLSI, 2013; CLSI, 2015). Samples not susceptible to either or both of the two drugs and samples with an unclear inhibition zone were excluded from the study.

Time-kill curves: Time-kill curves were performed in all ten selected *E. coli* isolates to determine killing activities and times according to CLSI standard (CLSI, 1999; Amsterdam, 2015). In this study, five treatment-based groups were assigned: Group 1, control (no drug); Group 2, enrofloxacin (ENR at $t=0$ hr); Group 3, doxycycline (DOX at $t=0$ hr); Group 4, simultaneous combination (ENR+DOX at $t=0$ hr); and Group 5, separated combination (DOX at $t=0$ followed by ENR at $t=12$ hr). The antibacterial stocks of enrofloxacin and doxycycline (Sigma, Nucleos, Singapore) were properly prepared in accordance with CLSI standard (CLSI, 2015).

E. coli inoculum was incubated in cation-adjusted Mueller-Hinton broth (BBL™, New Jersey, USA) for 2 hr, to reach its logarithmic phase, before the beginning of the study. After that, the treatment corresponding to the experimental group was added to the actively-growing inoculum to a final antibacterial concentration of 1xMIC of each drug in a final bacterial concentration of 5×10^5 CFU/ml. Aliquots were made for seven time points (0.5, 1, 2, 3, 6, 12, and 24 hr) in Groups 1-4, and six time points (12.5, 13, 14, 15, 18, and 24 hr) in Group 5. To determine the number of bacterial viable cells (CFU/ml) at each time point, a 100-µl aliquot from appropriate dilutions was plated and thoroughly spread onto Mueller-Hinton agar (Difco™, New Jersey, USA) in triplicate.

For each isolate, time to 3 log reduction (T3K) was estimated by linear regression out to the 6 hr time point of the line graph between log reduction and time. Another time indicator, time to elimination (TE), was defined as the time when viable cells were below the detectable level (< 100 CFU/ml) (Mueller et al., 2004). This parameter was derived from linear regression out to the 6 hr time point of the line graph between log of viable cells and time.

Statistical analysis: Killing activity at each time point was expressed as mean \pm SE of log reduction and percentage of killing. If the decrease in bacterial cell counts was greater than 3 log reduction or 99.9% kill at

24 (normal endpoint) or 12 hr (adjusted endpoint), bactericidal activity was indicated. Otherwise, bacteriostatic activity was stated (CLSI, 1999; Pankey and Sabath, 2004).

As for killing times, both T3K and TE of the enrofloxacin-exposed groups (Groups 2, 4 and 5) were expressed as mean \pm SE and compared using Kruskal-Wallis test followed by Dunn-Bonferroni pairwise comparison using SPSS software (SPSS statistic 22,

IBM, Chicago, IL, USA). P-value less than 0.05 ($p < 0.05$) indicated the level of statistical significance.

Results

Susceptibility of *E. coli* to enrofloxacin and doxycycline: A total of 10 *E. coli* isolates susceptible to both drugs were selected with MIC values ranging from 0.047 to 0.19 $\mu\text{g}/\text{ml}$ for enrofloxacin, and 0.75 to 1.5 $\mu\text{g}/\text{ml}$ for doxycycline (Table 1).

Table 1 Information and MIC values of *E. coli* samples used in this study

Sample ID	Species	Source	MIC ENR ($\mu\text{g}/\text{ml}$)	MIC DOX ($\mu\text{g}/\text{ml}$)
C8	Feline	Pus	0.094	1
R3	Canine	Urine	0.19	1
AB	Feline	Necrotic tissue	0.094	1
B1	Canine	Wound	0.19	0.75
N5	Canine	Prostate gland	0.047	0.75
Q3	Canine	Prostate gland	0.125	1.5
S2	Canine	Wound	0.125	1.5
CG	Canine	Mass	0.094	1
N3	Feline	Wound	0.094	1
T4	Feline	Wound exudate	0.094	1

Time-kill curves:

Killing activities: A normal growth curve was seen in Group 1 (Fig. 1). In Group 3, doxycycline exhibited only bacteriostatic activity up to 12 hr before re-growth subsequently occurred, except for two isolates in which the bacteriostatic activities persisted throughout 24 hr (Fig. 1).

