PHARMACOKINETICS, PHARMACODYNAMICS AND DOSING REGIMEN OF LONG-ACTING OXYTETRACYCLINE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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เภสัชจลนศาสตร์ เภสัชพลศาสตร์ และแบบแผนการใช้ยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นานในปลานิล *(Oreochromis niloticus)*

น.ส.กานต์อนุช วสุนธารักษ์

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กานต์อนุช วสุนธรารักษ์ : เภสัชจลนศาสตร์ เภสัชพลศาสตร์ และแบบแผนการใช้ยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นานในปลานิล *(Oreochromis niloticus*). (PHARMACOKINETICS, PHARMACODYNAMICS AND DOSING REGIMEN OF LONG-ACTING OXYTETRACYCLINE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)) อ.ที่ปรึกษาหลัก : ผศ. สพ.ญ.ดร.นิภัทรา สวนไพรินทร์, อ.ที่ปรึกษาร่วม : ศ. สพ.ญ.ดร.เจนนุช ว่องธวัชชัย

ปัญหาการดื้อยาต้านจุลชีพเป็นเรื่องสำคัญและมีอุบัติการณ์เพิ่มมากขึ้นทั่วโลก เนื่องจากยาต้านจุลชีพที่ใช้รักษาโรคติดเชื้อในการเพาะเลี้ยงสัตว์น้ำนั้นมีอยู่อย่างจำกัด ดังนั้นจึงมีความจำเป็นในการกำหนดแบบแผนการใช้ยาที่มีอยู่ให้ได้ผลการรักษาสูงสุดและเกิดการดื้อยาน้อยที่สุด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาเภสัชจลนศาสตร์ของยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นาน โดยการฉีดเข้าช่องท้องครั้งเดียวในปลานิล ทำการศึกษาในปลานิลเพศผู้ (450±37.47 กรัม) จำนวน 120 ตัว แบ่งเป็น 2 กลุ่มโดยการสุ่มกลุ่มละ 60 ตัว ปลาได้รับยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นาน โดยการฉีดเข้าช่องท้อง ขนาด 50 มก./กก. และ 100 มก./กก. ในกลุ่มที่ 1 และ 2 ตามลำดับ จากนั้นเก็บตัวอย่างเลือดจากปลาทั้ง 2 กลุ่ม ในช่วงเวลาต่าง ๆ และนำไปวิเคราะห์ด้วยวิธี high performance liquid chromatography (HPLC) การศึกษาทางเภสัชพลศาสตร์ ทำในเชื้อ *สเตรปโตคอคคัส อะกาแลคเทีย* จากปลานิลติดเชื้อ จำนวน 56 ตัวอย่าง ทดสอบหาค่า minimum inhibitory concentration (MIC) และ minimum prevention concentration (MPC) ด้วยวิธี agar dilution ผลการศึกษาพบว่าความเข้มข้นยาสูงสุดในพลาสมาเท่ากับ 110.70 ± 5.61 ไมโครกรัม/มล. ที่เวลา 2 ชั่วโมง สำหรับขนาดยา 50 มก./กก. และ 287.85 ± 8.03 ไมโครกรัม/มล. ที่เวลา 4 ชั่วโมง สำหรับขนาดยา 100 มก./กก. ระดับยาในพลาสมาลดลงอย่างช้าๆ และยังคงพบระดับยาในพลาสมาหลังได้รับยานาน 168 ชั่วโมง (7 วัน) ที่ระดับความเข้มข้น 3.99 ± 0.48 ไมโครกรัม/มล. และ 23.00 ± 2.51 ไมโครกรัม/มล. ในปลาที่ได้รับยาในขนาด 50 มก./กก. และ 100 มก./กก. ตามลำดับ ผลการวิเคราะห์เชื้อ *สเตรปโตคอคคัส อะกาแลคเทีย* 56 ตัวอย่าง พบว่าค่า MIC ของยาออกซิเตตร้าไซคลินต่อเชื้อ *เสเตรปโตคอคคัส อะกาแลคเทีย* อยู่ระหว่าง 0.5-2 ไมโครกรัม/มล. ค่า MIC_{50} และ MIC_{90} เท่ากับ 0.5 และ 1 ไมโครกรัม/มล. ตามลำดับ ค่า MPC อยู่ระหว่าง 4-512 ไมโครกรัม/มล. ค่า MPC_{50} และ MPC_{90} เท่ากับ 32 และ 128 ไมโครกรัม/มล. ตามลำดับ สำหรับอัตราส่วนระหว่าง MPC และ MIC และ mutant selection window (MSW) นั้น พบว่า MPC_{50}/MIC_{50} เท่ากับ 64 (MSW: 0.5 - 32 µg/ml) และ MPC_{90}/MIC_{90} เท่ากับ 128 (MSW: 1 -128 µg/ml) จากการการบูรณาการค่าทางเภสัชจลนศาสตร์และเภสัชพลศาสตร์ การให้ยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นานในขนาด 50 มก./กก. และ 100 มก./กก. ให้ระดับยาในพลาสมาเพียงพอที่จะออกฤทธิ์ต้านแบคทีเรียที่มีค่า MIC ≤ 1 ไมโครกรัม/มล. ได้อย่างน้อย 7 วัน เมื่อพิจารณาค่าทางเภสัชจลนศาสตร์ และเภสัชพลศาสตร์จากค่า MPC พบว่าออกซิเตตร้าไซคลินชนิดออกฤทธิ์นานในขนาด 100 มก./กก. เท่านั้นที่สามารถให้ปริมาณยาในพลาสมาสูงเพียงพอที่จะป้องกันการพัฒนาของ resistant-mutant subpopulation ดังนั้นจากการศึกษานี้ แบบแผนการให้ยาที่แนะนำของยาออกซิเตตร้าไซคลินชนิดออกฤทธิ์นาน คือ ขนาด 100 มก./กก. ให้โดยการฉีดเข้าช่องท้องเพียงครั้งเดียว เพื่อรักษาการติดเชื้อ และป้องกันการพัฒนาของ resistant-mutant subpopulation ของเชื้อ*เสเตรปโตคอคคัส อะกาแลคเทีย*

สาขาวิชา	ชีวศาสตร์ทางสัตวแพทย์	ลายมือชื่อนิสิต .....
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KEYWORD: antimicrobial activity, long-acting oxytetracycline, pharmacodynamics, pharmacokinetics, Streptococcus agalactiae, tilapia

Kananuch Vasuntrarak : PHARMACOKINETICS, PHARMACODYNAMICS AND DOSING REGIMEN OF LONG-ACTING OXYTETRACYCLINE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*). Advisor: Asst. Prof. NIPATTRA SUANPAIRINTR, Ph.D. Co-advisor: Prof. JANENUJ WONGTAVATCHAI, Ph.D.

