การเปรียบเทียบฤทธิ์ฆ่าเชื้อในหลอดทดลองของยาเอนโรฟลอกซาซินเมื่อใช้เดี่ยวและใช้ร่วมกับ
ดอกซีซัยคลินต่อเชื้อ อี. โคไลที่ได้จากสุนัขและแมว

นายศุภภัทร คีรีวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยาทางสัตวแพทยศาสตร์ ภาควิชาเภสัชวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

COMPARATIVE *IN VITRO* KILLING ACTIVITIES OF ENROFLOXACIN ALONE AND IN COMBINATION WITH DOXYCYCLINE AGAINST *E. COLI* ISOLATED FROM DOGS AND CATS

Mr. Supapatt Kireewan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Pharmacology

Department of Veterinary Pharmacology

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2016

Thesis Title	COMPARATIVE *IN VITRO* KILLING ACTIVITIES OF ENROFLOXACIN ALONE AND IN COMBINATION WITH DOXYCYCLINE AGAINST *E. COLI* ISOLATED FROM DOGS AND CATS
By	Mr. Supapatt Kireewan
Field of Study	Veterinary Pharmacology
Thesis Advisor	Instructor Nipattra Suanpairintr, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., Ph.D.)

THESIS COMMITTEE

....Chairman

(Associate Professor Piyarat Chansiripornchai, D.V.M., Ph.D.)

....Thesis Advisor

(Instructor Nipattra Suanpairintr, D.V.M., Ph.D.)

....Examiner

(Instructor Chenphop Sawangmake, D.V.M., Ph.D.)

....External Examiner

(Assistant Professor Kanjana Imsilp, D.V.M., Ph.D.)

ศุภภัทร คีรีวรรณ : การเปรียบเทียบฤทธิ์ฆ่าเชื้อในหลอดทดลองของยาเอนโรฟลอกซาซินเมื่อใช้เดี่ยว และใช้ร่วมกับ ดอกซีซัยคลินต่อเชื้อ อี. โคไลที่ได้จากสุนัขและแมว (COMPARATIVE *IN VITRO* KILLING ACTIVITIES OF ENROFLOXACIN ALONE AND IN COMBINATION WITH DOXYCYCLINE AGAINST *E. COLI* ISOLATED FROM DOGS AND CATS) อ.ที่ปรึกษาวิทยานิพนธ์ หลัก: อ. สพ.ญ. ดร.นิภัทรา สวนไพรินทร์, 76 หน้า.

เอนโรฟลอกซาซิน (enrofloxacin) เป็นยาต้านแบคทีเรียที่มีฤทธิ์ฆ่าเชื้อแบคทีเรีย (bactericidal activity) โดยเฉพาะแบคทีเรียแกรมลบ ส่วนดอกซีซัยคลิน (doxycycline) เป็นยาต้านแบคทีเรียที่ออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย (bacteriostatic activity) และเป็นยาหลักที่ใช้ในการรักษาการติดเชื้อแบคทีเรียในเซลล์ (intracellular bacteria) โดยเฉพาะ *Ehrlichia canis* (*E. canis*) บางครั้งเอนโรฟลอกซาซินถูกนำมาใช้ร่วมกับดอกซีซัยคลินเพื่อรักษาการติดเชื้อแบคทีเรียแกรมลบร่วมกับ *E. canis* อย่างหลีกเลี่ยงไม่ได้ ซึ่งในทางทฤษฎีการใช้ยาลักษณะดังกล่าวอาจทำให้เกิดปฏิกิริยาระหว่างยา (drug interaction) โดยเฉพาะการต้านฤทธิ์กัน (antagonism) อย่างไรก็ตาม ยังไม่มีการศึกษาปฏิกิริยาระหว่างยาสองชนิดนี้ต่อเชื้อแบคทีเรียก่อโรคที่พบได้ทั่วไป โดยเฉพาะ อี. โคไล (*E. coli*) ดังนั้นการศึกษานี้มีวัตถุประสงค์ เพื่อเปรียบเทียบฤทธิ์และเวลาฆ่าเชื้อในหลอดทดลอง ของ enrofloxacin แบบใช้เดี่ยวและใช้ร่วมกับ doxycycline ต่อเชื้อ *E. coli* ที่แยกได้จากสุนัขและแมว จำนวน 10 ตัวอย่าง ด้วยวิธี time-kill curves โดยแบ่งวิธีศึกษาการใช้ยาสองชนิดร่วมกัน เป็น 2 วิธี ได้แก่การให้ยาสองชนิดพร้อมกันและการให้ยาสองชนิดห่างกัน 12 ชั่วโมง ผลการศึกษา พบว่า การใช้ enrofloxacin แบบใช้เดี่ยวฆ่าเชื้อได้มากที่สุด คือ 3.96±0.49 log reduction (97.96-100.00%) ภายใน 2 ชั่วโมง รองลงมาคือ แบบใช้ enrofloxacin พร้อมกับ doxycycline ฆ่าเชื้อได้ 3.78±0.37 log reduction (98.73-100.00%) ภายใน 6 ชั่วโมง และน้อยที่สุดคือ แบบใช้ยาสองชนิดห่างกัน 12 ชั่วโมง โดยฆ่าเชื้อได้ 3.51±0.47 log reduction (99.19-100.00%) ภายใน 12 ชั่วโมง นอกจากนี้ เมื่อเปรียบเทียบระยะเวลาที่ใช้ฆ่าเชื้อ (time to 3 log reduction, T3K) พบว่า enrofoxacin เมื่อใช้ร่วมกับ doxycycline แบบให้ยาสองชนิดห่างกันฆ่าเชื้อได้ช้ากว่า enrofloxacin แบบใช้เดี่ยวอย่างมีนัยสำคัญทางสถิติ (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, $P<0.05$) จากการศึกษานี้ สรุปได้ว่า การใช้ enrofloxacin ร่วมกับ doxycycline ทำให้การฆ่าเชื้อลดลงและช้าลง โดยเฉพาะเมื่อให้ยาสองชนิดห่างกัน 12 ชั่วโมง ซึ่งข้อมูลดังกล่าวนี้สามารถนำไปประยุกต์ใช้ ในการประเมินผลทางคลินิก (clinical outcomes) ที่จะเกิดขึ้นกับสัตว์ป่วยที่ได้รับยาสองชนิดนี้ และศึกษาปฏิกิริยาระหว่างยาสองกลุ่มนี้ในเชิงลึกต่อไปในอนาคต

ภาควิชา   เภสัชวิทยา   ลายมือชื่อนิสิต _____

สาขาวิชา   เภสัชวิทยาทางสัตวแพทยศาสตร์   ลายมือชื่อ อ.ที่ปรึกษาหลัก _____

ปีการศึกษา   2559

5775311631 : MAJOR VETERINARY PHARMACOLOGY

KEYWORDS: CATS / DOGS / DOXYCYCLINE / DRUG INTERACTION / E. COLI / ENROFLOXACIN / IN VITRO KILLING

SUPAPATT KIREEWAN: COMPARATIVE *IN VITRO* KILLING ACTIVITIES OF ENROFLOXACIN ALONE AND IN COMBINATION WITH DOXYCYCLINE AGAINST *E. COLI* ISOLATED FROM DOGS AND CATS. ADVISOR: NIPATTRA SUANPAIRINTR, D.V.M., Ph.D., 76 pp.

Enrofloxacin and doxycycline are two of the most frequently used antibacterial agents in the veterinary practice. Enrofloxacin exhibits bactericidal activity especially against Gram-negative bacteria, while doxycycline demonstrates only bacteriostatic effect and is the drug of choice for treating intracellular bacteria, *Ehrlichia canis* (*E. canis*) in particular. On the occasion of Gram-negative bacteria and *E. canis* co-infection, using enrofloxacin in combination with doxycycline is inevitable. Theoretically, the antagonism between bactericidal and bacteriostatic agents has been well established. However, the drug interaction between enrofloxacin and doxycycline has not been yet identified especially against a common pathogen, *Escherichia coli* (*E. coli*). Therefore, the objective of this study was to compare *in vitro* killing activities and times of enrofloxacin alone to its combination with doxycycline, against ten *E. coli* isolates derived from dogs and cats using time-kill curves. The combinations were tested into two ways - simultaneous and separated combinations (12-hour interval). The results showed that enrofloxacin alone could kill *E. coli* best with a log reduction of 3.96±0.49 (97.96-100.00% kill) within two hours, followed by the simultaneous combination with a log reduction of 3.78±0.37 (98.73-100.00% kill) within six hours, and the separated combination with a log reduction of 3.51±0.47 (99.19-100.00% kill) after 12 hours of enrofloxacin exposure. Moreover, when comparing time to 3 log reduction (T3K), the separated combination killed *E. coli* significantly slower than that of enrofloxacin alone (8.04±0.94 Vs 2.47±0.40 hours, $P<0.05$), which was consistent with the results of time to elimination (TE) (14.97±1.35 Vs 5.83±0.58 hours, $P<0.05$). From this study, it can be concluded that the killing activity of enrofloxacin is reduced and delayed especially by the presence of doxycycline 12 hours prior to enrofloxacin. The findings in this study can be applied to predicting clinical outcomes from the use of enrofloxacin and doxycycline combination and to studying more about drug interaction between these two classes of antibacterial drugs in the future.

Department:	Veterinary Pharmacology	Student's Signature ....
Field of Study:	Veterinary Pharmacology	Advisor's Signature ....
Academic Year:	2016	

ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

µl	=	microliter
°C	=	degree of Celcius
ATCC	=	American type cell collection
CAMHB	=	cation-adjusted Mueller-hinton broth
CFU	=	colony forming unit
DNA	=	deoxyribonucleic acid
DXT	=	doxycycline
E. canis	=	*Ehrlichia canis*
E. coli	=	*Escherichia coli*
ENR	=	enrofloxacin
FQs	=	fluoroquinolones
M	=	molar (mol/L)
mg	=	milligram
MIC	=	minimum inhibitory concentration
MHA	=	Mueller-hinton agar
ml	=	milliter
NRRG	=	nonoptimal regulation of ribosomal genes
PAE	=	postantibiotic effect
PSDP	=	protein-synthesis dependent pathway
ROS	=	reactive oxygen species
T3K	=	time to 3 log kill
TE	=	time to elimination
TSA	=	tryptic soy agar

CHAPTER I

INTRODUCTION

Importance and Rationale

Nowadays, antibacterial agents have been widely used in both companion (Watson and Maddison, 2001; Guardabassi and Prescott, 2015) and food animals (McEwen and Fedorka-Cray, 2002). One of the most frequently used groups of antibacterials is fluoroquinolones (Escher *et al.,* 2011). This group of antibacterial agents acts by inhibiting DNA replication of bacterial cells (Schmitz *et al.,* 2002), leading to bacterial cell death (bactericidal activity). As a veterinary approved drug, enrofloxacin is the most prescribed fluoroquinolone for treating bacterial infections in companion animals (Wayne *et al.,* 2011). It exhibits a broad spectrum activity, particularly against Gram-negative bacteria (Watson and Maddison, 2001; Boothe, 2012). Another group of antibacterial drugs frequently selected is tetracyclines, which can inhibit bacterial protein synthesis and in turn inhibits bacterial growth by keeping bacterial cells in the stationary phase (bacteriostatic activity). Tetracyclines has a broad spectrum of activity covering various types of microbes, mainly Gram positive bacteria and rickettsia (MacDougall and Chambers, 2011; Boothe, 2012). The most chosen drug in this group is doxycycline, mainly to treat intracellular bacterial

infections especially *Ehrlichia canis* (*E. canis*) (Riviere and Papich, 2009; Wayne *et al.*, 2011)

In fact, an infection in companion animals can be caused by more than one type of microbes (polymicrobial infection), and only one antibacterial drug may not be effective for all types of the infectious agents. Therefore, combination antibacterial therapy should be considered applying (Gumbo, 2011), and thus drug interaction must be concerned. The general principle of combination antibacterial therapy is that drug interaction resulting in synergism or addition and avoidance of antagonism should be achieved. In doing so, bacteriostatic agents such as tetracyclines, phenicols, and macrolides should not be used together with bactericidal drugs like beta-lactams and fluoroquinolones because the killing mechanisms of the latter drugs depend on the bacterial growth. If bacteria is inhibited by a bacteriostatic agent or kept in the stationary phase, the killing activity of a bactericidal drug will be diminished (Jawetz and Gunnison, 1953; Jawetz *et al.*, 1954; Martinez *et al.*, 2006; Boothe, 2012; Podos *et al.*, 2012).

In the general veterinary practice, *E. canis* infection can be found along with Gram-negative bacterial infection like *E. coli*, partly due to the tropical climate in Thailand supporting microbial growth (Kelly, 2000; Normand *et al.*, 2000; Neer *et al.*, 2002; Ariyawutthiphan *et al.*, 2005). Hence, when the polymicrobial infection occurs, the use of enrofloxacin in combination with doxycycline is inevitable, which contradicts the principle of combination antibacterial therapy previously mentioned.

However, to the best of our knowledge, no research has been carried out to identify drug interaction between these two drugs against *E. coli*. So, the researchers are interested in studying the drug interaction between enrofloxacin and doxycycline by using *E. coli*, a common pathogen causing problems in both humans and animals, as a model (Sanchez *et al.*, 2002; Belanger *et al.*, 2011). The purpose of this study was to evaluate *in vitro* killing activities and *in vitro* killing times based on the hypothesis that doxycycline could antagonize the killing activity of enrofloxacin. The results from this study may shed light on the drug interaction between enrofloxacin and doxycycline and provide scientific information for combination antibacterial therapy in the general veterinary practice and for further studies on drug interactions.

Objectives

1. To compare *in vitro* killing activities (bactericidal activities) of enrofloxacin alone and in combination with doxycycline against *E. coli* isolated from dogs and cats.

2. To compare *in vitro* killing times of enrofloxacin alone and in combination with doxycycline against *E. coli* isolated from dogs and cats.

Keywords (Thai) : แมว สุนัข ดอกซีซัยคลิน ปฏิกิริยาระหว่างยา อี. โคไล เอนโรฟลอกซาซิน ฤทธิ์ฆ่าเชื้อในหลอดทดลอง

Keywords (English) : cats, dogs, doxycycline, drug interaction, *E. coli,* enrofloxacin, *in vitro* killing

Research Questions

1. Does *in vitro* killing activity (bactericidal activity) of enrofloxacin alone differ from that of its combination with doxycycline against *E. coli*?

2. Does *in vitro* killing time of enrofloxacin alone differ from that of its combination with doxycycline against *E. coli*?

Hypotheses

1. *In vitro* killing activity (bactericidal activity) against *E. coli* of enrofloxacin alone is greater than that of its combination with doxycycline

2. *In vitro* killing time against *E. coli* of enrofloxacin alone is shorter than that of its combination with doxycycline.

Merits, Significance and Implications

1. The obtained results will provide us with information on *in vitro* killing activities (bactericidal activity) of enrofloxacin, when used alone and in combination with doxycycline, against *E. coli* isolated from dogs and cats in order to assess drug interaction and predict clinical outcomes that may occur.