For the enrofloxacin-exposed groups, Group 2 showed the best bactericidal activities in most isolates with a log reduction of 3.96 ± 0.49 (97.96-100.00% kill)

following 2 hr of drug administration, except for four isolates in which the bactericidal activities were found at 3 hr for one isolate and 6 hr for the other three isolates (Fig. 2). However, the bactericidal activities and bacterial elimination were still found in all isolates (100%) at the normal endpoint (Figs. 1 and 2).

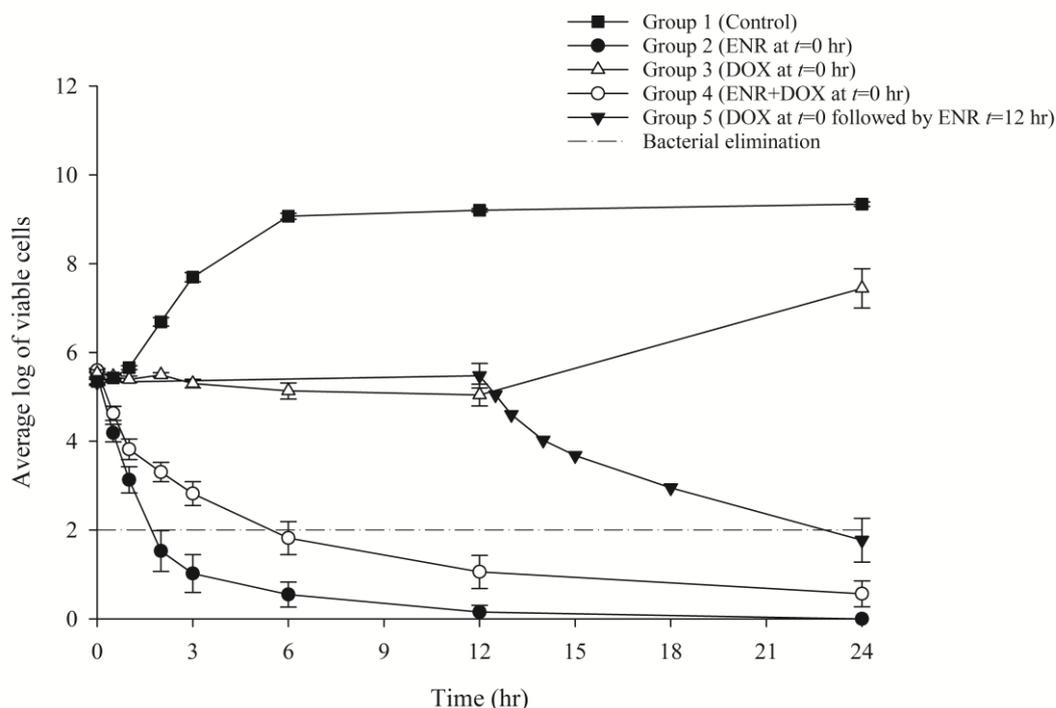


Figure 1 Growth curves of five experimental groups of 10 *E. coli* isolates

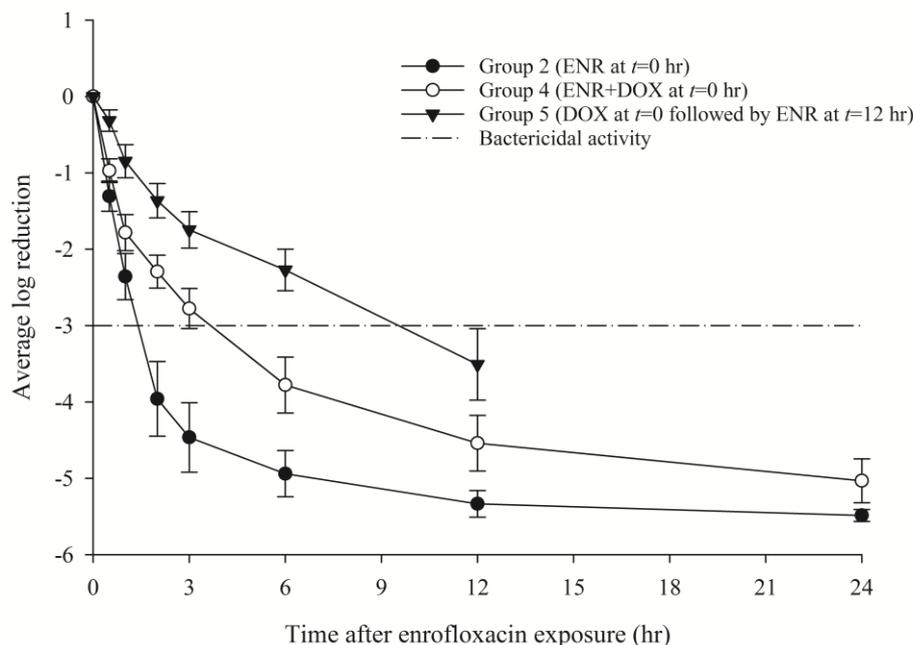


Figure 2 Average log reduction of enrofloxacin-exposed groups

Group 4, with the simultaneous combination of enrofloxacin and doxycycline, bactericidal activities were seen in most isolates with a log reduction of 3.78 ± 0.37 (98.73-100.00% kill) at 6 hr of drug exposure, except for two isolates in which the bactericidal activities were found at 12 and 24 hr, respectively (Fig. 1). At the normal endpoint, the bactericidal activities were still achievable in all isolates (100%), but only seven isolates (70%) exhibited bacterial elimination (Figs. 1 and 2).

In Group 5, with 12-hour doxycycline addition prior to enrofloxacin, bactericidal activities were not seen until reaching the normal endpoint (12 hr after enrofloxacin exposure), with a log reduction of 3.51 ± 0.47 (99.19-100.00% kill) (Fig. 1). Despite the endpoint, such bactericidal activities were seen in only five isolates (50%).

Considering the adjusted endpoint of 12 hr, the results were consistent with the normal endpoint, but to a lesser extent of isolates in which bactericidal activities (100%, 90% and 50%) and bacterial eliminations (90%, 50% and 40%) were found for Groups 2, 4 and 5, respectively (Figs. 1 and 2).

Killing times: The bactericidal activity was fastest in Group 2, followed by Groups 4 and 5, respectively. However, no significant difference in T3Ks between Groups 2 (2.47 ± 0.40 hr), and 4 (4.44 ± 0.63 hr) was detected ($p = 0.23$) while such difference between Groups 2 and 5 (8.04 ± 0.94 hr) was found ($p < 0.05$). For bacterial elimination, the fastest was observed in Group 2, followed by Groups 4 and 5, respectively. TE of Group 2 (5.83 ± 0.58 hr) was statistically different from that of Group 5 (14.97 ± 1.35 hr) ($p < 0.05$), but not from that of Group 4 (9.48 ± 1.15 hr) ($p = 0.16$). The results of TEs were consistent with those of T3Ks.