Antimicrobial resistance has become a serious global problem and is steadily increasing worldwide. In aquaculture, there are limited antimicrobial options for treatment. Thus, there are growing needs for more specific dosing regimens of existing antimicrobial drugs that are not only to obtain therapeutic efficacy but also to minimize the resistance of pathogens. The purposes of this study were to determine pharmacokinetics (PK) of long-acting oxytetracycline (OTC) after intraperitoneal (IP) administration in Nile tilapia. One hundred and twenty healthy male tilapia (450±37.47 g) were divided into two experimental groups (60 fish/group). Each group received OTC-LA single IP injection at dosage of 50 mg/kg or 100 mg/kg bodyweight. Blood samples were collected at various times post-dosing and plasma OTC were analyzed using high performance liquid chromatography (HPLC). For pharmacodynamics (PD) study, 56 *S. agalactiae* isolates from diseased tilapia were determined for minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) by agar dilution method. The results showed that the C_{max} and T_{max} of OTC were 110.70 ± 5.61 µg/ml at 2 h for the dosage of 50 mg/kg, and 287.85 ± 8.03 µg/ml at 4 h for the dosage of 100 mg/kg. OTC level in plasma was slowly depleted and remained at 3.99 ± 0.48 µg/ml and 23.00 ± 2.51 µg/ml at 168 h (7 day) after administration OTC-LA at the dosages of 50 and 100 mg/kg, respectively. From 56 *S. agalactiae* samples, MIC range was 0.5 to 2 µg/ml, with MIC_{50} and MIC_{90} at 0.5 µg/ml and 1 µg/ml, respectively. MPC range was 4 to 512 µg/ml, with MPC_{50} and MPC_{90} at 32 µg/ml and 128 µg/ml, respectively. For the ratio of MPC and MIC and mutant selection window (MSW) results, MPC_{50}/MIC_{50} ratio was 64 (MSW: 0.5 - 32 µg/ml) and MPC_{90}/MIC_{90} ratio was 128 (MSW: 1 -128 µg/ml). From the integrated PK/PD parameters, both OTC-LA at the dosages of 50 mg/kg and 100 mg/kg dosages achieved the target values and provided plasma OTC level above MIC for at least 7 days. While PK/PD parameters based on MPC, only OTC-LA at 100 mg/kg dosage can prevent the resistant-mutant subpopulation. Therefore, OTC-LA treatment at 100 mg/kg bodyweight IP administration would be suggested as the optimal dosing regimen to attain therapeutic efficacy and prevent the emergence of resistant-mutant subpopulation and possible to be used as a single administration for the infection caused by *S. agalactiae*.

Field of Study:	Veterinary Biosciences	Student's Signature .....
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ABBREVIATION AND SYMBOL

Abbreviation/ Symbol	Definition
AMR	Antimicrobial resistance
AUC	Area under the plasma drug concentration-time curve
AUC_{0-24}	Area under the plasma drug concentration-time curve from 0 to 24 hours
AUC_{0-168}	Area under the plasma drug concentration-time curve from 0 to 168 hours
$AUC_{0-\infty}$	Area under the plasma drug concentration-time curve from 0 to infinity
AUMC	Area under the first moment of the plasma drug concentration-time curve
AUC/MIC	The ratio of the area under the plasma drug concentration-time curve and the minimum inhibitory concentration
AUC_{0-168}/MIC_{50}	The ratio of the area under the plasma drug concentration-time curve from 0 to 168 hours and the 50^{th} percentile of minimum inhibitory concentration
AUC_{0-168}/MIC_{90}	The ratio of the area under the plasma drug concentration-time curve from 0 to 168 hours and the 90^{th} percentile of minimum inhibitory concentration
AUC/MPC	The ratio of the area under the plasma drug concentration-time curve and the mutant prevention concentration
AUC_{0-168}/MPC_{50}	The ratio of the area under the plasma drug concentration-time curve from 0 to 168 hours and the 50^{th} percentile of mutant prevention concentration

Abbreviation/ Symbol	Definition
$AUC_{0\text{-}168}/MPC_{90}$	The ratio of the area under the plasma drug concentration-time curve from 0 to 168 hours and the 90^{th} percentile of mutant prevention concentration
cfu/ml	Colony forming unit per milliliter
C_{max}	Peak plasma drug concentration
C_{max}/MIC	The ratio of peak plasma drug concentration and the minimum inhibitory concentration
CL/F	Apparent total body clearance
CTC	Chlortetracycline
CV	Coefficient of variation
h	Hour
HPLC	High performance liquid chromatography
HQC	High concentration quality control sample
IM	Intramuscular
IP	Intraperitoneal
IS	Internal standard
K_{el}	Elimination rate constant
LLOQ	Lower limit of quantification
LQC	Low concentration quality control sample
mg/kg	Milligram per milliliter
min	Minute
ml	Milliliter
µg/ml	Microgram per milliliter
µl	Microliter
M	Molar, mol per liter
MIC	Minimum inhibitory concentration
MIC_{50}	50^{th} percentile of minimum inhibitory concentration

Abbreviation/ Symbol	Definition
MIC_{90}	90^{th} percentile of minimum inhibitory concentration
MPC	Mutant prevention concentration
MPC_{50}	50^{th} percentile of mutant prevention concentration
MPC_{90}	90^{th} percentile of mutant prevention concentration
MPC_{50}/MIC_{50}	The ratio of 50^{th} percentile of mutant prevention concentration and minimum inhibitory concentration
MPC_{90}/MIC_{90}	The ratio of 90^{th} percentile of mutant prevention concentration and minimum inhibitory concentration
MQC	Medium concentration quality control sample
MRT	Mean residence time
$MRT_{0\text{-}168}$	Mean residence time from 0 to 168 h
$MRT_{0\text{-}\infty}$	Mean residence time from 0 to infinity
MSW	Mutant selection window
MSW_{50}	MSW from 50^{th} percentile of mutant prevention concentration and minimum inhibitory concentration
MSW_{90}	MSW from 90^{th} percentile of mutant prevention concentration and minimum inhibitory concentration
nm	Nanometer
OTC	Oxytetracycline
OTC-LA	Long-acting formulations of OTC
PAE	Post-antibiotic effect
PK	Pharmacokinetic
PD	Pharmacodynamic
PK/PD	The ratio of pharmacokinetic and pharmacodynamic
QC	Quality control
R^2	Coefficient of determination
SD	Standard deviation

Abbreviation/ Symbol	Definition
SEM	Standard error of the mean
T>MIC	The time that plasma drug concentration remains above the minimum inhibitory concentration
$T>MIC_{50}$	The time that plasma drug concentration remains above the 50^{th} percentile of minimum inhibitory concentration
$T>MIC_{90}$	The time that plasma drug concentration remains above the 90^{th} percentile of minimum inhibitory concentration
T>MPC	The time that plasma drug concentration remains above the mutant prevention concentration
$T>MPC_{50}$	The time that plasma drug concentration remains above the 50^{th} of mutant prevention concentration
$T>MPC_{90}$	The time that plasma drug concentration remains above the 90^{th} of mutant prevention concentration
$t_{1/2}$	Elimination half-life
T_{max}	Time of peak concentration
TS	Test standard
V_d/F	Apparent volume of distribution after non-intravenous administration

CHAPTER I
INTRODUCTION

Importance and Rationale

Antimicrobial resistance or AMR has become a serious global problem. Inappropriate use of antimicrobials in livestock production and aquaculture is one of the causes of antimicrobial resistance. Food and Agriculture Organization of the United Nations (FAO) has established the action plan on AMR. In the area of practitioner, the prudent use of antimicrobials is one of the measures to fight with AMR suggested by FAO. The prudent use of antimicrobials is to maximize therapeutic effect of the antimicrobial agent while minimizing the development of antimicrobial resistance (FAO, 2016). In aquaculture, there are limited antimicrobial options for treatment. Thus, there is a growing need for more specific dosing regimens of existing antimicrobial drugs that are not only to obtain therapeutic efficacy but also to minimize the resistance of pathogens.