2. The collected data will give us the information about *in vitro* killing times of enrofloxacin, when used alone and in combination with doxycycline, against *E. coli* isolated from dogs and cats so as to evaluate possible drug interaction which is useful for those studying the mechanisms of drug interaction between antibacterial drugs.

CHAPTER II

LITERATURE REVIEW

Enrofloxacin

Enrofloxacin is a first-generation fluoroquinolone with high lipid solubility (Lizondo *et al.*, 1997). Fluoroquinolones (FQs) can act by directly inhibiting two enzymes crucial to the DNA replication. The first target enzyme is DNA gyrase, which is an important target for FQs in Gram-negative bacteria. When DNA gyrase is bound by FQs, the movement of DNA through the replication process is blocked in a supercoiling state. The second target for FQs is topoisomerase IV, an important enzyme for FQs in Gram-positive bacteria. When this enzyme is compromised by FQs, daughter chromosomes produced by a round of replication are unable to separate from each other. Either way, the result is the formation of FQs-topoisomerase-DNA complexes which provide only bacteriostatic activity at a low concentration (Drlica *et al.*, 2008). More importantly, if such formation continues especially at a higher concentration, it will cause chromosome fragmentation and the accumulation of reactive oxygen species (ROS) through Fenton reaction, leading to rapid bacterial cell death (bactericidal activity) (Schmitz *et al.*, 2002; Kohanski *et al.*, 2010; Wang *et al.*, 2010). If the FQs concentration in the blood increases, the bactericidal activity will be

also enhanced (concentration-dependent activity), which is probably due to the more numbers of topoisomerase molecules being interfered by FQs. Moreover, with an increased level of FQs, the period of inhibiting bacterial growth when the drug concentration is below minimum inhibitory concentration (MIC) (post antibiotic effect, PAE) is also extended, making dosing interval longer (Wetzstein and Trenti, 1994; Carbone *et al.*, 2001; Martinez *et al.*, 2006; Boothe, 2012).

Based on pharmacokinetic study, enrofloxacin could be well absorbed through the gastrointestinal tract and partially metabolized by de-ethylation into an active metabolite, ciprofloxacin, up to 40% of the total drug concentration (Cester and Toutain, 1997). As a result, a success in clinical treatments would be due to the additive effect between enrofloxacin and ciprofloxacin (Blondeau *et al.*, 2012).

As a fluoroquinolone, not only does enrofloxacin use both a porin- and a lipid-mediated pathways to enter bacterial cells, but also utilizes self-promoted uptake pathway to increase its penetration as aminoglycosides and polymyxin B do (Chapman and Georgopapadakou, 1988; Delcour, 2009). In terms of antibacterial activity, enrofloxacin has been considered broad spectrum against Gram-negative bacteria such as *E. coli*, *Pasteurella* spp. *Klebsiella* spp.*, *Proteus mirabilis*, *Campylobacter* spp.*, *Vibrio* spp. and *Pseudomonas aeruginosa*, but limited spectrum against Gram-positive bacteria like *Staphylococcus* spp. and *Corynebacterium* spp.. Unfortunately, enrofloxacin is ineffective against anaerobic bacteria (Ihrke *et al.*, 1999; Watson and Maddison, 2001; Prescott *et al.*, 2002; Boothe, 2012). Moreover,

enrofloxacin can be used to treat many infections caused by some intracellular pathogens such as *Chlamydia psittaci*, *Haemobartonella felis*, *Leishmania infant*, and *Mycoplasma gallisepticum*, but it is ineffective against *E. canis* (Butaye *et al.*, 1997; Barbour *et al.*, 1998; Neer *et al.*, 1999; Dowers *et al.*, 2002; Bianciardi *et al.*, 2004)

Doxycycline

Doxycycline is a long-acting semi-synthetic tetracycline with high lipid solubility (Riviere and Papich, 2009). Tetracyclines can inhibit bacterial protein synthesis by binding with 16s ribosomal RNA (16s rRNA) on 30s ribosomal subunit (A site), which normal transfer RNA (tRNA) normally binds (Gale and Folkes, 1953; Suzuka *et al.*, 1966; Boothe, 2012), leading to incomplete protein synthesis and eventually inhibiting bacterial growth (bacteriostatic activity). The bacteriostatic activity of tetracyclines can be enhanced by the extension of the period that the drugs remain above MIC (time-dependent activity). The PAE of tetracyclines exerts in a concentration-dependent fashion (2.1-4.2 hours for doxycycline), and so do bactericidal activities against some bacteria such as *Streptococcus pneumoniae* (Cunha *et al.*, 2000; Riviere and Papich, 2009).

Doxycycline penetrates bacterial cell wall and membrane by simple diffusion, but sometimes involves with porins in Gram-negative bacteria (Stratton, 2015). As for antibacterial properties, the spectrum of doxycycline covers both Gram-positive

bacteria like *Staphylococcus* and *Streptococcus* spp., and Gram-negative bacteria such as *Escherichia coli*, *Pasteurella* spp., *Klebsiella* spp., *Salmonella* spp. including anaerobes, but it is not effective against *Pseudomonas aeruginosa* (Cunha *et al.*, 2000; Boothe, 2012). Moreover, with the ability to penetrate into cells, this drug can be effectively used to treat many systemic infections caused by intracellular microorganisms such as *Rickettsia rickettsia*, *Mycoplasma* spp*., Babesia gibsoni*, *Hemobartonella* spp. and *Ehrlichia canis*, making doxycycline considered to be the drug of choice for ehrlichiosis (van Heerden and Immelman, 1979; Breitschwerdt *et al.*, 1997; Neer *et al.*, 2002; Lin and Huang, 2010; Boothe, 2012). Furthermore, doxycycline is effective against bacterial endosynbiont *Wolbachia*, leading to indirectly killing *Dirofilaria immitis* in both microfilarial and adult stages (Bazzocchi *et al.*, 2008).
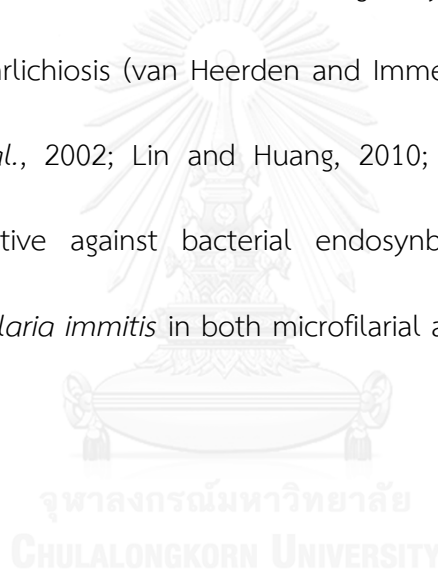
Combination antibacterial therapy

Combination antibacterial therapy is an application of assigning more than one antibacterial drug to a patient. The objectives of the therapy (Gumbo, 2011; MacDougall and Chambers, 2011) are as follows:

1) To treat polymicrobial infection in which only one antibacterial agent does not have enough spectrum of activity against all of the target microbes.

2) To accelerate rate of bacterial eradication, resulting in better and faster clinical outcomes.

3) To reduce likelihood of antibacterial resistance imposed by a single drug (monotherapy).

4) To reduce drug toxicity by minimizing doses of possibly toxic antibacterials being employed.

In order to attain such objectives, the general principle of the antibacterial combination therapy is to take advantage of favorable drug interactions, either addition or synergism, and to avoid unfavorable drug interaction, or antagonism (Yeh *et al.*, 2009; Boothe, 2012). Regarding the favorable ones, there are several modes to develop a new additive or synergistic combination (Cottarel and Wierzbowski, 2007).

1) A secondary drug prevents the primary drug (antibacterial) from being degraded or modified by bacteria such as the combination of Beta-lactams with a Beta-lactamase inhibitor (Barry *et al.*, 1984).

2) A secondary drug inhibits efflux pumps to enhance the accumulation of the primary drug (antibacterial). For example, phenylalanine arginylb-naphthylamide (PAβN) competitively inhibits MexAB pump in *Pseudomonas aeruginosa*, leading to the accumulation of ciprofloxacin and levofloxacin (Askoura *et al.*, 2011).

3) A secondary agent inhibits or blocks intrinsic repair pathways or tolerance mechanisms to the primary drug (antibacterial). For instance, curcumin can inhibit

SOS response through RecA-LexA system, an important system for DNA repair in *E. coli* (Bellio *et al.*, 2014).

4) A secondary drug itself has an antibacterial property through another mechanism similar to or different from that of the primary antibacterial such as the combination of cell-wall inhibitor agents acting through different targets, the combination of aztreonam and fosfomycin for example (Pillai *et al.*, 2005; Hickman *et al.*, 2014).

On the other hand, an antagonistic interaction can cause many disastrous outcomes such as increased mortality rates and cost, therapy failures and toxicities of drugs being used, all of which are the reasons why one should avoid using the antagonistic combination, especially in immunocompromised patients (Bodey, 1985; Githaiga *et al.*, 2005).

At present, there are still unknown underlying mechanisms explaining how these antagonistic phenomena predominantly happen between bactericidal and bacteriostatic agents (Ocampo *et al.*, 2014). One of the reasons why antagonism occurs is the use of a bactericidal agent in combination with a bacteriostatic drug (Jawetz and Gunnison, 1953; Jawetz *et al.*, 1954; Garrod, 1972; Bollenbach, 2015). For example, a bactericidal drug which acts by inhibiting cell wall synthesis should not be used together with a bacteriostatic agent because the killing mechanisms of the former drug depend on bacterial growth such as the use of penicillin in combination with chlortetracycline (Pillai *et al.*, 2005) or chloramphenicol (Jawetz *et al.*, 1951).

An underlying mechanism that explains the antagonistic interaction between penicillin and chloramphenicol may be involved with the fact that chloramphenicol inhibits the activity of autolysin, an enzyme important for penicillin-induced cell lysis (Tomasz and Waks, 1975). However, as for the antagonism between DNA synthesis inhibitors and bacteriostatic agents, especially protein synthesis inhibitors, the mechanisms behind this are still underexplored (Bollenbach *et al.*, 2009; Yeh *et al.*, 2009).

Drug-drug interaction assessment

Drug-drug interaction assessment is a process of identifying the drug interaction (addition, synergism or antagonism), but sometimes no interaction or indifference can be observed. In microbiology, there are five conventional *in vitro* methods to evaluate drug-drug interaction (Pillai *et al.*, 2005; Punam, 2007) as follows:

1) Checkerboard titration

This technique refers to the pattern of tubes or microtiter wells formed by multiple dilutions of the two antibacterial agents. The concentrations of the dilutions are equal to, above, and below MICs of the bacteria being tested. After incubation of bacteria of interest with two antibacterial agents in different concentrations for 18-24 hours, MICs of the

drugs are used to calculate fractional inhibitory concentration (FIC) for further interpretation.

2) Disk diffusion synergy test

This method is applied from the conventional disk diffusion test by placing two antibacterial disks, instead of one. The distance between the disks should be equal to or slightly greater than the combined inhibition zone of the two disks. After incubation for 18-24 hours, the inhibition zone between two disks is interpreted.

3) Paper strip diffusion

In this technique, filter paper strips are soaked in the antibacterial drugs of interest; one strip for one antibacterial. A drug strip is placed at the right angle to one another on an agar plate. After incubation for 18-24 hours, the inhibition zone at the joint angle is interpreted.

4) Kinetic spectrophotometry

This method utilizes the concept of spectrophotometry by measuring the absorbance of broth containing bacteria and antibacterial drug(s) of interest over time. It can be further modified to measure bactericidal activity and PAE by performing bacterial counts (Domínguez *et al.,* 2001)

5) Time-kill curves

This technique is done by mixing bacterial cells with two antibacterial drugs of interest in broth to reach desirable concentrations for both.

Then, repetitive bacterial counts are performed to determine bacterial viable cells over time for further interpretation by comparing bactericidal activity of the combination to that of the most active drug alone.

The advantages and disadvantages of each method are tabulated in Table 1. As a matter of fact, most of the methods are used to measure only the ability of antibacterial drugs to inhibit bacterial growth (bacteriostatic activity), except for time-kill curves which can measure bactericidal activity of the drugs (Mueller *et al.*, 2004; Pillai *et al.*, 2005). Nonetheless, when one tests the same pair of drugs with a different method, the results may be inconsistent with each other. For example, the results from checkerboard titration are interpreted as synergism but those of time-kill curves indicate no interaction. One of the reasons behind this inconsistency is because these two methods are designed to measure different parameters. That is, checkerboard titration is designed to measure bacteriostatic effect, while time-kill curves technique is used to assess bactericidal activity (CLSI, 1999; Punam, 2007).

Time-kill curves technique is an *in vitro* method to determine the number of bacterial cells being killed at each specific time under a strictly-controlled environment and a fixed concentration. This method is usually used to test bactericidal activity of a new antibacterial drug and to assess drug interaction between antibacterial agents by comparing killing activity of a particular combination with that of the most active drug featured in the combination (CLSI, 1999; Punam, 2007). The results derived from this method are correlated with those of bacterial

eradication (Dagan *et al.*, 2001). If the results show that the reduction of bacterial cells is at least 99.9% ($3\log_{10}$ reduction) from the initial count, it means that the tested drug or combination can exhibit bactericidal activity (CLSI, 1999). If the number of bacterial cells is constant and 99.9% reduction ($3\log_{10}$ reduction) is not achieved at the end of experiment, it refers to bacteriostatic activity (Pankey and Sabath, 2004; Tenover *et al.*, 2004).

As a class of bactericidal agents, there have been many studies of quinolones using time-kill curves. When tested with 8xMIC or clinical drug concentrations (2.1-4.1 µg/ml), enrofloxacin was able to kill *E. coli* within three hours, which was enough to determine bactericidal activity of quinolones especially with a high concentration being employed (Norcia *et al.*, 1999; Blondeau *et al.*, 2012). Gatifloxacin, trovafloxacin and levofloxacin at 1xMIC could demonstrate bactericidal activity within 0.6-1.2 hours (Fung-Tomc *et al.*, 2000).