Discussion

Enrofloxacin alone exhibited rapid bactericidal activity and bacterial elimination against *E. coli* within 24 hr even at 1xMIC (Norcia et al., 1999). The killing pattern in this study is similar to ciprofloxacin and tobramycin (Craig and Ebert, 1991; Blondeau et al., 2012). At 1xMIC, doxycycline demonstrated bacteriostatic activity against *E. coli*, which is consistent with previous studies (Cunha et al., 2000; Blondeau and Shebelski, 2016).

As expected, when both drugs were simultaneously combined at concentrations of 1xMIC of each drug, the bactericidal activity of enrofloxacin tended to be reduced and delayed, and so did bacterial elimination, compared to enrofloxacin alone. This agrees with previous studies describing that an active metabolite of enrofloxacin, also ciprofloxacin, can be antagonized by doxycycline (Cester and Toutain, 1997; Chait et al., 2007).

There are a few explanations responsible for this antagonism. First, DNA synthesis inhibitors like nalidixic acid and ciprofloxacin are solely able to reduce and even kill bacteria by interrupting optimal regulation of ribosomal genes, leading to nonoptimality and DNA stress. However, the addition of a protein synthesis inhibitor such as tetracycline can adjust such nonoptimality close to optimal level, possibly helping bacteria to survive from the DNA synthesis inhibitor (Bollenbach et al., 2009). Second, it has been known that reactive oxygen species (ROS) contribute to the killing activity of bactericidal drugs (Kohanski et al., 2007). For some quinolones, the production of ROS can be completely disrupted through protein-synthesis dependent pathway as can be seen in the combination of nalidixic acid and tetracycline, resulting in remarkably reduced killing (Ocampo et al., 2014). However, in this study, enrofloxacin in the simultaneous combination with

doxycycline still killed bacteria. This means enrofloxacin may kill bacteria by utilizing self-mediated pathway to get access to the target faster than doxycycline and perform its killing action on the ROS counterpart (Delcour, 2009). Moreover, the remaining killing activity of enrofloxacin may be due to protein-synthesis independent pathway as ciprofloxacin and moxifloxacin (Wang et al., 2010).