Nile tilapia (*Oreochromis niloticus*) is a popular farmed freshwater fish species in Thailand for both export and local consumption. The fast expansion of the tilapia farming industry has been accompanied by recurrent problems of bacterial infectious disease (Pereira et al., 2010). Streptococcosis is an important disease that can cause great losses in fish farm stocks and commercial growth of tilapia production. *Streptococcus agalactiae* (*S. agalactiae*) is the dominant species causing streptococcosis in farmed tilapia in Thailand (Jantrakajorn et al., 2014; Dangwetngam et al., 2016). It can cause disease in tilapia reared in several culture systems, hatchery, nursery and grow-out phase and can infect the fish at any stages of life, including fry, juvenile and broodstock (Wongtavatchai and Maisak, 2008; Jantrakajorn et al., 2014). Although vaccination is an effective control

of this disease, antimicrobial therapy is an essential management during the disease outbreak (Vinarukwong et al., 2018).

Oxytetracycline (OTC), a member of tetracyclines, is a broad-spectrum bacteriostatic antibiotic that inhibits bacterial protein synthesis. OTC has frequently been used in veterinary medicine, especially in livestock industry due to its broad-spectrum activity, good penetration into body fluids and tissues, low cost as well as low toxicity risks (Riviere and Spoo, 1995). Moreover, the rational use of OTC made its maximum residue limits be established for all food-producing species including fish (EMEA, 1995). OTC has been approved in the USA for treatment of bacterial diseases such as furunculosis (*Aeromonas salmonicida*) and columnaris disease (*Flavobacterium columnare*) in salmon and rainbow trout (USFDA, 2020). OTC has also been authorized in Thailand for the treatment of infection in fish caused by susceptible bacteria (FDA, 2020).

The most common route of drug administration in aquatic farming is oral administration. However, pharmacokinetic (PK) information of OTC is limited to certain fish species, for example, rainbow trout (Bjorklund and Bylund, 1991) and Atlantic salmon (Elema et al., 1996). Most of the PK studies of OTC in tilapia were depletion kinetics and withdrawal time determination following in-feed drug administration (Chen et al., 2004; Chen et al., 2005; Paschoal et al., 2012). However, antibiotic treatment *via* medicated feed is not usually successful as the sick fish might reject the medicated feed either due to poor palatability or loss of appetite. Inefficiency of drug levels in fish circulation results in the treatment failure and economic loss (Rigos et al., 1999). Parenteral drug administration, such as intramuscular (IM) and intraperitoneal (IP), is labor intensive but provides more beneficial in terms of achieving high tissue drug levels (Samuelsen et al., 2002) and can be used to treat valuable individuals, such as broodstock or ornamental fish. In addition, it is environmentally friendly because the quantity of the drug is not directly released into the environment (Rigos et al., 2010).

The use of antimicrobial drug should be based on antimicrobial susceptibility testing results. The minimum inhibitory concentration (MIC) values are the standard used to evaluate antimicrobial susceptibility and to calculate the important PK/PD indices of antimicrobial drugs. While MIC values are apparently less appropriate in preventing the emergence of resistant strains, mutant prevention concentration (MPC) values defined as the lowest drug concentration that prevents the growth of the least susceptible first-step resistant mutants have been considered (Xu et al., 2013). Long-acting formulations of OTC (OTC-LA) have widely been used in veterinary medicine to provide prolonged drug release in treated animal plasma, which provide extended long-lasting effects for days. This advantage is desired in clinical situations where repeated handling of infected individuals for drug administration should be minimized (Rigos et al., 2010).

Therefore, the purposes of this study were to investigate the pharmacokinetic parameters of a commercially available long-acting OTC formulation following IP injection in tilapia along with the *in vitro* antibacterial activity in terms of MIC and MPC of OTC against pathogenic bacteria *S. agalactiae* from tilapia. The pharmacokinetics and *in vitro* pharmacodynamics will be then integrated to determine the optimal dosing regimens of OTC-LA against *S. agalactiae* in tilapia.

Objectives of study

This study aims to determine PK parameters of OTC-LA after IP administration in healthy tilapia and to investigate the *in vitro* antibacterial activities; MIC and MPC; against clinical *S. agalactiae* isolates from diseased tilapia. In addition, PK parameters and *in vitro* PD values were integrated to determine optimal dosing regimens of OTC-LA against *S. agalactiae* in tilapia.

Keywords (Thai): ฤทธิ์ต้านเชื้อแบคทีเรีย ออกซิเตตร้าไซคลินชนิดออกฤทธิ์นาน เภสัชพลศาสตร์ เภสัชจลนศาสตร์ *สเตรปโตคอคคัส อะกาแลคเทีย* ปลานิล

Keywords (English): antimicrobial activity, long-acting oxytetracycline, pharmacodynamics, pharmacokinetics, *Streptococcus agalactiae*, tilapia

Hypotheses

1. Pharmacokinetics of OTC-LA after IP administration in tilapia exhibit prolonged effect similar to those of domestic species.
2. OTC exerts its antibacterial activity against *S. agalactiae* isolated from diseased tilapia.
3. Dosing regimen of OTC-LA obtained from PK/PD integration is suitable for achieving therapeutic efficacy against *S. agalactiae* in tilapia.

CHAPTER II

LITERATURE REVIEW

1. Streptococcosis in Nile tilapia

Nile tilapia (*Oreochromis niloticus*) is a popular farmed freshwater fish species in Thailand. Tilapia was traditionally reared in rice field, ditches and co-cultured with other livestock animals. Nowadays, the rearing styles have been changed to intensive culture with many strategies to promote production such as male-monosex culture, using commercial feed, dietary supplement as well as medicine (Wongtavatchai, 2017). The industrial farming provides products for both export and local consumption. There were about 335,441 tilapia farms in Thailand with the total production of 208,635 tons in 2019 (Nhurith, 2020). The fast expansion of the tilapia farming industry has been accompanied by recurrent problems of bacterial infectious diseases (Pereira et al., 2010). The major pathogenic bacteria responsible for mortalities in tilapia include *Aeromonas hydrophila* (Tipmongkolsilp et al., 2012), *Francisella noatunensis* subsp. *orientalis* (Soto et al., 2013), *Flavobacterium columnare* (Dong et al., 2015), *Vibrio vulnificus* (Chen et al., 2006), *Streptococcus iniae* and *Streptococcus agalactiae* (Jantrakajorn et al., 2014).

Streptococcosis is an important disease that can cause great losses in fish farm stocks and commercial growth of tilapia production. *Streptococcus iniae* and *Streptococcus agalactiae* are the causes of streptococcosis in tilapia around the world. In Thailand, *Streptococcus agalactiae* (*S. agalactiae*) has been the dominant species causing streptococcosis in farmed tilapia (Jantrakajorn et al., 2014; Dangwetngam et al., 2016). *S. agalactiae* is a gram-positive coccus in chain, facultative anaerobe, non-spore forming and non-motile species. It is a fastidious organism that required 5-10% of blood in media for the growth. This pathogen can cause disease in tilapia reared in several culture systems, hatchery, nursery and grow-out phase and can infect the fish at any stages of life, including fry, juvenile and broodstock (Wongtavatchai and Maisak, 2008;

Jantrakajorn et al., 2014). The main clinical signs observed in tilapia with streptococcosis are loss of appetite, unilateral or bilateral exophthalmos, eye hemorrhage, corneal opacity, distended abdomen, curvature of the spinal cord, stiffness, erratic swimming, and scattered hemorrhage around the operculum, mouth, fin and body. However, some fish may not show clinical signs before death (Azmai and Saad, 2011; Jantrakajorn et al., 2014). The mortality rate is very high (up to 80–100%) especially under stress conditions and inappropriate managements (Mian et al., 2009).