Table 1 Comparisons of advantages and disadvantages among five drug interaction

(modified from Domínguez, 2001, Pillai, 2005 and Punam, 2007)

Method	Advantages	Disadvantage
1. Checkerboard titration	- Can test multiple drugs with multiple dilutions within a test - Requires readily available equipment for microbiology laboratories - Easy to understand in terms of mathematical calculation for interpretation	- Measures only bacteriostatic activity - Assumes a Linear dose-response curve for all antibacterial drugs - Examines only one time point
2. Disk diffusion synergy test	- Has commercially designed antibacterial disks similar to Bauer-Kirby susceptibility test - Easy to perform	- Measures only bacteriostatic activity - Examines only one time point - Yields only qualitative results - Difficult to differentiate between indifferent and synergistic interactions.
3. Paper strip diffusion	- Has some commercially designed antibacterial strip available (Epsilometer Etest strip) - Can be modified to provide quantitative determination of the potential synergistic combination	- Measures only bacteriostatic activity - Examines only one time point
4. Kinetic spectrophotometry	- Provides a dynamic picture of an antibacterial action by multiple time points - Can be modified to provide bactericidal activity and PAE	- Measures only bacteriostatic activity
5. Time-kill curves	- Measures bactericidal activity - Provides a dynamic picture of an antibacterial action - Useful in guiding therapy especially when bactericidal activity is required	- Tedious and costly because of repetitive bacterial counts - Can be performed with limited number of antibacterial drugs and their concentrations being tested

Antagonism between quinolones and other bacteriostatic drugs

Regarding antagonism prevailing among bactericidal-bacteriostatic interactions, as a group of bactericidal agents, quinolones have also been studied with many bacteriostatic drugs (Table 2). For instance, when tested against *S. aureus, B. subtilis* and *E. coli* by disk diffusion synergy test, ciprofloxacin showed antagonism with fusidic acid (Uri, 1993). Additionally, ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin showed antagonistic interactions with fusidic acid by disk synergy test (Ertek *et al.*, 2002), which is consistent with the previous study by Uri (1993). As for time-kill technique, the killing activity of nalidixic acid (25 µg/ml; 2xMIC) against *E. coli* could be mitigated by the presence of many bacteriostatic agents such as tetracycline (12.5 µg/ml; 0.83xMIC), erythromycin (200 µg/ml; 0.4xMIC), and trimethoprim (10 µg/ml; 0.66xMIC) (Ocampo *et al.*, 2014). When tested against *E. coli* in logarithmic phase of growth, the killing actions of ciprofloxacin and norfloxacin (0.1 and 10 µg/ml; 1x and 10xMIC) were reduced when chloramphenicol (4 µg/ml, unknown MIC) was added, but this antagonistic phenomenon did not happen when the same experiment was done in stationary-phase *E. coli* (Zeiler, 1985). Moreover, when ciprofloxacin (0-35.2 ng/ml; a range of sub-MICs) was tested with doxycycline (0-81.92 ng/ml; a range of sub-MICs), the killing effect against *E. coli* in the logarithmic phase of growth was diminished defined as suppressive antagonism. The killing activities of gatifloxacin and ciprofloxacin (8xMIC) against *E. faecalis* were reduced

100-fold when they were combined with bacteriostatic drugs, either rifampicin or chloramphenicol (Gradelski *et al.*, 2001).

Nevertheless, the underlying mechanisms behind antagonistic interactions between quinolones and bacteriostatic drugs have not been clearly understood yet. One of the possible reasons is due to the facts that a bacteriostatic agent reduces bacterial growth rate or keeps bacterial cells in the stationary phase, and that the killing activities of quinolones are partly dependent on protein and RNA synthesis during bacterial cell division (Martinez *et al.*, 2006). As a result, using a quinolone in combination with a protein synthesis inhibitor (or with an RNA synthesis inhibitor) against actively-growing bacteria or using a quinolone such as flerofloxacin alone against stationary-phase bacteria will reduce its bactericidal activity (Table 3) (Widmer *et al.*, 1991; Sulochana *et al.*, 2009; Podos *et al.*, 2012). However, the previous assumption is inconsistent with some research suggesting that some quinolones such as ciprofloxacin, norfloxacin and ofloxacin were still be able to kill bacteria for both *in vitro* and *in vivo* experiments, even if a bacteriostatic agent such as chloramphenicol was added to the logarithmic inoculum or bacterial cells were in the stationary phase (Zeiler, 1985; Zeiler *et al.*, 1988).

According to the studies above, it is clearly that drug interactions between quinolones and bacteriostatic drugs are still controversial depending on quinolones, phase of bacterial growth, concentrations including drug interaction assessing method being employed (Yeh *et al.,* 2006; Chait *et al.,* 2007; Boothe, 2012; Ocampo *et al.,* 2014).

Table 2 *In vitro* studies on the drug interactions between quinolones and other antibacterial agents

Quinolones	Combined drugs	Tested Bacteria	Method	Results	References
Ciprofloxacin	Chloramphenicol	*E. coli*	Time-kill curves	Antagonism[1], No interaction[2]	Zeiler, 1985
Norfloxacin	Chloramphenicol	*E. coli*	Time-kill curves	Antagonism[1], No interaction[2]	Zeiler, 1985
Ciprofloxacin	Fusidic acid	*S. aureus, B. subtilis, E. coli*	Disk diffusion synergy test	Antagonism	Uri, 1993
Ciprofloxacin Gatifloxacin	Rifampicin Chloramphenicol	*E. faecalis*	Time-kill curves	Antagonism[1]	Gradelski, 2001
Ofloxacin, Ciprofloxacin, Levofloxacin, Moxifloxacin	Fusidic acid	*S. aureus*	Disk diffusion synergy test	Antagonism	Ertek, 2002
Ciprofloxacin	Doxycycline	*E. coli*	Time-kill curves	Antagonism[1]	Chait, 2007
Enrofloxacin	Ciprofloxacin	*E. coli* *S. pseudintermedius* *P. aeruginosa*	Time-kill curves	Addition[1]	Blondeau, 2012
Nalidixic acid	Tetracycline, Erythromycin Trimethoprim	*E. coli*	Time-kill curves	Antagonism[1]	Ocampo, 2014
Ciprofloxacin	Fusidic acid, Doxycycline, Tetracycline, Chloramphenicol	*E. coli*	Time-kill curves	Antagonism[1]	Ocampo, 2014
Lomefloxacin	Fusidic acid, Doxycycline, Tetracycline, Chloramphenicol Erythromycin, Sulfamonomethoxine	*E. coli*	Time-kill curves	Antagonism[1]	Ocampo, 2014

[1]Logarithmic-phase bacteria; [2]Stationary-phase bacteria

Table 3 Time-kill studies on quinolones against stationary-phase bacteria

Quinolones	Tested Bacteria	Method/Model	Results Overall activity[1]	Results Compared with logarithmic phase bacteria	References
Enoxacin, Amifloxacin, Pefloxacin, Norfloxacin		Time-kill curves[2]	Bacteriostatic (<2log kill)	-	Zeiler, 1988
Ofloxacin	*E. coli*		Bacteriostatic (2log kill)	-	
Ciprofloxacin			Bacteriostatic (2.5log kill)	-	
Norfloxacin, Pefloxacin		Murine model of granuloma pouch[2]	Bacteriostatic (<2log kill)	-	
Ciprofloxacin			Bacteriostatic (<2log kill)	-	
Flerofloxacin	*E. coli*	Time-kill curves	Bacteriostatic (<2log kill)	→	Widmer, 1991
Ciprofloxacin			Bactericidal (>3log kill)	→ (slightly)	
Moxifloxacin	*M. tuberculosis*	Time-kill curves[2]	Bacteriostatic (2.5log kill)	→	Sulochana, 2009
Moxifloxacin	*S. aureus*	Time-kill curves	Bacteriostatic (<2log kill)	→	Podos, 2012

[1] If 3log kill is achievable at the end point, it can be interpreted as bactericidal activity.

[2] End point was not set at 24 hours

CHAPTER III

METHODOLOGY

Conceptual framework
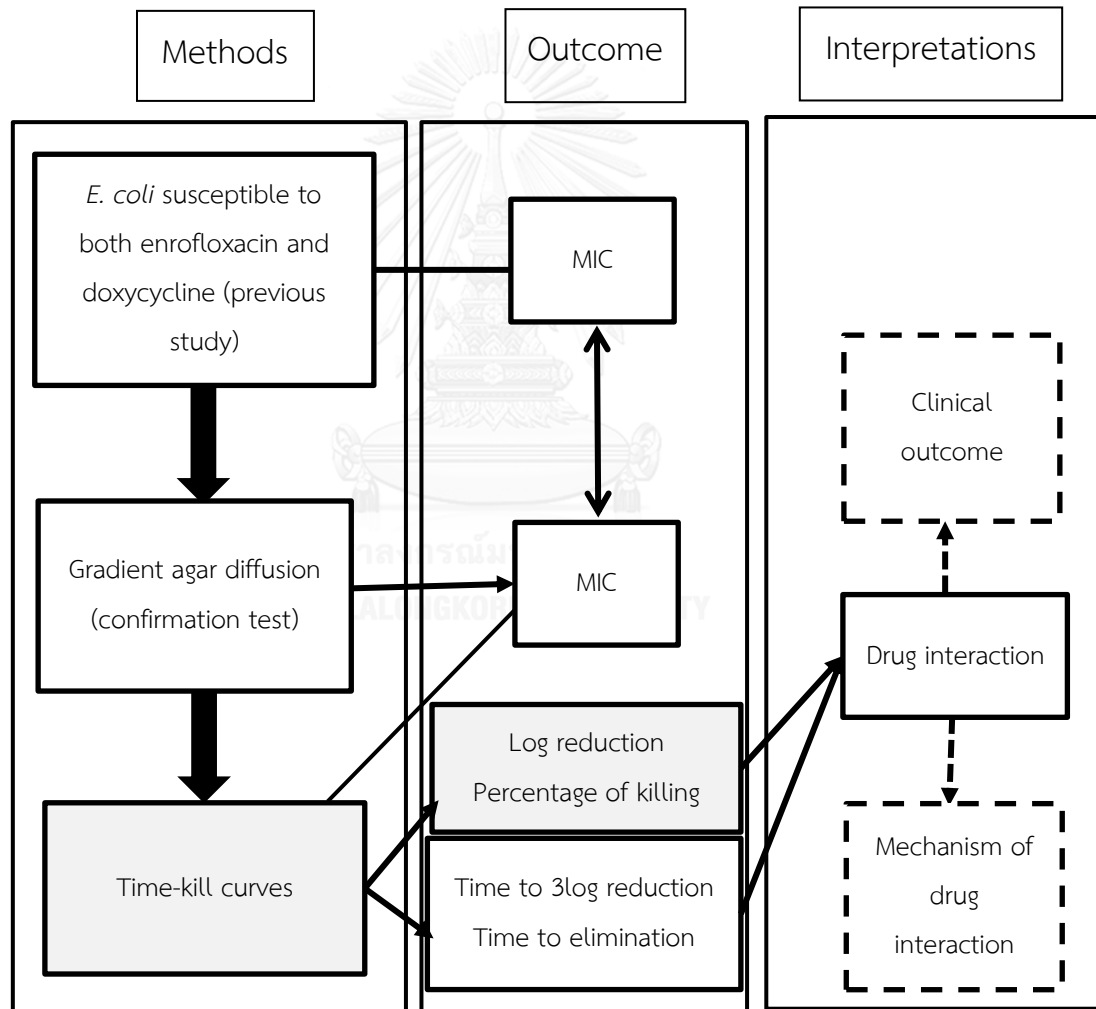
Research Instruments and Equipment

1. Instruments and equipment

    1.1 Petri dishes

    1.2 Inoculation loop

    1.3 50 ml graduated cylinder

    1.4 5 ml and 10 ml serological pipettes

    1.5 Autoclavable glass tubes, size 16x100 mm

    1.6 Autoclavable glass tubes , size 20x100 mm

    1.7 Autoclavable test tube caps, size 16 mm

    1.8 Cryovials and cryoboxes

    1.9 Alcohol burner

    1.10 Micropipettes and micropipette tips

    1.11 Sterile cotton swabs

    1.12 Disposable spreaders

    1.13 Non-toothed forceps

    1.14 Vortex mixer

    1.15 Densitometer (suspension turbidity detector) (DEN-1B, Biosan, Latvia)

    1.16 Incubator (Memmert, Germany)

    1.17 Autoclave machine

    1.18 -80 °C freezer

2. *Chemicals*

    1.1 Doxycycline powder (Sigma, USA)

    1.2 Enrofloxacin powder (Sigma, USA)

    1.3 Doxycycline MIC strips (Liofilchem®, Italy)

    1.4 Enrofloxacin MIC strips (Liofilchem®, Italy)

    1.5 0.9% sodium chloride solution (0.9% NaCl)

    1.6 Glycerol

    1.7 0.5 McFarland standard solution

    1.8 Mueller Hinton agar (MHA) (Difco™, USA)

    1.9 Cation-adjusted Mueller Hinton broth (CAMHB) (BBL™, USA)

    1.10 Tryptic soy agar (TSA) (Oxoid Ltd., UK)

    1.11 Sterile distilled water
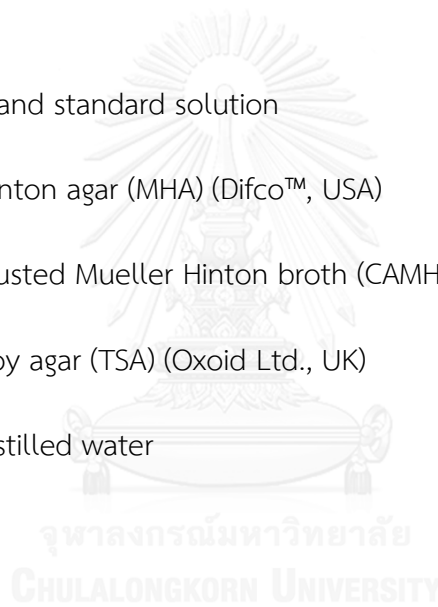
3. *Biological sample*

    3.1 *E. coli* ATCC® 25922

Materials and Methods

Experiment 1 : Susceptibility of *E. coli* to enrofloxacin and doxycycline

1.1 *E. coli* isolates

E. coli isolates used in this experiment were clinical isolates from dogs and cats submitted to veterinary diagnostic laboratory, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, for bacterial identification and antibiograms. Ten isolates susceptible to both enrofloxacin and doxycycline were selected and stored at -80 °C in the storage media composed of tryptic soy broth (TSB) 70% and glycerol 30% until further analyses.

1.2 Gradient agar diffusion susceptibility test

Each *E. coli* isolate was thawed and subcultured on TSA and incubated at 37 °C for 18-24 hours. Susceptibility tests to enrofloxacin and doxycycline were performed in all ten isolates using MIC strips according to the manufacturer's instructions. Briefly, after incubated for 18-24 hours, one or two growth colonies were suspended in 0.9% sodium chloride solution and adjusted to a McFarland standard of 0.5. Then, the bacterial suspension was thoroughly spread onto MHA using a sterile cotton swab. The inoculated plates were allowed to dry before enrofloxacin and doxycycline MIC strips were carefully placed onto the agar. The antibacterial concentration ranges determined on MIC strips were 0.002 to 32 µg/ml for

enrofloxacin and 0.016 to 256 µg/ml for doxycycline. All plates were incubated at 37

°C for 18-24 hours (Figures 1 and 2). After incubation, the inhibition zones of the

tested isolate were read and interpreted based on susceptible MIC breakpoints ($\leq$ 0.5

µg/ml for enrofloxacin and $\leq$ 4 µg/ml for doxycycline) according to Performance

standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated

from animals; VET01S, 3rd ed. (CLSI, 2015). *E. coli* ATCC[®] 25922 was used as the

quality control isolate in accordance with Performance standards for antimicrobial

disk and dilution susceptibility tests for bacteria isolated from animals, VET01-A4

(CLSI, 2013). Samples not susceptible to both enrofloxacin and doxycycline and

samples with unclear inhibition zones were excluded from the study.