In general veterinary practice, the use of enrofloxacin and doxycycline together at separated times has been done in order to avoid antagonism between the drugs. However, both drugs circulate in the blood for 24 hr and can accumulate in various tissues due to their lipid solubility, so exploiting such strategy may be a futile attempt (Cester and Toutain, 1997; Boothe, 2012; Gutiérrez et al., 2012). In order to simulate such condition, *E. coli* was treated with doxycycline 12 hr prior to enrofloxacin in this study. It showed that the bactericidal activity and bacterial elimination of enrofloxacin were significantly reduced and delayed compared to enrofloxacin alone. This may be because the protein synthesis inhibition effect of doxycycline is enhanced in a time-dependent manner, similar to some protein synthesis inhibitors such as azithromycin, clarithromycin and erythromycin. Nonoptimal regulation may play a less important role in this antagonism because doxycycline was still able to perform its inhibitory action under the condition without DNA stress. However, for both combinations, the possibility that either drug may trigger efflux pump cannot be excluded, leading to reduced and delayed killing (Li and Nikaido, 2009).

In this study, the discrepancy between the combinations was addressed. This is consistent with a previous study in which chloramphenicol was added prior to penicillin. The bactericidal activity of penicillin was less than that of the other two groups in which both drugs were similarly added at the beginning, and penicillin was added before chloramphenicol. Moreover, the addition of chloramphenicol 15 min after ciprofloxacin exposure could kill *E. coli* more than the addition of both drugs at the same time, but less than ciprofloxacin alone (Zeiler, 1985). However, the addition of enrofloxacin prior to doxycycline was not performed in the present study. According to our pilot study using *E. coli* ATCC® 25922, enrofloxacin alone and in combination with doxycycline at 1xMIC exhibited bacterial elimination within 2 hr (unpublished data). Thus, no bacterial cells were available by the time of doxycycline addition (12 hr) as can also be observed with enrofloxacin alone of this study (Fig. 1).

In spite of no standardized protocol, time-kill curve is one of the most suitable methods to evaluate the bactericidal activity of an antibacterial drug like enrofloxacin and its combinations with doxycycline due to the measurement of bactericidal activities with various time points (Verma, 2007). Moreover, the disadvantage of inability to measure drug-drug interactions with various concentrations can be overlooked. It is because only the bactericidal activity of the combination at 1xMIC was of our interest. The 1xMIC concentration is also a legitimate concentration to test on account of being a clinically achievable concentration for both drugs (Cester and Toutain, 1997;

Gutiérrez et al., 2012). Besides, T3K and TE were introduced in this study because these parameters have been associated with clinical outcomes (Blondeau, 2016). Even though they are in the same direction, only T3K determination may be insufficient for some cases such as endocarditis (Upton et al., 2005). In hope of filling this gap, TE was applied to provide a more accurate prediction of clinical outcome in cases for which T3K might not be satisfying and in patients with sepsis and immunocompromised condition (Craig et al., 1988; Jacobs, 2003).

Despite the efforts, there were still some limitations in this study. Sometimes results from *in vitro* study may not be extrapolated to *in vivo* study (Pillai et al., 2005). Unlike *in vitro* study, many factors such as protein binding, host immunity, fluctuating drug concentration, biofilm and other factors may affect killing activity in *in vivo* settings (Haritova and Russenova, 2010; Jung et al., 2012; Amsterdam, 2015). Therefore, more studies of the complexity of the combination of enrofloxacin and doxycycline are needed for both *in vitro* and *in vivo* aspects to gain insights into such interaction accordingly. Additionally, MIC of resistant isolates (the survivors) after each treatment needs to be further evaluated to explain if it was due to the selection of resistant mutants, inactivation of the antibacterial treatment, or regrowth of susceptible bacterial cells which have escaped the antibacterial activity by adhering to the wall of the culture tube (CLSI, 1999).

In conclusion, enrofloxacin has been widely used in small animals and prone to be antagonized by bacteriostatic agents such as doxycycline. This study showed this antagonism using time-kill studies and found that the antagonism was clearly present with the separated administration of doxycycline prior to enrofloxacin, but less with the simultaneous administration. As a result, splitting administration aggravates the antagonism, instead of avoiding drug interaction as intended. Along with many unpredictable *in vivo* factors, the use of enrofloxacin in either of the combinations with doxycycline should be judiciously applied and should be avoided in patients with sepsis or immunocompromised condition.