Farm hygiene management and disease prevention system are the most important strategies for prevention of disease outbreak. Commercial vaccine that protects against *S. agalactiae* is available and has been used in several countries. However, it has a limitation because the immunity stimulated by vaccine has no cross protection against other *S. agalactiae* serotypes. Antimicrobial therapy plays an essential role in the management of outbreak. It helps reduce streptococcosis severity and spread. The selection of antimicrobial drug should be based on antimicrobial susceptibility testing results (Wongtavatchai, 2017). However, the use of antimicrobial drugs is directly linked to the emergence of drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (Ventola, 2015) which has limited antimicrobial options for treatment. Therefore, there is a growing need for more specific dosing regimens of existing antimicrobial drugs that are not only to obtain therapeutic efficacy but also to minimize the resistance of pathogens.

2. Oxytetracycline

Oxytetracycline (OTC), a member of tetracyclines, is one of the oldest antibiotics still in use in medicine. It is a yellow amphoteric crystalline compound with a molecular weight of 460.44. It is a low water solubility and low octanol/water partition coefficient substance. It is stable as a powder but unstable in solution, therefore injections of

oxytetracycline are often formulated as hydrochloride or dihydrate (Treves-Brown, 2000). The chemical structure of OTC is given in figure 1.

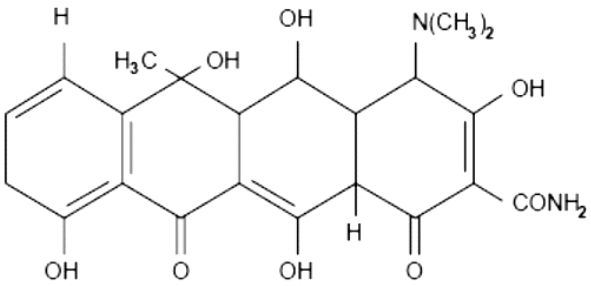


Figure1: Chemical structure of OTC

OTC has frequently been used in veterinary medicine, especially in livestock industry, due to its broad-spectrum activity, good penetration into body fluids and tissues, low cost, as well as low toxicity risks (Riviere and Spoo, 1995). Moreover, the rational use of OTC made its maximum residue limits (MRLs) established for all food-producing species including fish (EMEA, 1995).

2.1 Mechanism of action and spectrum of activity

OTC exerts its antimicrobial activity by binding to the 16S rRNA and S7 protein of the 30S ribosomal subunit of susceptible organisms. Upon binding, OTC interferes with tRNA binding to mRNA and subsequently preventing bacterial protein synthesis (del Castillo, 2013). OTC is a broad-spectrum bacteriostatic antibiotic. In domestic animals, it has been used for the treatment of gastrointestinal and respiratory infections, mainly for aerobic microorganisms including gram positive/negative bacteria, *Rickettsia*, *Mycoplasma*, and *Chlamydia* species (Aktas and Yarsan, 2017).

In aquaculture, it was approved in the USA to treat ulcer disease (*Hemophilus piscium*), furunculosis (*Aeromonas salmonicida*), cold-water disease (*Flavobacterium psychrophilum*), columnaris disease (*F. columnare*), bacterial hemorrhagic septicemia (*Aeromonas hydrophila*), and pseudomonas disease (*Pseudomonas* spp.). The target fish

species are rainbow trout, catfish and salmonids (USFDA, 2020). OTC has also been authorized in Thailand for the treatment of infection in fish caused by susceptible bacteria (FDA, 2020).

2.2 Mechanism of resistance

Resistance to tetracyclines can be mediated by different mechanisms. The most common mechanism arises from acquisition of genes that either encode transporters of the major facilitator superfamily (MFS), which remove the antibiotics from the cell (e.g., *TetB*, *TetK*), or encode proteins that dissociate the tetracyclines from their binding sites (e.g., *TetM*, *TetO*) (Chopra and Roberts, 2001).

2.3 Pharmacokinetics of OTC

Oxytetracycline has a low toxicity and a high ability to readily disperse into blood and most tissues (del Castillo, 2013). However, OTC has a rather limited bioavailability because it chelates or forms complexes with polyvalent cations such as Ca^{2+}, Fe^{2+}, Al^{3+}, and Mg^{2+} (Riviere and Spoo, 1995). These electrically charged complexes, which are microbiologically inert, are not able to easily traverse the lipid-rich biological membranes thereby causing a several fold decrease in the absorption of oxytetracycline (Riviere and Spoo, 1995; Treves-Brown, 2000). The absorption of OTC may vary depending on pharmaceutical dosage form and its salts. The oral bioavailability of OTC is 5% in non-fasting calves and pigs. Bioavailability is further reduced when fed with milk or milk replacer, but it is much higher in fasted calves and pigs. The long-acting injectable formulation delays the absorption because it contains some excipients that retain the drug at the injection site via different mechanisms. The OTC distribution is the highest in richly perfused organs such as kidneys, liver, and lungs. Plasma protein binding capacity of OTC is lower than other drugs in this group. The excretion of OTC is primarily by glomerular filtration, followed by biliary secretion and intestinal excretion, respectively.

OTC also undergoes enterohepatic circulation which contributes to its long half-life (del Castillo, 2013).

2.4 Pharmacokinetics of OTC in fish

Pharmacokinetics of OTC in fish are generally like those in terrestrial animals. The bioavailability is low when administered orally but good distribution. OTC is not metabolized or biotransformed to a significant extent by fish. Thus, almost all the administered doses may be excreted into the environment (Treves-Brown, 2000). However, in some fish species, it persists in bone tissues, scales and in the pronephros (Grondel et al., 1987). It was reported that approximately 60% of the OTC is eliminated in the urine by glomerular filtration and the remaining 40% being eliminated in the feces (Riviere and Spoo, 1995). Moreover, it has suggested that the environmental temperature likely plays an important role in the rate of OTC excretion. The significant slower of OTC elimination at lower water temperatures were reported in rainbow trout (Bjorklund and Bylund, 1990).

Pharmacokinetic information of OTC is limited to certain fish species. For aquatic species, there have been reported in different fish species, for example, rainbow trout (Bjorklund and Bylund, 1991; Miller et al., 2012), channel catfish (Luzzana et al., 1994), Atlantic salmon (Elema et al., 1996), sea bass (Rigos et al., 2010), grass carp (Zhang and Li, 2007), olive flounder (Jung et al., 2008) and tilapia (Sidhu et al., 2018). Most of the PK studies of OTC in tilapia were depletion kinetics and withdrawal time determination following in-feed drug administration in healthy fish (Chen et al., 2004; Paschoal et al., 2012). A study examined plasma and tissue depletion of OTC after intravenous administration in tilapia challenged with pathogens; *Streptococcus iniae* and *Vibrio vulnificus* (Chen et al., 2005). The PK of OTC after single oral administration in tilapia maintained at three different salinities were recently reported. The results demonstrated that OTC was rapidly absorbed and slowly excreted in freshwater and brackish water

tilapia. The fastest absorption and elimination of OTC were found in tilapia maintained in salt water. This indicated that the more water salinity, the greater increase in clearance of OTC in tilapia (Sidhu et al., 2018).

The special consideration of PK in aquatic animals is the variety of rearing environment. The water temperature and water salinity play an important role in the pharmacokinetics of OTC, especially in the rate of elimination (Bjorklund and Bylund, 1990; Sidhu et al., 2018). Therefore, PK data are variable among fish species and could not be extrapolated.