Figure 1 The inhibition zone after the application of MIC strip for enrofloxacin

Figure 2 The inhibition zone after the application of MIC strip for doxycycline
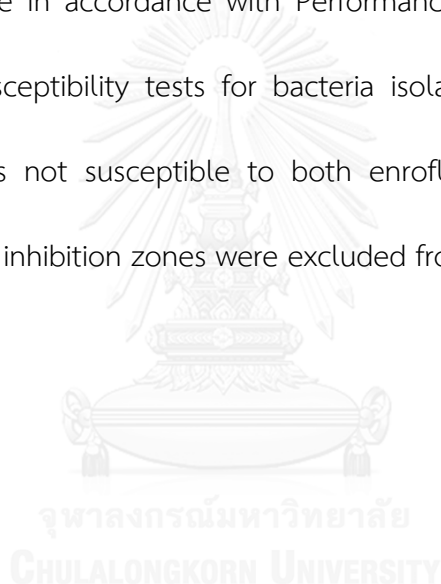
Experiment 2 : Time-kill curves

2.1 *E. coli* inoculum preparation

Ten *E. coli* isolates susceptible to both enrofloxacin and doxycycline from the previous experiment were each subcultured on TSA and incubated at 37 °C for 18-24 hours. In order to achieve an actively growing inoculum (logarithmic inoculum), one or two bacterial colonies on TSA were suspended in CAMHB and incubated again at 37 °C for 2 hours. Then, the inoculum was adjusted to a McFarland standard of 0.5, which was approximately equivalent to 1.5×10^{8} CFU/ml, and further diluted by 10-fold dilution technique to attain the inoculum size of 1.5×10^{6} CFU/ml.

2.2 Antibacterial stock solution preparation

The initial concentration of antibacterial stock solutions for both enrofloxacin and doxycycline was 10 mg/ml. Both antibacterial stocks were prepared and diluted properly according to Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; VET01S, 3rd ed. (CLSI, 2015). In doing so, a 10 mg of enrofloxacin powder was solubilized in 0.5 ml of distilled water and 0.1 M sodium hydroxide dropwise until the powder was completely dissolved. The solution was further adjusted to a volume of 1 ml by distilled water. As for doxycycline, a 10 mg of doxycycline was completely

solubilized in 1 ml of distilled water. For both antibacterials, the prepared solutions could be further diluted by sterile CAMHB to achieve the desirable concentrations of the particular isolate being tested.

2.3 Time-kill curves

In this experiment, each *E. coli* inoculum was prepared for five groups based on the tested drugs as follows:

1. Group 1 control (no drugs)

2. Group 2 enrofloxacin

3. Group 3 doxycycline

4. Group 4 enrofloxacin and doxycycline added at the same time

5. Group 5 enrofloxacin and doxycycline in which enrofloxacin was added after 12-hour doxycycline exposure

To begin with, the prepared antibacterial stock was added to the inoculum of each group to achieve a final antibacterial concentration of 1 MIC of each drug in a final inoculum concentration of 5×10^5 CFU/ml. The mixed inoculum was equally divided into seven 1-ml aliquots for 0.5 ml sampling at each time point. The time points of sampling were 0, 0.5, 1, 2, 3, 6, 12 and 24 hours in Groups 1-4, but 0, 12, 12.5, 13, 14, 15, 18 and 24 hours in Group 5. Additionally, one aliquot was used only once for sampling at one time point.

Then, a 100-µl aliquot from each diluted tube at each time point was plated and thoroughly spread onto MHA in triplicates. All plates were incubated at

37 °C for 18-24 hours. The bacterial count was performed with the countable plates containing 20-200 bacterial colonies on each of the three plates. The viable bacterial cells were expressed as CFU/ml.

Statistical analyses

1. Bactericidal activities

At each time point, the bactericidal activity of each treatment group was expressed in mean±SE of log reduction which were calculated from the following equation:

$$Log\ reduction_T = log_{10}(initial\ cell\ count) - log_{10}(viable\ cell\ count\ at\ time\ T)$$

Where *log reduction$_T$* was the number of bacterial cells killed in base-10 logarithm (base 10) at time T, *log$_{10}$(initial cell count)* was the number of bacterial cells available in base-10 logarithm at time 0, and *log$_{10}$(viable cell count) at time T* was the number of bacterial cells alive in base-10 logarithm at time T (Mueller *et al.*, 2004; Blondeau *et al.*, 2012).

Moreover, the data at each time point were also expressed as percentage of killing calculated from the equation as follows:

$$\text{Percentage of killing}_T = \frac{\textit{Initial cell count} - \textit{viable cell count at time T}}{\textit{Initial cell count}} \times 100$$

Where *percentage of killing$_T$* was the percentage of the bacterial cells killed at time T compared to the initial cell count, *initial cell count* was the number of bacterial cells available at time 0 and *viable cell count at time T* was the number of bacterial cells alive at time T (Blondeau *et al.*, 2012; Blondeau and Shebelski, 2016).

After 24 hours of drug exposure, the decrease of bacterial cell counts more than 99.9% or 3log reduction indicated the bactericidal activity. However, if 99.9% or 3log reduction was not achieved during drug exposure, the bacteriostatic activity was stated in accordance with Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline, M26-A (CLSI, 1999).

2. Killing times

The time indicators for bactericidal activity in this experiment were as follows:

(1) The time when 99.9% killing or 3 log kill was achieved (time to 3log reduction, T3K). In order to find T3K, the straight-line graph of a sample was depicted by the equation as follows:

$$y = ax+b$$

Where x was the time point of drug exposure, and y was the log of bacterial cells that was killed at each time point (log reduction). Then, T3K was assessed by using slopes from the linear regression out to the 6 hour time point of the line graph, and T3K was then calculated from the linear regression equation.

(2) The time when the number of bacterial cells was below detectable level (<100 CFU/ml) or assumed to be equivalent to bacterial elimination (time to elimination, TE) (McKellar *et al.*, 2004). The line of a sample was built by the equation as follows:

$$y_1 = cx_1+d$$

Where x_1 was the time point of drug exposure, and y_1 was the log of bacterial viable cells at each time point (log of viable cells). TE was estimated by

using slopes from the linear regression out to the 6 hour time point of the line graph, and TE was then calculated from the linear regression equation.

After T3K and TE had been determined, each of which was expressed as mean±SE. The differences among the three enrofloxacin-exposed groups (Groups 2, 4 and 5) were performed using Kruskal-Wallis test (ANOVA on ranks) followed by Dunn-Bonferroni pairwise comparison using SPSS software (SPSS version 22, licensed by Chulalongkorn University). The level of statistical significance was addressed when P-value was less than 0.05 ($P<0.05$).

CHAPTER IV

RESULTS

Experiment 1 : Susceptibility of *E. coli* to enrofloxacin and doxycycline

The MICs of each isolate were displayed in Table 4. After tested with enrofloxacin and doxycycline MIC strips, 10 isolates susceptible to both drugs were selected, with MIC values ranging from 0.047 to 0.19 μg/ml for enrofloxacin, and 0.75 to 1.5 μg/ml for doxycycline.

Experiment 2 : Time-kill curves

2.1 Bactericidal activities

The overall growth curve of each group is shown in Figure 3. Without drugs added, normal growth curve was seen in Group 1. As for Group 3, doxycycline exhibited only bacteriostatic activity up to 12 hours before regrowth subsequently occurred, except for two isolates (R3 and AB) in which the bacteriostatic activity persisted throughout 24 hours.

Regarding the normal endpoint of 24 hours, log reductions, percentages of killing and the numbers of isolates with bactericidal activity and bacterial elimination were achieved are shown in Tables 5, 6 and 7, respectively. For Group 2, the bactericidal activities of enrofloxacin were seen in most isolates with a log reduction

of 3.96±0.49 (97.96-100.00% kill) following two hours of drug exposure (Table 5), except for four isolates (C8, AB, N5 and Q3) that the bactericidal activities were found at 3 hours for one isolate and 6 hours for the other three isolates. However, the bactericidal activities were still found in these isolates at the endpoint (Table 7). For Group 4, when tested with the simultaneous combination of enrofloxacin and doxycycline, the bactericidal activities were seen in most isolates with a log reduction of 3.78±0.37 (98.73-100.00% kill) at six hours of drug exposure (Table 5), except for two isolates (AB and Q3) that the bactericidal activities were found at 12 and 24 hours respectively. At the endpoint, 100% bactericidal activity was still achievable (Table 7). For Group 5, after enrofloxacin was added, the bactericidal activities were not seen until the normal endpoint (12 hours after enrofloxacin exposure), with a log reduction of 3.51±0.47 (99.19-100.00% kill). Despite the endpoint, such bactericidal activities were seen in only five isolates (Table 7), except for N3, N5, Q3, S2 and CG that the bactericidal activities were not achieved.

For the adjusted endpoint of 12 hours post enrofloxacin exposure, the numbers of isolates in which bactericidal activity and bacterial elimination were attained are presented in Table 8. The overall bactericidal activity was still in the same direction as the normal endpoint. For all enrofloxacin-treated groups, bacterial elimination was not seen in one isolate (Q3). In addition to Q3, bacterial elimination was not seen in other two isolates of Group 4 (C8 and AB) at the adjusted endpoint.

Table 4 The samples selected from Experiment 1 and their MIC values

Sample ID	Species	Source	MIC ENR (µg/ml)	MIC DXT (µg/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
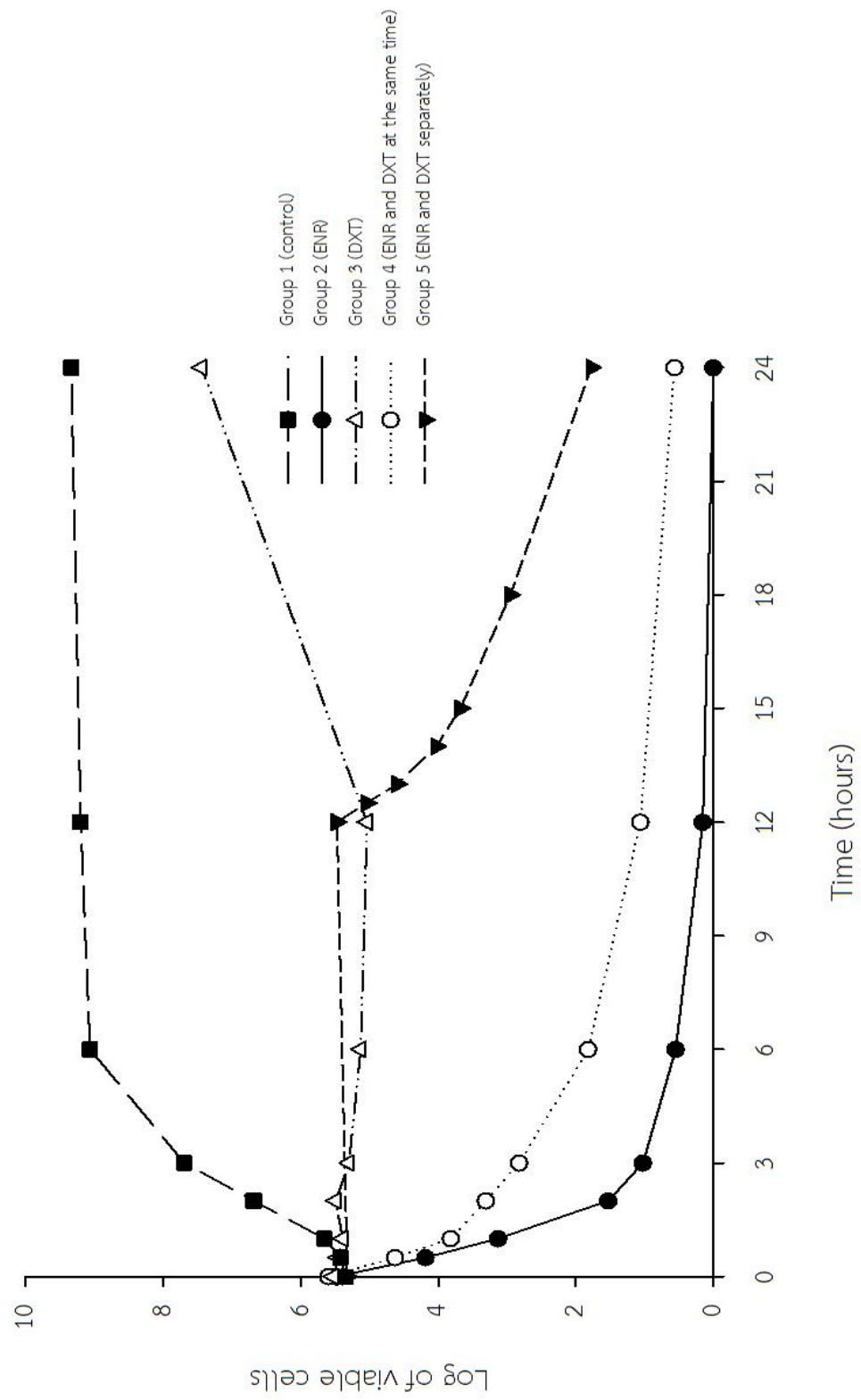
Figure 3 Average log of viable counts from 10 *E. coli* isolates in each treatment group

Table 5 Log reduction (mean±SE) at each time point after enrofloxacin exposure

Group	Time after enrofloxacin exposure (hours)							
	0	0.5	1	2	3	6	12	24
Group 2 (ENR)	0.00±0.00	1.30±0.30	2.36±0.30	3.96±0.49	4.47±0.46	4.94±0.30	5.33±0.17	5.49±0.08
Group 4 (ENR+DXT same time)	0.00±0.00	0.97±0.16	1.78±0.24	2.29±0.22	2.78±0.26	3.78±0.37	4.54±0.36	5.03±0.29
Group 5 (ENR+DXT separately)	0.00±0.00	0.31±0.14	0.85±0.22	1.36±0.22	1.75±0.24	2.27±0.27	3.51±0.47	NA

Table 6 Percentages of killing (minimum-maximum) at each time point after enrofloxacin exposure

Group	Time after enrofloxacin exposure (hours)							
	0	0.5	1	2	3	6	12	24
Group 2 (ENR)	0.00	72.69-99.37	92.84-99.98	97.96-100.00	99.51-100.00	99.95-100.00	99.99-100.00	100.00
Group 4 (ENR+DXT same time)	0.00	60.13-98.00	89.34-99.91	96.00-99.97	96.92-99.99	98.73-100.00	99.58-100.00	99.95-100.00
Group 5 (ENR+DXT separately)	0.00	-38.26-93.51	46.46-98.98	71.47-99.83	93.05-99.92	96.56-99.99	99.19-100.00	NA

Table 7 The numbers of *E. coli* isolates with bactericidal activity and bacterial elimination achieved at 24 hours

Observed effects	Group (n=10 in each group)				
	1 (control)	2 (ENR)	3 (DXT)	4 (ENR and DXT at the same time)	5 (ENR and DXT separately)
Bactericidal activity (99.9% kill or 3log reduction)	0 (0%)	10 (100%)	0 (0%)	10 (100%)	5 (50%)
Bacterial elimination (<2 log of viable cells)	0 (0%)	10 (100%)	0 (0%)	7 (70%)	4 (40%)

Table 8 The numbers of *E. coli* isolates with bactericidal activity and bacterial elimination achieved at 12 hours after enrofloxacin exposure

Observed effects	Group (n=10 in each group)		
	2 (ENR)	4 (ENR and DXT at the same time)	5 (ENR and DXT separately)
Bactericidal activity (99.9% kill or 3log reduction)	10 (100%)	9 (90%)	5 (50%)
Bacterial elimination (<2 log of viable cells)	9 (90%)	5 (50%)	4 (40%)

2.2 Killing times

2.3.1 Time to 3 log reduction (T3K)

From the log reductions in Figure 4 and estimated times to reach 3log reduction (T3Ks) in Table 9, bactericidal activity was fastest in Group 2, followed by Groups 4 and 5. However, there was no significant difference between T3Ks of Groups 2 (2.47±0.40), and 4 (4.44±0.63) (P=0.226) while T3Ks were significantly different between Groups 2 and 5 (8.04±0.94) (P<0.05).