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บทคัดย่อ

การเปรียบเทียบฤทธิ์ฆ่าเชื้อในหลอดทดลองของยาเอนโรฟลอกซาซินเมื่อใช้เดี่ยวและใช้ร่วมกับ ดอกซีซัยคลินต่อเชื้อ อี. โคลิที่ได้จากสุนัขและแมว

ศุภภัทร ศิริวรรณ นภัตรา สวนไพรินทร์*

เอนโรฟลอกซาซิน (enrofloxacin) เป็นยาต้านแบคทีเรียที่มีฤทธิ์ฆ่าเชื้อแบคทีเรีย (bactericidal activity) โดยเฉพาะแบคทีเรียแกรมลบ ส่วนดอกซีซัยคลิน (doxycycline) เป็นยาต้านแบคทีเรียที่ออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย (bacteriostatic activity) และเป็นยาหลักที่ใช้ในการรักษาการติดเชื้อ *Ehrlichia canis* (*E. canis*) บางครั้งเอนโรฟลอกซาซินถูกนำมาใช้ร่วมกับดอกซีซัยคลินเพื่อรักษาการติดเชื้อแบคทีเรียแกรมลบร่วมกับ *E. canis* อย่างหลีกเลี่ยงไม่ได้ การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบฤทธิ์และเวลาฆ่าเชื้อในหลอดทดลองของเอนโรฟลอกซาซินแบบใช้เดี่ยวและใช้ร่วมกับดอกซีซัยคลินต่อเชื้ออี. โคลิ (*E. coli*) ที่แยกได้จากสุนัขและแมว จำนวน 10 ตัวอย่าง ด้วยวิธี time-kill curves โดยแบ่งวิธีศึกษาการให้ยาสองชนิดร่วมกันเป็น 2 วิธี คือ การให้ยาสองชนิดพร้อมกันและการให้ยาสองชนิดห่างกัน 12 ชั่วโมง การศึกษาพบว่า เอนโรฟลอกซาซินแบบใช้เดี่ยวฆ่าเชื้อได้มากที่สุด คือ 3.96 ± 0.49 log reduction (97.96-100.00%) ภายใน 2 ชั่วโมง รองลงมา คือ เอนโรฟลอกซาซินแบบให้พร้อมกับดอกซีซัยคลิน ซึ่งฆ่าเชื้อได้ 3.78 ± 0.37 log reduction (98.73-100.00%) ภายใน 6 ชั่วโมง และน้อยที่สุด คือ เอนโรฟลอกซาซินแบบให้ร่วมกับดอกซีซัยคลินห่างกัน 12 ชั่วโมง ซึ่งฆ่าเชื้อได้ 3.51 ± 0.47 log reduction (99.19-100.00%) ภายใน 12 ชั่วโมง นอกจากนี้ เมื่อเปรียบเทียบระยะเวลาที่ใช้ฆ่าเชื้อ (time to 3 log reduction, T3K) พบว่าเอนโรฟลอกซาซินแบบให้ร่วมกับดอกซีซัยคลินห่างกัน 12 ชั่วโมงฆ่าเชื้อได้ช้ากว่าเอนโรฟลอกซาซินแบบใช้เดี่ยวอย่างมีนัยสำคัญทางสถิติ (8.04 ± 0.94 และ 2.47 ± 0.40 ชั่วโมง; $p < 0.05$) ซึ่งสอดคล้องกับระยะเวลาที่ใช้ฆ่าเชื้อสมบูรณ์ (time to elimination, TE) (14.97 ± 1.35 และ 5.83 ± 0.58 ชั่วโมง; $p < 0.05$) จากการศึกษาสรุปได้ว่า การใช้เอนโรฟลอกซาซินร่วมกับดอกซีซัยคลินทำให้การฆ่าเชื้อลดลงและช้าลง โดยเฉพาะเมื่อให้ยาสองชนิดห่างกัน 12 ชั่วโมง ซึ่งแสดงให้เห็นถึงปฏิกริยาด้านฤทธิ์กันระหว่างเอนโรฟลอกซาซินและดอกซีซัยคลินต่อการฆ่าเชื้ออี. โคลิที่ได้จากสุนัขและแมว

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