3. Dosing regimens in aquaculture

The most common route of drug administration in aquatic farming is in-feed medication because of its low cost, ease of use and less fish stress. In-feed medication is the standard treatment regimen of OTC for fish. However, antibiotic treatment *via* medicated feed have not usually been successful as the sick fish might reject the medicated feed either due to poor palatability or loss of appetite. Inefficiency of drug levels in fish circulation results in the treatment failure and economic loss (Rigos et al., 1999). Recommended OTC doses of in-feed medication for fish range from 55 to 83 mg/kg bodyweight per day for 10 days (Treves-Brown, 2000).

Injection is an administration method used almost exclusively for experimental purposes. It is rarely used in routine fish management because it is both labor-intensive and stressful to the fish (Treves-Brown, 2000). For intraperitoneal administration, injection is made into the peritoneal cavity between the pelvic and anal fins to the right of the ventral midline. This administration method can be less stressful on the fish and make handling easier if the fish are sedated or anaesthetized which is usually accomplished by immersion in anesthetic-medicated water. Although IP injection is not used in routine fish management. However, it provides more beneficial in terms of achieving high tissue drug levels (Samuelsen et al., 2002) and can be used to treat valuable individuals, such as sick

broodstock or ornamental fish. In addition, it is environmentally friendly because the quantity of the drug is not directly released into the environment (Rigos et al., 2010). The extra-label dose suggestion of OTC injection in fish is 25-50 mg/kg bodyweight as single administration. (Stoffregen et al., 1996; Noga, 2010; Stoskopf, 2011).

4. Long-acting formulations of OTC (OTC-LA)

Long-acting formulations of OTC (OTC-LA) have widely been used in veterinary medicine to provide prolonged drug release into treated animal plasma, which provide extended long-lasting effects for days (AliAbadi and Lees, 2000). This advantage is desired in clinical situations where repeated handling of infected individuals for drug administration should be minimized. The dosing regimen of OTC-LA in cattle, sheep and pigs is 20 mg/kg bodyweight administration by deep intramuscular injection. The drug is released more slowly from the depot at the injection site, thus giving rise to a prolonged action lasting 3-5 days after a single injection (SPC of Terramycin/LA; Vm: 42058/4151).

Apart from the long half-life which is a specific property of OTC compound (del Castillo, 2013), OTC-LA formulation could enhance the long action activity. Long-acting mechanism of OTC-LA has been explained by the depot injectable formulation, which involves the slow drug release from the injection site into the blood. Moreover, this formulation contains excipient, 2-pyrrolidone or dimethylacetamide, that slows down the absorption from the site of injection. El Korchi et al., (2001) studied the disposition of two long-acting OTC formulations in pigs after IM administration. They found that the slow absorption from muscle delays the disposition of the drug and decreases the drug elimination from the kidney. OTC disposition behaves the flip-flop kinetics which the rate of absorption limits the plasma pharmacokinetics and the terminal phase of the plasma concentration-time curve. There have many PK reports of OTC-LA injection in livestock animals including claves (Kumar and Malik, 1998), goats (Aktas and Yarsan, 2017), pigs (El Korchi et al., 2001) and buffaloes (Poapolathep et al., 2017). Moreover, the PK studies

of OTC-LA were expanded to some wildlife, tammar wallabies (McLelland et al., 2011) and reptiles such as American alligators (Helmick et al., 2004) and freshwater crocodiles (Poapolathep et al., 2020).

Although the extra-label use of OTC-LA injection have been applied in the fish broodstock. There are very limited scientific data about the therapeutic use and the optimal dosing regimens. Recently, Ali et al. (2019) studied the single IP injection of OTC-LA at a dose of 100 mg/kg bodyweight in white sea bream broodstock. They found that serum OTC concentration was high and long-lasting. The concentration remained at more than 5 μg/ml until seven days post-administration. Those were adequate to control of *Staphylococcus epidermidis* and *Bacillus cereus* infection in white sea bream broodstock. Pharmacokinetics of OTC-LA after IM injection at 50 mg/kg bodyweight in grouper were reported by Rigos et al. (2010). The results showed that high peak serum OTC concentration and high OTC levels were maintained through the whole experiment (48 h after administration).

Despite the necessity of a long-acting OTC injection in bacterial disease control in tilapia broodstock, the pharmacokinetic data of OTC-LA after IP injection in tilapia have not been established. The pharmacokinetic characteristics of OTC-LA in tilapia need to be more thoroughly understood to determine the optimal dosing regimens for achieving and maintaining therapeutic drug levels as well as minimizing antimicrobial resistance.

5. Antimicrobial susceptibility testing

5.1 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism (CLSI, 2019). MIC

is now being more commonly used to evaluate the susceptibility of bacteria to antimicrobials. The MIC procedure mainly utilizes antibiotic dilution assay including agar dilution, broth dilution as well as broth micro-dilution methods. Serial dilutions of antibiotics are prepared and inoculated into the agar plates, wells or tubes alongside a standard inoculum of a test organism in each dilution. Antibiotic susceptibility is stated as the lowest concentration of antibiotic that completely inhibits visible growth in medium. Another MIC procedure is diffusion-based method that the agar plates are inoculated with diffusion strip containing an antibiotic concentration gradient. After incubation, the MIC value can be read at the point the ellipse edge intersects the MIC strip (Jorgensen and Ferraro, 2009).

MIC values can be interpreted as susceptible (S), intermediate (I) or resistance (R) to an antimicrobial drug based on established MIC breakpoints by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). For fish, CLSI approved only two fish-specific breakpoints for *Aeromonas salmonicida* and established epidemiological cutoff values (ECVs) of some antimicrobial drugs for *Aeromonas salmonicida, Aeromonas hydrophila*, *Flavobacterium psychrophilum* and *Flavobacterium columnare*. (CLSI, 2020). Specific breakpoints of antimicrobials for other fish pathogens are scarce, therefore, the breakpoints from a terrestrial animal species or humans were used to extrapolate instead (Lukkana et al., 2015; Dangwetngam et al., 2016; Chideroli et al., 2017). MIC breakpoints for Streptococcus group B isolates are susceptible (S) at MIC $\leq$ 2, intermediate (I) at 4 and resistance (R) at MIC $\geq$ 8 µg/ml (CLSI, 2018).

5.2 Mutant prevention concentration (MPC)

The mutant prevention concentration or MPC is the lowest antimicrobial concentration that prevents the growth of resistant sub-population in heterogeneous

bacteria (Blondeau, 2009). In general, resistant mutant sub-populations spontaneously arise in bacterial densities of 10^7-10^9 cfu/ml (Dong et al., 1999; Blondeau et al., 2004). MPC is a new concept meant to confront the increased prevalence of antimicrobial resistance by using antimicrobial at the concentrations that are able to prevent the selection of resistant bacteria populations (Caron and Mousa, 2010). The MPC procedure is carried out using agar dilution method which high bacterial inoculum are applied to agar plates. Bacterial inoculum more than or equal to 10^{10} cfu/ml is required to provide resistant mutants for testing (Blondeau et al., 2001).

5.3 Mutant selection window (MSW)

MPC is typically higher than MIC indicating that higher antimicrobial concentration is required to prevent the growth of mutant sub-populations from high density bacteria (Blondeau, 2009). The range of antimicrobial concentration between the MIC and MPC is called the mutant selection window (MSW) in which selective amplification of resistant sub-populations may occur (Caron and Mousa, 2010). For antimicrobial concentrations falling within the MSW, susceptible cells are likely inhibited but not the mutant cells. Therefore, therapeutic drug concentrations may be the same drug concentration that selectively amplifies the mutant portion. Antimicrobial concentrations higher than the MPC may block both susceptible and mutant cell growth (Blondeau, 2009).