2.3.2 Time to elimination (TE)

According to the log of viable cells in Figure 5 and estimated times to elimination in Table 9, bacterial elimination (or <2 from log of viable cells) was seen first in Group 2, followed by Groups 4 and 5. Based on the estimation from linear regressions, however, TE of Group 2 (5.83±0.58) was statistically different from that of Group 5 (14.97±1.35) (P<0.05), but not Group 4 (9.48±1.15) (P=0.161). These results are in the same direction as those of T3K.
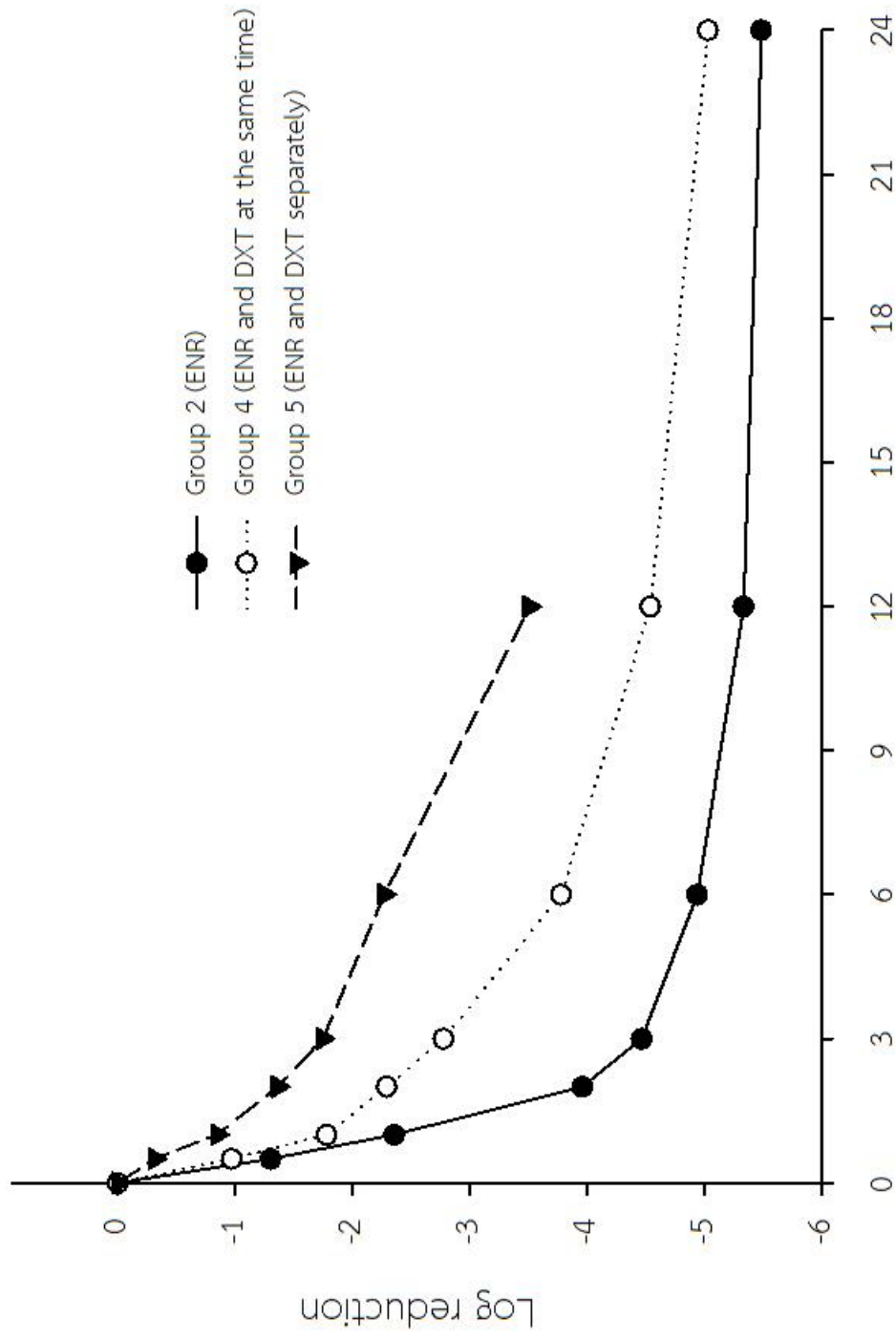
Figure 4 Average log reductions of 10 *E. coli* isolates after enrofloxacin exposure

Table 9 Estimated T3Ks and TEs from linear regressions out of 6 hours after enrofloxacin exposure in each sample

Sample ID	Estimated T3Ks (hours)			Estimated TEs (hours)		
	Group 2 (ENR)	Group 4 (ENR+DXT same time)	Group 5 (ENR+DXT separately)	Group 2 (ENR)	Group 4 (ENR+DXT same time)	Group 5 (ENR+DXT separately)
C8	3.15	5.38	3.91	5.85	11.64	8.47
R3	1.55	2.41	12.67	4.71	5.44	18.45
AB	4.23	6.11	8.08	8.18	12.27	13.93
B1	1.18	4.62	4.06	4.47	10.96	9.34
N5	4.03	3.94	10.70	8.03	8.05	18.71
Q3	4.14	8.95	10.64	8.92	17.45	20.66
S2	1.71	4.04	7.35	4.48	7.82	12.69
CG	1.81	2.56	7.13	4.78	7.20	14.00
N3	1.31	2.57	5.78	4.36	5.93	13.64
Mean$\pm$SE	2.47$\pm$0.40	4.44$\pm$0.63	8.04$\pm$0.94	5.83$\pm$0.58	9.48$\pm$1.15	14.97$\pm$1.35
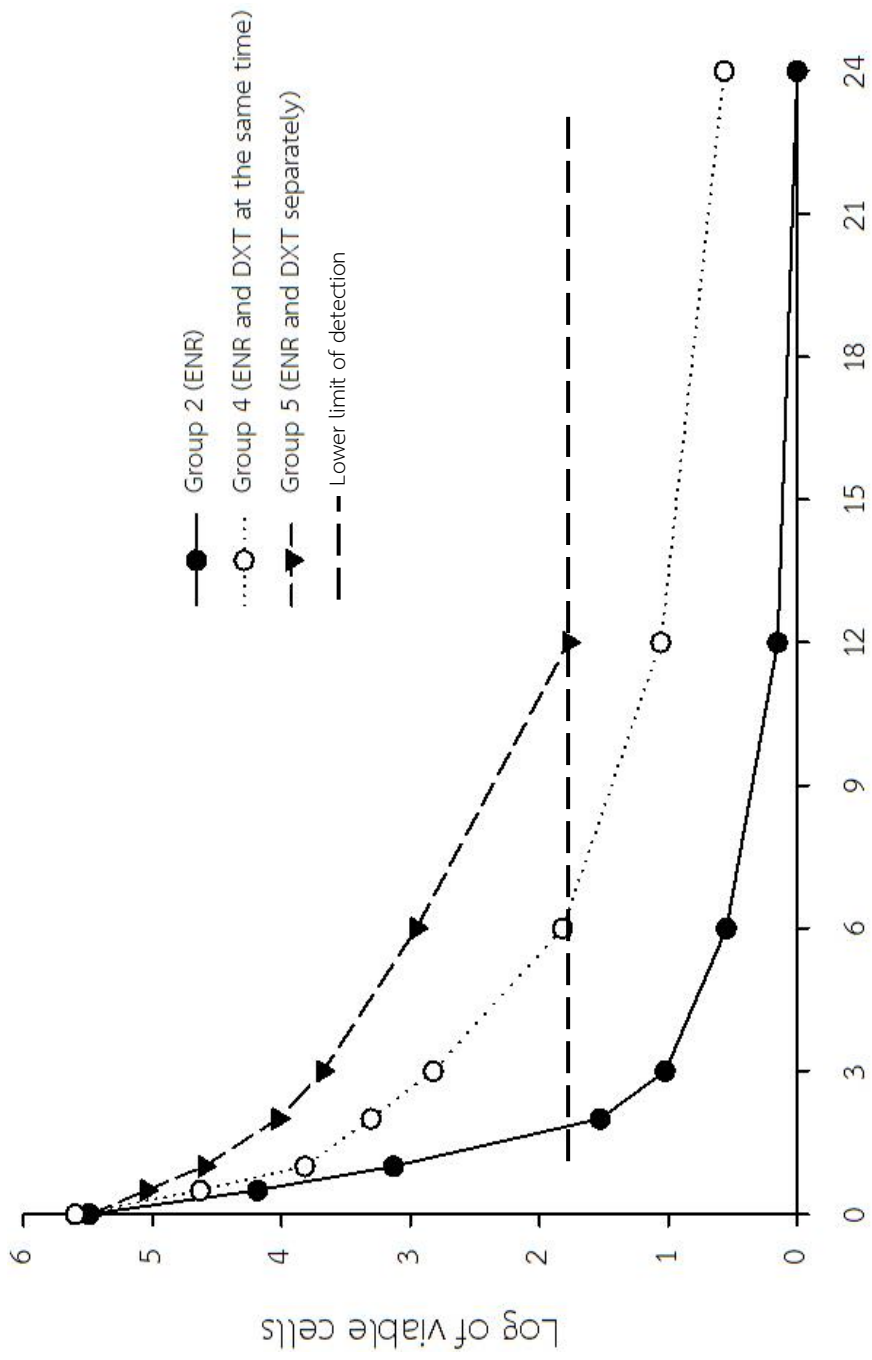
Figure 5 Average logs of viable cells from 10 *E. coli* isolates after enrofloxacin exposure

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

Using time-kill curves as a drug-drug interaction assessing method

Time-kill technique is not the best but a suitable method for

enrofloxacin

Even though none of the time-kill protocols has been standardized yet, time-kill curve technique has been considered to be one of the most suitable methods to evaluate the bactericidal effect of an antibacterial drug like enrofloxacin and its combinations with doxycycline, due to the measurements of bactericidal activities with various time points (Punam, 2007). According to the clinical use of the combination of enrofloxacin with doxycycline, with enrofloxacin for bacteria and doxycycline for *E. canis*, only bactericidal activity of the combination at 1xMIC is of our interest. Therefore, we overlook the fact that this technique is not suitable for measuring drug-drug interactions with various concentrations, like the other four methods (checkerboard titration, disk diffusion synergy test, paper strip diffusion and kinetic spectrophotometry). Moreover, unlike time-kill curves, the other four methods measure only bacteriostatic activities, not bacterial elimination, which has been

linked to a better clinical outcome (Dagan *et al.*, 2001). Besides, in some circumstances such as aerobic and anaerobic conditions, in which MIC may be equal to each other, but killing activities from time-kill study are different (Malik *et al.*, 2007). So, the time-kill curve may be more useful in this dilemma.

T3K and TE are in the same direction but refer to slightly different outcomes

Regarding experimental procedures, the protocol used in Experiment 2 was adopted from Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline, M26-A (CLSI, 1999). In contrast to the other drug-drug interaction assessing techniques described in Table 1, time-kill curve technique provides us with a dynamic picture of drug-bacteria responses over a certain period. Aside from bactericidal activity, the rate of killing can be expressed as either T3K or slope between time and log reduction (Fung-Tomc *et al.*, 2000; Schafer *et al.*, 2006). However, as a simple unit like hours, the former seems to be easier to understand than log reduction/hour of the latter. Moreover, the killing rate plays an important role in reducing the number of bacteria in a patient. In other words, the more rapidly and the higher amount of bacteria an antibacterial drug kills, the less chances bacteria can develop antimicrobial resistance (Boothe, 2012).

In this study, T3K refers to the time when bactericidal activity was achieved, indicating how fast a bactericidal effect occurred. However, regardless of virulence factors, an infection present in a patient is indicated by the number of infecting

bacteria expressed in colony-forming unit per milliliter (CFU/ml). In order to clear infection, the number of bacteria must be decreased to a certain level which varies among organs, types of bacteria and stages of bacterial growth (König *et al.,* 1998). This means, even a T3K is attainable during a course of treatment, the infection in some organs may not be resolved such as endocarditis (Upton *et al.,* 2005). In hopes of fulfilling this discrepancy, TE was introduced in this study, referring to the time when bacterial cells are assumed to be eliminated. This parameter must be extrapolated and located between the last time when bacteria are still present and the first time when there is no bacterial growth on agar. In fact, the reason of the absence of bacterial growth is because the number of bacteria is so infinitesimal that the remaining bacterial cells cannot be sampled to grow on agar (probably less than lower limit of detection, which is <100 CFU/ml in this study). TE is of importance especially in patients with sepsis and immunocompromised conditions (Craig *et al.,* 1988). In sum, even both parameters are in the same direction, TE is deemed to be a better predictor of clinical outcomes than T3K.

E. coli is a good candidate to determine drug interaction between enrofloxacin and doxycycline.

While doxycycline is the only drug of choice to treat ehrlichiosis, enrofloxacin is a good choice to treat Gram-negative bacterial infection in which *E. coli* seems to be a primary suspect (Neer *et al.,* 1999; Neer *et al.,* 2002; Prescott *et al.,* 2002). However, in the clinical setting, a pet can be co-infected by *E. canis* and Gram-

negative bacteria, so using enrofloxacin in combination with doxycycline is inevitable.

In our opinion, there are many reasons why *E. coli* is one of the most common

Gram-negative bacteria that needs to be tested with enrofloxacin. First, *E. coli* resides

as normal flora in gastrointestinal and urogenital systems (Blount, 2015). It is still

capable of infecting various body systems by utilizing its virulence factors (Johnson *et*

al., 2008), so it is more likely to cause infections. Second, once an infection occurs, *E.*

coli can be transmitted from a pet to its owner(s), so a veterinarian has to assure that

the infection in the pet is properly treated (Belanger *et al.*, 2011). Third, *E. coli* has

the potential abilities to develop antimicrobial resistance, which has been

extensively studied during recent years (Normand *et al.*, 2000; Sanchez *et al.*, 2002;

Belanger *et al.*, 2011). Last, thanks to broad spectrum and rapid bactericidal activity

of enrofloxacin, it is one of the most frequently used drugs, but not every case of

enrofloxacin use is judiciously applied, especially to pets with gastroenteritis for

which enrofloxacin is used as an empirical treatment (Escher *et al.*, 2011). As a result,

E. coli is a good candidate for testing drug interaction between enrofloxacin and

doxycycline.

1xMIC is not the best but a legitimate concentration.

In general, the concentration used for each drug in time-kill experiment is

usually chosen by considering two aspects. First, it should be a concentration

achievable at the site of infection. Second, it can be chosen from concentrations that

have produced an interesting interaction derived from another drug-drug interaction

assessing method, checkerboard titration for example (Arce *et al.*, 2006; Punam, 2007). In this study, a minimum inhibitory concentration (1xMIC) is chosen for both enrofloxacin and doxycycline because it is achievable in most organs (Cester and Toutain, 1997; Boothe, 2012), which probably helps predict clinical outcomes more accurately.

In relation to the prediction of clinical outcome, MIC, the lowest concentration at which bacterial growth is visibly inhibited in an *in vitro* setting, is said to be a good indicator for bacteriostatic activity. However, MIC is still a legitimate target for bactericidal drugs to attain as well because success of antimicrobial therapy depends on many factors involved with host-drug-microbe relationships (Boothe, 2012). In this study, both enrofloxacin and doxycycline were tested against *E. coli* at 1xMIC. Despite the fact that time-kill technique is usually used to determine the killing effect of a drug combination, 1xMIC seems to be an appropriate concentration for a bacteriostatic agent like doxycycline for two reasons. First, it is hard for doxycycline to achieve bactericidal concentrations in the clinical environments. With a normal dose in dogs (10 mg/kg), maximum serum concentration (C_{max}) of doxycycline hyclate from oral administration is only 5.8 ± 0.5 µg/ml (Gutiérrez *et al.*, 2012). However, according to Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; VET01S, 3rd ed., the susceptible breakpoint of doxycycline against *E. coli* is ≤ 4 µg/ml, thus doxycycline is less likely to exhibit bactericidal activity against *E. coli* with

such C_{max} (CLSI, 2015). Second, doxycycline has high protein binding capacity (87.16±2.43 %) (Davis *et al.*, 2006), so only small amount of unbound, active fraction is available making it harder to demonstrate the bactericidal effect. As a result, a bacteriostatic concentration like 1xMIC would be a sensible concentration to test for doxycycline, and the emphasis should be put on the change in bactericidal effects of enrofloxacin more than the clinically-unattainable bactericidal activity of doxycycline.

As for enrofloxacin, even though MBC seems to be a better choice due to its bactericidal effect, there are three reasons why 1xMIC is still a reasonable concentration to be tested. First, based on the results in Experiment 2, enrofloxacin could still show bactericidal activity at 1xMIC, which is in agreement with the fact that bactericidal drugs have MBCs (0.015-0.06 µg/ml) close to MICs (0.008-0.015 µg/ml) (Haritova and Russenova, 2010; Boothe, 2012). As a consequence, 1xMIC can be used to test for killing effect of a bactericidal agent as well. Second, as mentioned above, 1xMIC of enrofloxacin is easily achievable in clinical settings (Cester and Toutain, 1997). Last, in spite of the differences in MICs among individual bacterial populations and hosts, FQ dosing regimen is usually adjusted in accordance with MIC, possibly up to 8-10xMIC. This dose adjustment can optimize the use of FQs in terms of preventing bacteria from regrowing and selective pressure, making 1xMIC the least concentration to reach in most certain conditions (McKellar *et al.*, 2004). In sum, 1xMIC is a legitimate concentration for both enrofloxacin and doxycycline.

Bactericidal activity of enrofloxacin against *E. coli*

Overall, when tested with enrofloxacin alone, bactericidal activities and bacterial elimination were seen in all *E. coli* isolates within 24 hours. According to the killing times of enrofloxacin, it took 2.47 ± 0.40 hours to exhibit 3log kill and 5.83 ± 0.58 hours to show bacterial elimination. These findings are consistent with some studies showing that enrofloxacin exhibit rapid bactericidal activity and bacterial elimination within 24 hours (Norcia *et al.*, 1999; Blondeau *et al.*, 2012). As a bactericidal agent, the pattern observed is the same as ciprofloxacin and tobramycin (Craig and Ebert, 1991). In comparison with other fluoroquinolones, enrofloxacin shares the very rapid bactericidal activity against *Enterobacteriaceae* (Grobbel *et al.,* 2007)*.*

Bactericidal activity of enrofloxacin combined with doxycycline at the same time

When *E. coli* exposed to enrofloxacin and doxycycline simultaneously, the number of isolates showing bacterial elimination was lower than that when exposed to enrofloxacin alone. In terms of killing times, T3K and TE of the enrofloxacin administered with doxycycline at the same time tend to delay compared to the use of enrofloxacin alone. Even though there was no statistical significance, it was obvious that the killing effect of enrofloxacin given concurrently with doxycycline

was still present at the endpoint. To explain this unclear delay in killing, there are two possible reasons behind this observation.

For one thing, ciprofloxacin, an active metabolite of enrofloxacin, shows a strongly antagonistic or suppressive interaction with doxycycline (Chait *et al.*, 2007; Yeh *et al.*, 2009). This type of interaction results in the overall therapeutic effect to be lower, not only than the sum effects of the two individuals, but also than the effect of one drug alone. The combination of enrofloxacin with doxycycline may yield the same interaction to a certain extent. It is due to the fact that up to 30% of enrofloxacin can be transformed into ciprofloxacin, which can antagonize doxycycline as well (Cester and Toutain, 1997). To clarify this suppressive interaction, one of the underlying mechanisms is nonoptimal regulation of ribosomal genes (Bollenbach *et al.*, 2009).

In normal condition, bacteria have a negative feedback mechanism to maintain ribosome and DNA to be close to the optimal level, depending on the nutrients in the environment. This optimal condition can be interfered by a DNA synthesis inhibitor, leading to downregulation and nonoptimal control of ribosomal genes. In our study, the combination of enrofloxacin and doxycycline were simultaneously added. The entry of enrofloxacin into bacterial cells through porin-mediated, lipid-mediated and self-promoting pathways, facilitated it to its targets faster than doxycycline. (Delcour, 2009) This entry, however, drove bacterial cells to

be under DNA stress and nonoptimal condition. In this nonoptimal condition, bacterial cells cannot adjust ribosomal protein synthesis through ribosomal gene regulation, leading to the reduced bacterial growth. However, this nonoptimal situation can be rectified by a translation inhibitor like doxycycline. That is to say, this late comer reversed the situation by adjusting downregulated ribosomal genes, finally resulting in an increase in survival and growth of bacterial cells which had been exposed to a DNA synthesis inhibitor. However, this nonoptimality from suppression usually occurs under sub-MICs, but the combination of enrofloxacin with doxycycline in our study was at 1xMIC each. With this concentration, only a tendency in delayed killing was found, making the influence of nonoptimality on bacterial growth not as clear as other studies (Chait *et al.,* 2007; Cottarel and Wierzbowski, 2007; Ocampo *et al.,* 2014), or perhaps there are other mechanisms involved with this delayed killing effect other than nonoptimal regulation.

Second, more importantly, the killing mechanism of each quinolone may be different from each other. There are two major pathways contributing to rapid bacterial cell death. The first one is protein-synthesis dependent pathway, or chloramphenicol-sensitive pathway, which can be further divided into two pathways – aerobic and anaerobic pathways. Both aerobic and anaerobic pathways lead to the common pathway as in other bactericidal agents, the formation of hydroxyl radicals which are lethal to bacterial cells. The other pathway depends on neither protein

synthesis nor aerobic condition, called chloramphenicol-insensitive pathway. This pathway causes lethal effect to bacteria without the production of hydroxyl radicals. Pertaining to the relationship between these killing mechanisms and drug interactions, it has been found that nalidixic acid kills bacteria only through aerobic protein-synthesis dependent pathway, so protein synthesis inhibitors like tetracycline and chloramphenicol can completely antagonize its killing activity, by directly reducing the production of lethal hydroxyl products (Wang *et al.*, 2010; Ocampo *et al.*, 2014). However, with the chemically-structural modification of newer quinolones, their killing activities are altered and so are their bactericidal mechanisms. As can be seen in Table 10, ciprofloxacin, moxifloxacin and ciprofloxacin-like compound, PD161144, can kill bacteria through both protein-synthesis dependent and protein-synthesis independent pathways. Thus, they are still able to kill bacteria in the presence of a bacteriostatic agent, but require a higher concentration to do so (Malik *et al.*, 2007). As for enrofloxacin in combination with doxycycline, at the regular concentration, the killing effect was still observed, but it was reduced to be lower than that of enrofloxacin exposure alone. Therefore, it is possible that enrofloxacin exert its remaining bactericidal effect through protein-synthesis independent pathway, which cannot be interrupted by the presence of doxycycline.

Table 10 The different pathways which contribute to lethality of quinolones activities

Quinolone	Killing pathway			References
	Protein-synthesis dependent		Protein-synthesis independent	
	Aerobic	Anaerobic		
Nalidixic acid	Yes	No	No	Malik, 2007 and Kohanski, 2007
Norfloxacin	Yes	Yes*	No	
Ciprofloxacin	Yes	Yes*	Yes	
PD161144 (ciprofloxacin-like compound)	?	?	Yes	Malik, 2007 and Wang, 2010
Oxolinic acid	Yes	No	No	Wang, 2010
Moxifloxacin	Yes	?	Yes	

* at a higher concentration

(?) refers to uncertainty about the mechanism

Effect of 12-hours doxycycline exposure on the killing activity of enrofloxacin

In the general veterinary practice, the use of enrofloxacin and doxycycline together at the separated time has been done purposely to avoid the antagonism between the drugs. In order to simulate such condition, *E. coli* was treated with doxycycline 12 hours prior to enrofloxacin. The bactericidal activity and bacterial elimination seen in this combination were lower than those of enrofloxacin alone. Additionally, T3K and TE were significantly slower than those of enrofloxacin alone. All of these findings indicate that the antagonistic effect of doxycycline on the overall killing activity is enhanced by the 12 hours of doxycycline pretreatment. However, regarding the strategy to avoid antagonism between enrofloxacin and doxycycline by administering each drug separately at 12 hour interval, the antagonism found in this study is still inevitable by doing so. Because both drugs circulate in the blood for 24 hours and can accumulate in various tissues due to their lipid solubility (Cester and Toutain, 1997; Boothe, 2012; Gutiérrez *et al.*, 2012). To elaborate on this antagonistic phenomenon, there are a few explanations on this reduced and delayed killing activity.

First, as addressed in the partially antagonistic interaction between the simultaneous combination of enrofloxacin and doxycycline, the killing mechanisms of the combination may be reduced by the inhibitory effect of doxycycline on the protein-synthesis dependent pathway. Compared with the partial antagonism between the simultaneous combination and enrofloxacin alone, the magnitude of

the antagonistic phenomenon in the separated combination is much clearer. Unlike the simultaneous combination, this clear antagonism occurs probably due to doxycycline pretreatment which allows the drug itself to reach the target and perform its inhibitory effect on the protein-synthesis dependent killing pathway for a longer period before enrofloxacin does its killing duty. This has also happened in the case of prolonged azithromycin, clarithromycin and erythromycin treatments, which cause protein synthesis to decrease in a time-dependent manner, ultimately leading to bactericidal activity (Tateda *et al.*, 1996). However, after 12 hours of doxycycline pretreatment, the killing effect did not happen, indicating that 1xMIC is enough for doxycycline to inhibit but not kill *E. coli* in such time-dependent way (Cunha *et al.*, 2000).

Second, with reference to the optimal regulation of ribosomal genes, it is less likely to counteract killing effect in the separated combination. It is due to the fact that the nonoptimality requires DNA stress condition to take place prior to the addition of a protein synthesis inhibitor like doxycycline (Bollenbach *et al.*, 2009).

Either way, it does not exclude the possibility that efflux pump triggered by doxycycline may be in part responsible for decreased killing activity and delayed killing times of enrofloxacin (Poole, 2005). To sum up, the killing effects of the separated combination are reduced and killing times are delayed, due to the prolonged exposure of doxycycline, probably leading to markedly inhibitory effect on protein-synthesis dependent killing.

Discrepancy between the simultaneous and separated combinations

In this study, it has been discovered that adding a drug combination at the same time can produce the different result from that of the separated combination. This inconsistency has also been found in other studies. For example, antagonism between penicillin and chloramphenicol was found in a dog model of pneumococcal meningitis only when chloramphenicol was administered before penicillin. However, when both drugs were given simultaneously or when penicillin was given first, the antagonism was less marked. Not given the initial protocol, continued administration of both drugs still exhibited bacterial elimination of the organism in this model (Wallace *et al.*, 1967).

Conversely, as for adding a DNA synthesis inhibitor prior to a protein synthesis inhibitor, another study showed that the addition of chloramphenicol 15 minutes after ciprofloxacin exposure could kill *E. coli* more than adding both drugs at the same time, but less than ciprofloxacin alone (Zeiler, 1985). This may be because of the nonoptimal ribosomal regulation by the presence of ciprofloxacin prior to chloramphenicol. Nonetheless, in our study, this nonoptimality may not be the case for prolonged enrofloxacin exposure before doxycycline, because enrofloxacin alone can exhibit bacterial elimination within 12 hours in almost all isolates. Moreover, based on our pilot study in *E. coli* ATCC25922, enrofloxacin alone and in combination with doxycycline at 1xMIC exhibited bacterial eradication within two hours. Therefore, there must be no bacterial cells left at 12 hours of doxycycline

addition, which is the reason we decided to add doxycycline before enrofloxacin, not enrofloxacin before doxycycline.

With reference to killing activities and times, even though there is a gap between the two combinations, both still exhibit bactericidal activity but to a lower extent with delayed times compared to the use of enrofloxacin alone. Considering *in vivo* settings, there are several factors to be taken into account such as immune system, drug disposition and bacteria themselves (Boothe, 2012). Therefore, using either of the combinations may take more time to eliminate bacteria due to the reduced effects and delayed times, especially in immunocompromised patients whose immune system does not function properly (Githaiga *et al.*, 2005).

To wrap up, the discrepancy between the simultaneous combination and separated combination is addressed in this study. Moreover, it may raise the concern about testing drug interaction in which each drug is separately administered, but the effect of each drug persists through another in a clinical setting.

Limitations

Despite an effort that has been made so far, there are still some limitations of this study. For one thing, sometimes the results from an *in vitro* study may not be extrapolated to an *in vivo* study. For example, the antagonism between penicillin and chloramphenicol derived from time-kill studies and a dog model of pneumococcal meningitis fails to be addressed in a rabbit model of meningitis (Wallace *et al.*, 1967; Pillai *et al.*, 2005). There are many reasons behind this gap.

First, the killing activity of an antibacterial drug depends on the amount of free drug available. In other words, the amount of protein in blood and protein binding capacity of the drug can alter killing activity, resulting in different MICs and killing activities (Haritova and Russenova, 2010).