For limitation of MSW, MPC values are determined from bacterial population at 10^{10} cfu/ml that provide occasion for single step mutant. If density of bacterial population is greater than 10^{10} cfu/ml, MPC can only delay the amplification of antibacterial-resistant mutants but cannot completely prevent their growth because double step resistant mutants may occur. Thus, eradication of the mutants is depended on host defense mechanism (Drlica and Zhao, 2007). Antimicrobial drug dosages that provide plasma drug concentrations above MPC may be higher than many recommended dosages,

thereby, it may increase risk of adverse effects (Drlica and Zhao, 2007; Zhao and Drlica, 2008). Therefore, the practitioners should consider based on many factors such as margin of safety and adverse effects of antimicrobial drugs as well as health status of animals.

6. Pharmacokinetic/pharmacodynamic (PK/PD) properties

In vivo pharmacokinetic (PK) and *in vitro* pharmacodynamic (PD) experiments are used to explain a relationship between plasma drug concentrations and the effect (Ambrose et al., 2007). The application of PK/PD integration to establish the dosing regimens for antimicrobial drugs is one of the strategies to decrease the inappropriate used of antimicrobial drugs in veterinary medicine (Papich, 2014).

The aims of antimicrobial therapy are to eliminate pathogens, achieve clinical improvement, as well as minimize the antimicrobial resistant bacteria. However, inadequate antimicrobial drug concentrations or time of exposure to drugs can lead to drug resistant problems. Utilizing PK parameters of antimicrobial drugs of specific animal species together with PD data of the targeted pathogenic bacteria can provide optimum dosages with greater treatment efficacy and lower risk of antimicrobial drug resistance (McKellar et al., 2004).

The PK/PD indices are typically used for antimicrobials including the ratio of the maximum plasma drug concentration and the minimum inhibitory concentration (MIC) (C_{max}/MIC), the time the plasma drug concentration remains above MIC (T>MIC) expressed as a percent of the dosing interval, and the ratio of the area under the plasma drug concentration-time curve and MIC (AUC/MIC) (Toutain et al., 2002). These indices used to describe the shape of plasma concentration vs time profile was shown in Figure 2. Target PK/PD ratios that provide clinical efficacy are varied depending on bacterial strains and antibacterial drugs (Heffernan et al., 2018).

One of the factors used to define the best PK/PD indices for a particular antibacterial is the pattern of microbial kill exhibited by the compound. Many reports have

described the PK/PD properties of the major classes of antibiotics and three patterns of activity were observed (Craig, 2002; Craig, 2003; Hesje et al., 2007).

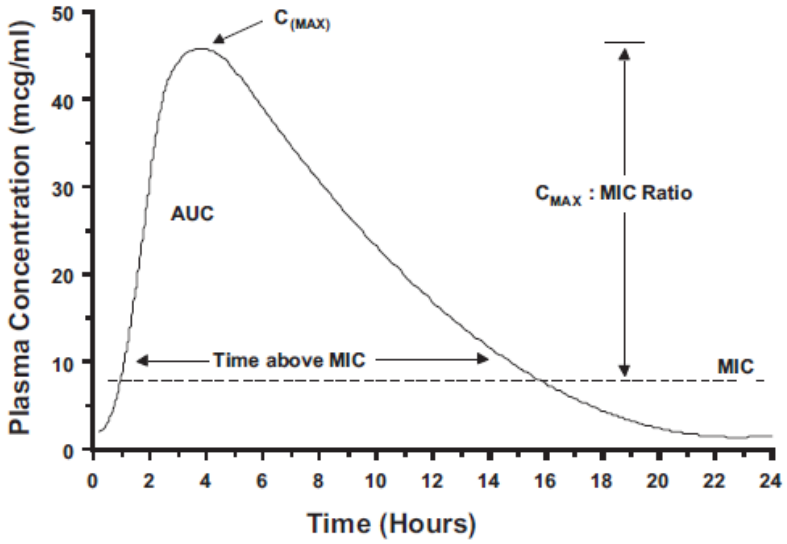


Figure 2: Pk/PD relationships (Papich, 2014)

Firstly, antibiotics display concentration-dependent killing with a prolonged post-antibiotic effect (PAE). Increasing of drug concentrations result in more rapid and extensive organism killing. The goal of dosing regimen for this class of drug would be to maximize the drug concentration, thus the C_{max}/MIC and/or the AUC/MIC ratios are the best PK/PD parameters correlating with the treatment efficacy. This pattern is predictive of the activity of aminoglycosides and fluoroquinolones (Craig, 2002).

Secondly, antibiotics exhibit time-dependent killing with minimal to moderate PAE. The different classes of beta-lactams (penicillins, cephalosporins, monobactams and carbapenems) exhibit this pattern of activity. The PK/PD index that correlates with bacterial killing and microbiological response is T>MIC. The duration of antimicrobial exposure should be extended to optimize the antimicrobial activity, The more frequent of dosing interval must be done for the drug with the shorter elimination half-life (Craig, 2003). The use of a continuous intravenous infusion to maintain the T>MIC at 100% may

be the most effective way of maximizing pharmacodynamic exposure, especially if higher T>MIC are required (Pea and Viale, 2006).

Thirdly, antibiotics demonstrate time-dependent killing with prolonged PAE. Increasing of drug concentrations not only slightly enhances the organism killing but also produces prolonged suppression of organism regrowth. The goal of dosing is to optimize the amount of drug, and the AUC/MIC ratio is the index most correlated with efficacy. This pattern is observed in glycopeptides, linezolid, tetracyclines, clindamycin and azithromycin (Craig, 2002; Craig, 2003; Hesje et al., 2007).

Tetracyclines display a time-dependent killing pattern and exhibit a moderate to prolonged PAE (van Ogtrop et al., 2000; Petersen et al., 2007; Noviello et al., 2008). Thus, AUC/MIC is suggested as the best PK/PD index to reflect their efficacy (Craig, 2007). However, T > MIC versus effect also had a high correlation coefficient (van Ogtrop et al., 2000).

When using AUC/MIC to predict the antimicrobial efficacy, it is assumed that the AUC is measured over a 24 h interval or AUC_{0-24}. The 24 h interval should be at steady state, but if the dosing interval is longer than 24 h, the AUC for the interval covered by the activity of the antimicrobial drug were used, such as 48 or 72 h (Papich, 2014). In general, AUC/MIC ratios are generally recommended at 125 or greater for gram-negative and 30-50 for gram-positive bacteria for high antibacterial efficacy (Hesje et al., 2007). However, these criteria may not fit all situations due to various factors such as host defense mechanism and health status (McKellar et al., 2004). Therefore, the ratios may differ even for the same drug and pathogen.

T > MIC is the time that plasma drug concentration is above MIC. The percentage of T > MIC is dependent on PK parameters such as $t_{1/2}$, CL and V_d. T > MIC should be more than 40-50% to optimize efficacy (Hesje et al., 2007; Mouton et al., 2012; Papich, 2014). As time-dependent killing property, the longer duration of drug concentration

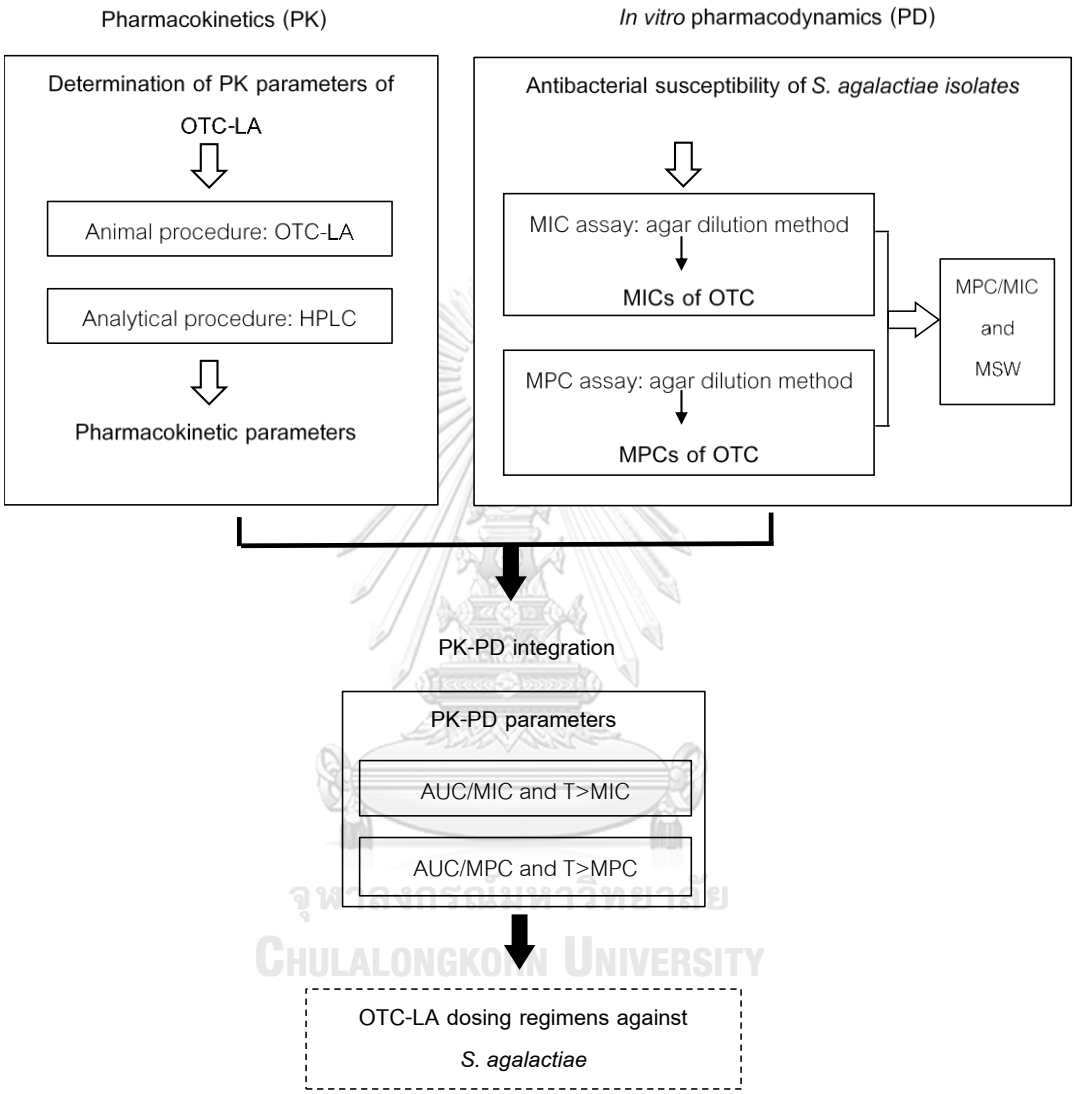
above the MIC the better bacteriological cure the antimicrobial drug can provide (McKellar et al., 2004). Some veterinary drugs are formulated with vehicles in solutions to prolong the absorption, and subsequently the half-life of the drugs to maintain the drug concentration above the MIC for an extended interval, (e.g., long-acting forms of tetracycline) (Papich, 2014).

Regarding the emergence of antimicrobial resistance, increased data from *in vitro* and animal infection models have demonstrated a strong relationship between the magnitude of PK/PD parameters and the prevention of resistance (Drusano, 2003). Although MIC is generally used as a standard to calculate the important PK/PD indices of an antimicrobial drug to get an optimal dosing regimen. It is apparently less appropriate in preventing the emergence of the resistant strains. The use of MPC has been considered. Therefore, the exposure time of drug concentration above MPC (T>MPC) serves as a more important factor that could prevent the selection of antimicrobial drug-resistant mutants (Xu et al., 2013).

CHAPTER III

METHODOLOGY

Conceptual framework



Research instruments and equipment

Instruments and equipment

1. Analytical balance
2. Autoclave machine (Systec, Germany)
3. Biological safety cabinet (BSC) class II
4. C18 column; 150 x 4.6 mm i.d., 5 µm (Symmetry®) (Waters Co., USA)
5. Centrifuge (Andreas Hettich GmbH & Co., Germany)
6. Cryovials and cryoboxes
7. Densitometer (Biosan, Latvia)
8. Disposable sterile spreaders
9. Glass test tubes (13x100 mm) and cap
10. HPLC guard column Inertsil ODS-3 (GL Sciences Inc., Japan)
11. HPLC Shimadzu 10AVP Series (Shimazu, Japan).
12. HPLC vials and inserts
13. Incubator (Memmert, Germany)
14. Lithium heparin blood collection tubes (1ml)
15. Loops
16. Loop sterilizer
17. Micropipettes and micropipette tips
18. Petri dishes
19. Spectrophotometry (Thermo Scientific, Canada)
20. Sterile centrifuge tubes with caps (50 and 1.5 ml)
21. Sterile cotton swabs
22. Sterile #22 and #23 needles
23. Sterile 1 ml syringes
24. Vortex (Scientific Industries, INC., USA)

25. -20°C freezer (Thermo Fisher Scientific, USA)
26. -80°C freezer (Thermo Fisher Scientific, USA)
27. 48-pin replicator (*Sigma* Aldrich, USA)
28. 96-well microplate

Chemicals and reagents

1. Acetonitrile (Fisher Chemical, USA)
2. Anesthetic solution (Aquanes®, Better Pharma, Thailand)
3. Chlortetracycline hydrochloride (Sigma Chemical Co., USA)
4. Glycerol
5. Hydrochloric acid (Merck, Germany)
6. Methanol (Sigma Chemical Co., USA)
7. Mueller Hinton agar (Difco, USA)
8. Mueller Hinton broth (Difco, USA)
9. Oxalic acid dihydrate (KemAus, Australia)
10. Oxytetracycline long-acting preparation (Terramycin®/LA, Zoetis Indonesia Ltd., Indonesia)
11. Oxytetracycline dihydrate (Certified reference standard) (Sigma Chemical Co., USA)
12. Oxytetracycline hydrochloride for microbiological (Sigma Chemical Co., USA)
13. Sodium hypochlorite
14. Sterile sheep blood
15. Trichloroacetic acid (Sigma Chemical Co., USA)
16. Trypticase soy agar (Oxiod Ltd, UK)
17. 95% Ethanol

18. 0.5 McFarland standard solution

Biological isolates

1. *E. coli* ATCC® 25922 (American type culture collection, USA)
2. *S. agalactiae*

Materials and methods

1. Pharmacokinetics of OTC-LA after intraperitoneal injection in tilapia

1.1 Animals

One hundred and twenty healthy male tilapia weighing 400 - 500 g were provided by a Good Agricultural practice (GAP)-certified farm in Chachoengsao province. All fish were kept in a concrete tank and acclimatized to the experimental condition (dissolved oxygen; >5 mg/L, temperature; 28-32 °C, pH; 7-7.5 and ammonia; <0.1 ppm) for 7 days before the study. On the day of the experiment, fish were randomly allocated into two groups (60 fish/group). Each group was kept in a separated tank and allocated into floating cages. Each of twelve fish was stored in a 1 x 1.5 m^2 with a water depth of 1 m floating cage. The fish were fed with commercial dry pellet (antimicrobial-free) twice a day at 3% bodyweight. Throughout the study, water quality parameters including dissolved oxygen, temperature, pH, ammonia and nitrite were monitored daily. All procedures were approved by the Experimental Ethics Committee of Faculty of Veterinary Science, Chulalongkorn University (approval number: 2031098).