Second, with an attempt to control the effect of some variables on the antibacterial activity such as cation concentration and pH, CAMHB was chosen and its pH was measured before conducting time-kill procedures (CLSI, 1999; Pillai *et al.*, 2005; Amsterdam, 2015). However, with the variation of such factors and increased amount of bacterial waste product during the experiment, the antibacterial activity may be altered (Parhad and Rao, 1974).

Third, *in vitro* killing activities might be overestimated. In the clinical practice, rapid killing sometimes is unachievable especially in some types of chronic infections such as osteomyelitis and endocarditis. This is because biofilm can act as a diffusion barrier to slow antibacterial down, resulting in lower killing activities compared with

that of logarithmic planktonic cells (Spoering and Lewis, 2001; Brady *et al.*, 2008; Jung *et al.*, 2012).

Last, unlike *in vivo* situation, another point worth considering is the drug concentration used is fixed while the fluctuation is seen in the blood. In addition, this time-kill study takes one single dosing of the combination into account because the killing activity of enrofloxacin can be varied up to the fluctuating concentrations (Cester and Toutain, 1997).

Conclusions

As an expert in killing bacteria, enrofloxacin has been widely used in the general veterinary practice and known to be antagonized by a bacteriostatic agent. However, this antagonistic interaction seems unavoidable, so this study has shed light on this antagonism using time-kill studies. It has been found that the antagonism is clearly found in the separated administration of enrofloxacin and doxycycline, but not much with simultaneous administration (Figure 6).

In this study, it seems that the interaction between enrofloxacin and doxycycline is suppressive interaction as found in the combination of ciprofloxacin and doxycycline (Chait *et al.*, 2007). According to killing mechanisms of quinolones, the ROS production of enrofloxacin can be disrupted by doxycycline through protein-synthesis dependent pathway (Kohanski *et al.*, 2010). Moreover, the nonoptimal regulation corrected by doxycycline also promotes bacterial growth to counteract

bactericidal activity. Therefore, the killing activity of the former drug tends to be reduced and delayed. However, in today's clinical practices, an arbitrary strategy to overcome this antagonism by splitting the combination should be pondered. It is due to the fact that such disruption can be enhanced by prolonging doxycycline exposure before adding enrofloxacin, making the antagonism more intense. Besides, both drugs can deposit and circulate throughout the body for almost 24 hours, so the antagonism is still inevitable by exploiting such strategy (Cester and Toutain, 1997; Boothe, 2012; Gutiérrez *et al.,* 2012).

To the best of our knowledge, the findings in this study indicate that bactericidal effect of enrofloxacin can be antagonized by the presence of doxycycline, although bactericidal activity and bacterial elimination still exhibit at a reduced extent and delayed rates (Figure 6). However, along with some limitations of this *in vitro* study, there are still many *in vivo* factors to be taken into consideration such as host immune system, fluctuation of drug concentration and infectious microbes. This means that although the use of enrofloxacin in combination with doxycycline is inevitable, it should be judiciously applied especially in patients with an immunocompromised condition.

Further studies

In order to expand our understanding of the suppressive interaction between enrofloxacin and doxycycline, here are some suggestions. As for *in vitro* studies, the killing activity of the combination should be tested at a higher MIC or a concentration achievable in an infected organ of interest. On the basis of the ROS contributing to bacterial cell death, the amounts of ROS between the combination and enrofloxacin alone should be compared as well. Aside from the protein-synthesis dependent pathway, some quinolones can kill bacteria through the protein-synthesis independent pathway, so it should be also studied. Moreover, according to the discrepancy observed between the simultaneous and separated combinations, the drug combination needs to be assessed accordingly, and so does the effect of prolonged protein inhibition on the killing activity of enrofloxacin.

For clinical translation, undertaking an *in vivo* study is essential to precisely predict clinical outcomes. In fact, a course of antibacterial therapy always deals with multiple/ repeat dosing. However, time-kill study only focuses on the killing effect of a single dosing, which does not provide enough information for the whole dosing regimen. With a fluctuation of drug concentration and concentration-dependent killing activity of enrofloxacin, this suggests that an *vivo* study with a multiple/ repeat dosing should be conducted to fulfill missing pieces of our intellectual knowledge about drug interactions.
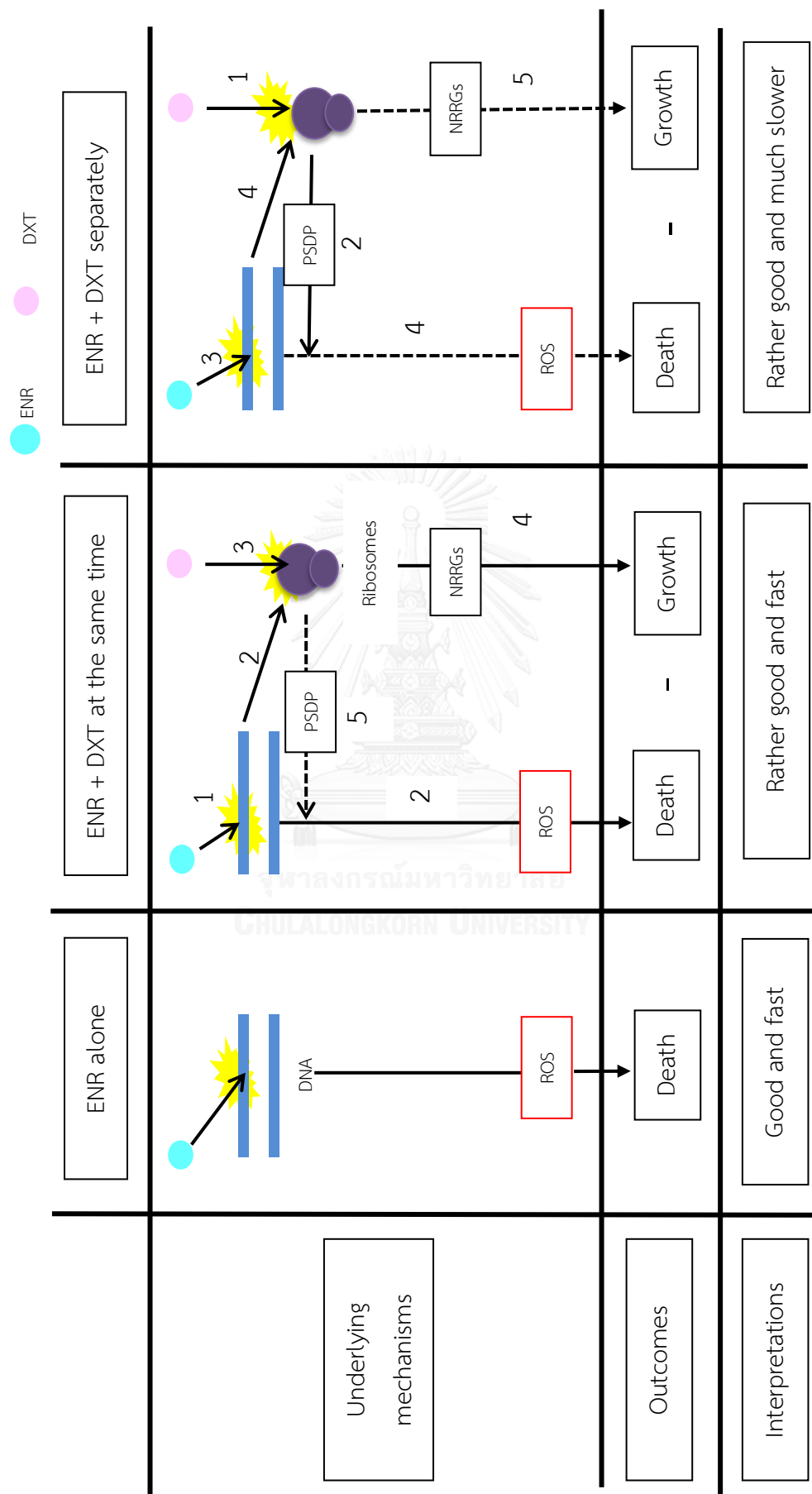
Figure 6 The relationship between the results from this study and the possible mechanisms involved based on conceptual framework

(bold line, strong effect; dash line, weak effect; PSDP, protein-synthesis dependent pathway; NRRGs, nonoptimal regulation of ribosomal genes)

REFERENCES

Amsterdam D 2015. Susceptibility Testing of Antimicrobials in Liquid Media. In: Antibiotics in Laboratory Medicine. 6 ed. D. Amsterdam (ed). Baltimore: Lippincott Williams & Willkins. 51-148.

Arce JS, Gainza EC, Brusola AG, Estévez RO, Canton E and Gobernado M 2006. *In vitro* activity of linezolid in combination with doxycycline, fosfomycin, levofloxacin, rifampicin and vancomycin against methicillin-susceptible *Staphylococcus aureus*. Rev Esp Quimioterap. 19(3): 252-247.

Ariyawutthiphan O, Shokshai-utsaha K, Sananmuang T, Chungpivat S, Sarikaputi M and Viseshakul N 2005. The Microscopic and Molecular Detections of Canine Ehrlichiosis. The Thai Journal of Veterinary Medicine. 38(2): 29-36.

Askoura M, Mottawea W, Abujamel T and Taher I 2011. Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. The Libyan Journal of Medicine. 6: 10.3402/ljm.v3406i3400.5870.

Barbour EK, Hamadeh S, Talhouk R, Sakr W and Darwish R 1998. Evaluation of an enrofloxacin-treatment program against *Mycoplasma gallisepticum* infection in broilers. Prev Vet Med. 35(2): 91-99.

Barry AL, Ayers LW, Gavan TL, Gerlach EH and Jones RN 1984. *In vitro* activity of ticarcillin plus clavulanic acid against bacteria isolated in three centers. European Journal of Clinical Microbiology. 3(3): 203-206.

Bazzocchi C, Mortarino M, Grandi G, Kramer LH, Genchi C, Bandi C, Genchi M, Sacchi L and McCall JW 2008. Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against Dirofilaria immitis in experimentally infected dogs. International Journal for Parasitology. 38(12): 1401-1410.

Belanger L, Garenaux A, Harel J, Boulianne M, Nadeau E and Dozois CM 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic E-coli. Fems Immunology and Medical Microbiology. 62(1): 1-10.

Bellio P, Brisdelli F, Perilli M, Sabatini A, Bottoni C, Segatore B, Setacci D, Amicosante G and Celenza G 2014. Curcumin inhibits the SOS response induced by levofloxacin in *Escherichia coli*. Phytomedicine. 21(4): 430-434.

Bianciardi P, Fasanella A, Manzillo VF, Trotta T, Pagano A, Sorino S, Gradoni L and Oliva G 2004. The efficacy of enrofloxacin, alone or combined with metronidazole, in the therapy of canine leishmaniasis. Parasitology research. 93(6): 486-492.

Blondeau JM, Borsos S, Blondeau LD and Blondeau BJ 2012. *In vitro* killing of *Escherichia coli*, *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* by enrofloxacin in combination with its active metabolite ciprofloxacin using clinically relevant drug concentrations in the dog and cat. Vet Microbiol. 155(2-4): 284-290.

Blondeau JM and Shebelski SD 2016. Comparative *in vitro* killing of canine strains of *Staphylococcus pseudintermedius* and *Escherichia coli* by cefovecin, cefazolin, doxycycline and pradofloxacin. Vet Dermatol. 27(4): 267-e263.

Blount ZD 2015. The unexhausted potential of E. coli. eLife. 4: e05826.

Bodey GP 1985. Overview of the problem of infections in the immunocompromised host. The American Journal of Medicine. 79(5, Supplement 2): 56-61.

Bollenbach T 2015. Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. Current Opinion in Microbiology. 27: 1-9.

Bollenbach T, Quan S, Chait R and Kishony R 2009. Nonoptimal microbial response to antibiotics underlies suppressive drug interactions. Cell. 139(4): 707-718.

Boothe DM 2012. Antimicrobial Drugs. 2 ed. In: Small animal clinical pharmacology and therapeutics. Elsevier Health Sciences. 1334.

Brady RA, Leid JG, Calhoun JH, Costerton JW and Shirtliff ME 2008. Osteomyelitis and the role of biofilms in chronic infection. FEMS Immunology & Medical Microbiology. 52(1): 13-22.

Breitschwerdt EB, Davidson MG, Hegarty BC, Papich MG and Grindem CB 1997. Prednisolone at anti-inflammatory or immunosuppressive dosages in conjunction with doxycycline does not potentiate the severity of Rickettsia

rickettsii infection in dogs. Antimicrobial agents and chemotherapy. 41(1): 141-147.

Butaye P, Ducatelle R, De Backer P, Vermeersch H, Remon JP and Haesebrouck F 1997. *In vitro* activities of doxycycline and enrofloxacin against European *Chlamydia psittaci* strains from turkeys. Antimicrobial Agents and Chemotherapy. 41(12): 2800-2801.

Carbone M, Pennisi MG, Masucci M, De Sarro A, Giannone M and Fera MT 2001. Activity and postantibiotic effect of marbofloxacin, enrofloxacin, difloxacin and ciprofloxacin against feline *Bordetella bronchiseptica* isolates. Veterinary Microbiology. 81(1): 79-84.

Cester CC and Toutain PL 1997. A comprehensive model for enrofloxacin to ciprofloxacin transformation and disposition in dog. J Pharm Sci. 86(10): 1148-1155.

Chait R, Craney A and Kishony R 2007. Antibiotic interactions that select against resistance. Nature. 446(7136): 668-671.

Chapman JS and Georgopapadakou NH 1988. Routes of quinolone permeation in *Escherichia coli*. Antimicrobial Agents and Chemotherapy. 32(4): 438-442.

CLSI 1999. Methods for determining antibactericidal activity of antimicrobial agents; Approved Guideline. CLSI document M26-A. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pensylvania, 19087, USA.

CLSI 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard - Fourth edition. CLSI document VET01-A4. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI 2015. Permance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated From Animals. 3rd ed. VET01S CLSI supplement. Wayne, PA: Clinical and Laboratory Standards Institute.

Cottarel G and Wierzbowski J 2007. Combination drugs, an emerging option for antibacterial therapy. Trends in Biotechnology. 25(12): 547-555.

Craig W, Leggett J, Totsuka K and Vogelman B 1988. Key pharmacokinetic parameters of antibiotic efficacy in experimental animal infections. J Drug Dev. 1(Suppl 3): 7-15.

Craig WA and Ebert SC 1991. Killing and regrowth of bacteria *in vitro*: a review. Scand J Infect Dis. 74(Suppl): S63-S70.

Cunha BA, Domenico P and Cunha CB 2000. Pharmacodynamics of doxycycline. Clinical Microbiology and Infection. 6(5): 270-273.

Dagan R, Klugman KP, Craig WA and Baquero F 2001. Evidence to support the rationale that bacterial eradication in respiratory tract infection is an important aim of antimicrobial therapy. Journal of antimicrobial chemotherapy. 47(2): 129-140.

Davis JL, Salmon JH and Papich MG 2006. Pharmacokinetics and tissue distribution of doxycycline after oral administration of single and multiple doses in horses. Am J Vet Res. 67(2): 310-316.

Delcour AH 2009. Outer Membrane Permeability and Antibiotic Resistance. Biochimica et biophysica acta. 1794(5): 808-816.

Domínguez MC, de la Rosa M and Borobio MV 2001. Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar. Journal of Antimicrobial Chemotherapy. 47(4): 391-398.

Dowers KL, Olver C, Radecki SV and Lappin MR 2002. Use of enrofloxacin for treatment of large-form *Haemobartonella felis* in experimentally infected cats. Journal of the American Veterinary Medical Association. 221(2): 250-253.

Drlica K, Malik M, Kerns RJ and Zhao X 2008. Quinolone-mediated bacterial death. Antimicrobial agents and chemotherapy. 52(2): 385-392.

Ertek M, Yazgi H, Erol S and Altoparlak U 2002. Demonstration of *in vitro* antagonism between fusidic acid and quinolones. J Int Med Res. 30(5): 525-528.

Escher M, Vanni M, Intorre L, Caprioli A, Tognetti R and Scavia G 2011. Use of antimicrobials in companion animal practice: a retrospective study in a veterinary teaching hospital in Italy. Journal of Antimicrobial Chemotherapy. 66(4): 920-927.

Fung-Tomc JC, Gradelski E, Valera L, Kolek B and Bonner DP 2000. Comparative Killing Rates of Fluoroquinolones and Cell Wall-Active Agents. Antimicrobial Agents and Chemotherapy. 44(5): 1377-1380.

Gale EF and Folkes JP 1953. The assimilation of amino-acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in Staphylococcus aureus. Biochemical Journal. 53(3): 493.

Garrod LP 1972. Causes of failure in antibiotic treatment. BMJ. 4(5838): 473-476.

Githaiga A, Ndirangu M and Peterson DL 2005. Infections in the immunocompromised patient. 5 ed. In: Textbook of critical care. Saunders, Philadelphia. 2358.

Gradelski E, Kolek B, Bonner D, Valera L, Minassian B and Fung-Tomc J 2001. Activity of gatifloxacin and ciprofloxacin in combination with other antimicrobial agents. International journal of antimicrobial agents. 17(2): 103-107.

Grobbel M, Lbbke-Becker A, Wieler LH, Froyman R, Friederichs S and Filios S 2007. Comparative quantification of the in vitro activity of veterinary fluoroquinolones. Veterinary Microbiology. 124(1-2): 73-81.

Guardabassi L and Prescott JF 2015. Antimicrobial Stewardship in Small Animal Veterinary Practice: From Theory to Practice. Veterinary Clinics of North America: Small Animal Practice. 45(2): 361-376.

Gumbo T 2011. General Principles of Antimicrobial therapy. In: Goodman & Gillman's The Pharmacological Basis of Therapeutics. 12 ed. Laureunce L. Brunton, Bruce A. Chabner, and Bijorn C. Knollmann (eds). New York: McGraw-Hill. 1365-1381.

Gutiérrez L, Velasco Z-H, Vázquez C, Vargas D and Sumano H 2012. Pharmacokinetics of an injectable long-acting formulation of doxycycline hyclate in dogs. Acta Veterinaria Scandinavica. 54(1): 35-35.

Haritova A and Russenova N 2010. *In vitro* antibacterial effect of enrofloxacin determined by time-killing curves analysis. Bulgarian Journal of Veterinary Medicine. 13(4): 218-226.

Hickman RA, Hughes D, Cars T, Malmberg C and Cars O 2014. Cell-wall-inhibiting antibiotic combinations with activity against multidrug-resistant *Klebsiella*

pneumoniae and *Escherichia coli*. Clinical Microbiology and Infection. 20(4): O267-O273.

Ihrke PJ, Papich MG and DeManuelle TC 1999. The use of fluoroquinolones in veterinary dermatology. Veterinary Dermatology. 10(3): 193-204.

Jawetz E and Gunnison JB 1953. Antibiotic synergism and antagonism: an assessment of the problem. Pharmacological reviews. 5(2): 175-192.

Jawetz E, Gunnison JB and Coleman VR 1954. Observations on the mode of action of antibiotic synergism and antagonism. Journal of general microbiology. 10(2): 191-198.

Jawetz E, Gunnison JB, Speck RS and Coleman VR 1951. Studies on antibiotic synergism and antagonism; the interference of chloramphenicol with the action of penicillin. AMA Arch Intern Med. 87(3): 349-359.

Johnson JR, Johnston B, Clabots CR, Kuskowski MA, Roberts E and DebRoy C 2008. Virulence genotypes and phylogenetic background of *Escherichia coli* serogroup O6 isolates from humans, dogs, and cats. Journal of clinical microbiology. 46(2): 417-422.

Jung CJ, Yeh CY, Shun CT, Hsu RB, Cheng HW, Lin CS and Chia JS 2012. Platelets enhance biofilm formation and resistance of endocarditis-inducing streptococci on the injured heart valve. J Infect Dis. 205(7): 1066-1075.

Kelly P 2000. Canine ehrlichioses: an update: review article. Journal of the South African Veterinary Association. 71(2): 77-86.

Kohanski MA, Dwyer DJ and Collins JJ 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Micro. 8(6): 423-435.

König C, Simmen H and Blaser J 1998. Bacterial concentrations in pus and infected peritoneal fluid--implications for bactericidal activity of antibiotics. Journal of Antimicrobial Chemotherapy. 42(2): 227-232.

Lin M-Y and Huang H-P 2010. Use of a doxycycline-enrofloxacin-metronidazole combination with/without diminazene diaceturate to treat naturally occurring canine babesiosis caused by *Babesia gibsoni*. Acta Veterinaria Scandinavica. 52(1): 27.

Lizondo M, Pons M, Gallardo M and Estelrich J 1997. Physicochemical properties of enrofloxacin. Journal of Pharmaceutical and Biomedical Analysis. 15(12): 1845-1849.

MacDougall C and Chambers HF 2011. Protein synthesis inhibitors and miscellaneous antibacterial agents. In: Goodman & Gilman's the pharmacological basis of therapeutics. 11th. ed. New York:. 11 ed. Laureunce L. Brunton, Bruce A. Chabner, and Bijorn C. Knollmann (eds). China: McGraw-Hill. 1521-1547.

Malik M, Hussain S and Drlica K 2007. Effect of anaerobic growth on quinolone lethality with *Escherichia coli*. Antimicrobial agents and chemotherapy. 51(1): 28-34.

Martinez M, McDermott P and Walker R 2006. Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. Veterinary Journal. 172(1): 10-28.

McEwen SA and Fedorka-Cray PJ 2002. Antimicrobial Use and Resistance in Animals. Clinical Infectious Diseases. 34(Supplement 3): S93-S106.

McKellar QA, Sanchez Bruni SF and Jones DG 2004. Pharmacokinetic/pharmacodynamic relationships of antimicrobial drugs used in veterinary medicine. J Vet Pharmacol Ther. 27(6): 503-514.

Mueller M, de la Pena A and Derendorf H 2004. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. Antimicrobial agents and chemotherapy. 48(2): 369-377.

Neer TM, Breitschwerdt EB, Greene RT and Lappin MR 2002. Consensus Statement on Ehrlichial Disease of Small Animals from the Infectious Disease Study Group of the ACVIM*. Journal of Veterinary Internal Medicine. 16(3): 309-315.

Neer TM, Eddlestone SM, Gaunt SD and Corstvet RE 1999. Efficacy of enrofloxacin for the treatment of experimentally induced *Ehrlichia canis* infection. Journal of Veterinary Internal Medicine. 13(5): 501-504.

Norcia LJ, Silvia AM and Hayashi SF 1999. Studies on time-kill kinetics of different classes of antibiotics against veterinary pathogenic bacteria including *Pasteurella, Actinobacillus* and *Escherichia coli*. J Antibiot (Tokyo). 52(1): 52-60.

Normand EH, Gibson NR, Reid SWJ, Carmichael S and Taylor DJ 2000. Antimicrobial-resistance trends in bacterial isolates from companion-animal community practice in the UK. Preventive Veterinary Medicine. 46(4): 267-278.

Ocampo PS, Lazar V, Papp B, Arnoldini M, Abel zur Wiesch P, Busa-Fekete R, Fekete G, Pal C, Ackermann M and Bonhoeffer S 2014. Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. Antimicrob Agents Chemother. 58(8): 4573-4582.

Pankey G and Sabath L 2004. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clinical infectious diseases. 38(6): 864-870.

Parhad N and Rao N 1974. Effect of pH on survival of *Escherichia coli*. Journal (Water Pollution Control Federation). 980-986.

Pillai SK, Moellering RC and Eliopoulos GM 2005. Antimicrobial combinations. In: Antibiotics in Laboratory Medicine. 5 ed. V Lorian (ed). Baltimore: Lippincott Williams and Wilkins. 365-440.

Podos SD, Thanassi JA, Leggio M and Pucci MJ 2012. Bactericidal activity of ACH-702 against nondividing and biofilm Staphylococci. Antimicrob Agents Chemother. 56(7): 3812-3818.

Poole K 2005. Efflux-mediated antimicrobial resistance. Journal of Antimicrobial Chemotherapy. 56(1): 20-51.

Prescott JF, Hanna WJB, Reid-Smith R and Drost K 2002. Antimicrobial drug use and resistance in dogs. The Canadian Veterinary Journal. 43(2): 107-116.

Punam V 2007. Methods for Determining Bactericidal Activity and Antimicrobial Interactions. In: Antimicrobial Susceptibility Testing Protocols. CRC Press. 275-298.

Riviere JE and Papich MG 2009. Veterinary Pharmacology and Therapeutics. 9 ed. In: John Wiley & Sons. 1524.

Sanchez S, McCrackin Stevenson MA, Hudson CR, Maier M, Buffington T, Dam Q and Maurer JJ 2002. Characterization of Multidrug-Resistant Escherichia coli Isolates Associated with Nosocomial Infections in Dogs. Journal of Clinical Microbiology. 40(10): 3586-3595.

Schafer JA, Hovde LB and Rotschafer JC 2006. Consistent rates of kill of *Staphylococcus aureus* by gentamicin over a 6-fold clinical concentration range in an in vitro pharmacodynamic model (IVPDM). J Antimicrob Chemother. 58(1): 108-111.

Schmitz F-J, Higgins P, Mayer S, Fluit A and Dalhoff A 2002. Activity of quinolones against gram-positive cocci: mechanisms of drug action and bacterial resistance. European Journal of Clinical Microbiology and Infectious Diseases. 21(9): 647-659.

Spoering AL and Lewis K 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. Journal of bacteriology. 183(23): 6746-6751.

Stratton WC 2015. Molecular Mechanisms of Action for Antimicrobial Agents. In: Antibiotics in Laboratory Medicine. 6 ed. D. Amsterdam (ed). Baltimore: Lippincott Williams & Willkins. 450-495.

Sulochana S, Mitchison DA, Kubendiren G, Venkatesan P and Paramasivan CN 2009. Bactericidal activity of moxifloxacin on exponential and stationary phase cultures of *Mycobacterium tuberculosis*. J Chemother. 21(2): 127-134.

Suzuka I, Kaji H and Kaji A 1966. Binding of specific sRNA to 30S ribosomal subunits: effect of 50S ribosomal subunits. Proceedings of the National Academy of Sciences of the United States of America. 55(6): 1483-1490.

Tateda K, Ishii Y, Matsumoto T, Furuya N, Nagashima M, Matsunaga T, Ohno A, Miyazaki S and Yamaguchi K 1996. Direct evidence for antipseudomonal activity of macrolides: exposure-dependent bactericidal activity and inhibition of protein synthesis by erythromycin, clarithromycin, and azithromycin. Antimicrobial agents and chemotherapy. 40(10): 2271-2275.

Tenover FC, Weigel LM, Appelbaum PC, McDougal LK, Chaitram J, McAllister S, Clark N, Killgore G, O'Hara CM and Jevitt L 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrobial agents and chemotherapy. 48(1): 275-280.

Tomasz A and Waks S 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc Natl Acad Sci U S A. 72(10): 4162-4166.

Upton A, Drinković D, Pottumarthy S, West T and Morris AJ 2005. Culture results of heart valves resected because of streptococcal endocarditis: insights into duration of treatment to achieve valve sterilization. Journal of Antimicrobial Chemotherapy. 55(2): 234-239.

Uri JV 1993. Antibacterial antagonism between fusidic acid and ciprofloxacin. Acta Microbiol Hung. 40(2): 141-149.

van Heerden J and Immelman A 1979. The use of doxycycline in the treatment of canine ehrlichiosis. Journal of the South African Veterinary Association. 50(4): 241-244.

Wallace JF, Smith RH, Garcia M and Petersdorf RG 1967. Studies on the pathogenesis of meningitis. VI. Antagonism between penicillin and chloramphenicol in experimental pneumococcal meningitis. J Lab Clin Med. 70(3): 408-418.

Wang X, Zhao X, Malik M and Drlica K 2010. Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. Journal of Antimicrobial Chemotherapy. 65(3): 520-524.

Watson ADJ and Maddison JE 2001. Systemic antibacterial drug use in dogs in Australia. Australian Veterinary Journal. 79(11): 740-746.

Wayne A, McCarthy R and Lindenmayer J 2011. Therapeutic antibiotic use patterns in dogs: observations from a veterinary teaching hospital. Journal of Small Animal Practice. 52(6): 310-318.

Wetzstein H and Trenti F 1994. The in vitro postantibiotic effect of enrofloxacin. Proc 18th World Buiatrics Congr. 615-618.

Widmer A, Wiestner A, Frei R and Zimmerli W 1991. Killing of nongrowing and adherent *Escherichia coli* determines drug efficacy in device-related infections. Antimicrobial agents and chemotherapy. 35(4): 741-746.

Yeh P, Tschumi AI and Kishony R 2006. Functional classification of drugs by properties of their pairwise interactions. Nature genetics. 38(4): 489-494.

Yeh PJ, Hegreness MJ, Aiden AP and Kishony R 2009. Drug interactions and the evolution of antibiotic resistance. Nat Rev Micro. 7(6): 460-466.

Zeiler H 1985. Evaluation of the *in vitro* bactericidal action of ciprofloxacin on cells of *Escherichia coli* in the logarithmic and stationary phases of growth. Antimicrobial agents and chemotherapy. 28(4): 524-527.

Zeiler HJ, Voigt WH and Endermann R 1988. Efficacy of Ciprofloxacin and Other Quinolones on Stationary-Phase Bacteria *in vitro* and *in vivo*. Reviews of Infectious Diseases. 10: S119-S121.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX

VITA

Supapatt Kireewan was born on May 17, 1989. He originally came from Saraburi province, Thailand. Regarding his educational background, he was graduated from secondary school, Suankularb Witthayalai Nonthaburi, in a mathematics-science program in 2008. After that, he was graduated a degree in Doctor of Veterinary Medicine (D.V.M.) from Chulalongkorn University with a first class honor in 2014. Now, he is pursuing a Master's degree in Veterinary Pharmacology Program, Department of Pharmacology, Faculty of Veterinary Science, Chulalongkorn University. Of his interest, antibacterial agents and their drug interactions between each other are the main topics.