1.2 Drugs and chemicals

1.2.1 Animal procedure

Oxytetracycline long-acting (OTC-LA) preparation ((Terramycin®/LA, Zoetis Indonesia Ltd., Indonesia) was used in this experiment. Five percent eugenol (Aquanes®, Better Pharma, Thailand) was used as an anesthetic agent.

1.2.2 Analytical procedure

Oxytetracycline (Certified reference standard) and Chlortetracycline hydrochloride (Certified reference standard) were purchased from Sigma Chemical Co., USA. Acetonitrile (Fisher Chemical, USA) and methanol (Sigma Chemical Co., USA) were HPLC-grade. The analytical grade trichloroacetic acid (Sigma Chemical Co., USA), hydrochloric acid (Merck, Germany) and oxalic acid (KemAus, Australia) were used.

1.3 Experimental design

The fish were randomly allocated into two experimental groups (60 fish/group). Each group was injected intraperitoneally either with OTC-LA at a dosage of 50 or 100 mg/kg bodyweight. Five fish were randomly sampled at each of the following time points after administration: 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 120 and 168 hours, respectively. All fish were anesthetized with 5% eugenol (Aquanes®, Better Pharma, Thailand) before handling. Blood (500 µl) was drawn from the caudal vein of each fish and transferred to heparinized tube and kept in cold container at 4°C. After blood collection, the fish were kept in the resting tanks until fully recover from anesthesia. Blood samples were centrifuged at 2000×*g* at 4°C for 10 min, plasma samples were collected and stored at -80°C until analysis.

1.4 OTC analytical procedure

1.4.1 Standard solution preparation

For the target drug, oxytetracycline; OTC (Sigma Chemical Co., USA), was dissolved in water with 1M hydrochloric acid to prepare stock solution at the final concentration of 2 mg/ml. The standard stock solution was stored at -20°C and protected from light. OTC working solutions were freshly prepared for calibration curve and quality control samples by diluting the stock solution with deionized water.

For internal standard (IS), chlortetracycline hydrochloride; CTC (Sigma Chemical Co., USA), was dissolved in water with 1M hydrochloric acid to prepare stock solution at the final concentration of 1 mg/ml. The standard stock solution was protected from light and kept at -20°C. CTC working solution at the concentration of 210 µg/ml was freshly prepared for calibration curve, quality control samples and sample analysis by diluting the stock solution with deionized water.

1.4.2 Sample extraction

The drug extraction method was modified from Sun et al. (2002); Miller et al. (2007). A 100 µl of each fish plasma sample was added into a 1.5 ml microcentrifuge tube, then spiked with 20 µl of internal standard working solution to reach the final concentration of 30 µg/ml. One hundred microliters acetonitrile and 50 µl 15% trichloroacetic acid were added and mixed by vortex for 30 sec. The tube was centrifuged at 12,000 x *g* 4°C for 15 min. Thereafter the supernatant was collected and kept in HPLC vials, and 20 µl was injected into the HPLC system.

1.4.3 High performance liquid chromatography (HPLC)

In this study, the HPLC procedure was performed using Shimadzu 10AVP Series HPLC System (Shimadzu, Japan). The chromatographic separation was achieved using Symmetry® C18 column (150 x 4.6 mm i.d., 5 µm) (Waters Co., Ltd., USA) with a Inertsil ODS-3 guard column (10 x 4 mm i.d., 5 µm) (GL Sciences Inc., Japan). The column was maintained at ambient temperature throughout the analysis.

The HPLC procedure was modified from Poapolathep et al. (2017). The mobile phase used in the binary gradient of elution was composed of 0.01M oxalic acid in deionized water (A) and acetonitrile: methanol (80:20 v/v) (B). The flow rate was 1.2 ml/min. The gradients were as follow: 0-1 min, 100% A; 1–6 min, from 100% to 70% A; 6–

9 min, kept at 70% A; 9-15 min, from 70% to 100% A, followed by re-equilibration at 100% A until 18 min. OTC was detected at wavelength of 360 nm.

1.4.4 Calibration curve

The quantitative determination was performed by the internal standard calibration that was prepared by spiking the same concentration of IS into a series of concentration of OTC standard solutions in blank plasma.

For the preparation of the calibration curve, blank plasma (80 µl) was spiked with 20 µl of OTC working solutions to reach the final concentrations of 0.1, 1, 10, 20, 40, 80, 160 and 320 µg/ml, respectively, then 20 µl of IS working solution was added to each dilution. The calibration curves were freshly prepared in every batch run of sample. Calibration curve was constructed using analytes/internal standard peak area ratio versus concentration of the analytes. The coefficient of determination (R^2) of calibration curve was determined.

The data was evaluated from peak area of the acquired chromatograms by using Lab solution software version 5.82 SP1. The concentration of each sample was calculated from the linear equation by using regression analysis of calibration curve as a weighting factor (1/C). The linear equation of calibration curve was as followed.

$$y = ax + b$$

y: peak area ratio

a: slope

x: concentration

b: intercept

The back-calculated concentrations were determined from the value of peak area ratios, intercept, and slope according to the following equation:

$$\text{Concentration} = \frac{\text{Peak area ratio - intercept}}{\text{slope}}$$

1.5 Method validation procedure

The analytical method was validated according to USFDA guideline (USFDA, 2018)

1.5.1 Selectivity

Method selectivity assessed interferences that may be caused by the matrix when using this method. The experiment was performed by analyzing blank plasma samples from six individual sources. Blank and zero calibrators were free of interference at the retention times of the analytes and the IS. The results were expressed as % interference. For the acceptance criteria, the response of interfering peaks at retention time of the drug peak were ≤ 20% of the response of the lower limit of quantification (LLOQ) sample. The response of interfering peaks at retention time of the IS peak were ≤ 5% of the response of the LLOQ sample.

1.5.2 Lower limit of quantification (LLOQ)

The LLOQ defines the method sensitivity. LLOQ is the lowest number of analytes in sample which can be quantified for acceptable accuracy and precision. Five replications of LLOQ were measured and calculated as mean and standard deviation (SD). The accuracies were within 80-120% of nominal concentration and the precisions were ≤ 20% of the coefficient of variation (CV).

1.5.3 Linearity and reproducibility of calibration curve

To evaluate linearity and reproducibility of calibration curve, three sets of calibration standards (8 concentrations) were examined. A linear regression equation was constructed and the R^2 value was calculated for each calibration curve. The reproducibility for calibration curve was determined on three different days. Calibration curve was processed and run along with each batch of samples that was analyzed on consecutive days using freshly prepared solution each day. The linearity of calibration curve was presented by the R^2 value that was ≥ 0.99. Back calculation of all standard concentrations was ±15% of nominal concentrations, except at LLOQ where the concentration was ±20% of nominal concentrations. Percent CV of the slope and R^2 from the three sets were not greater than 15%. At least 75% of the calibration curves with a minimum of six calibration standards met the criteria in each validation run.

1.5.4 Accuracy and precision

For accuracy and precision, quality control (QC) samples including LLOQ, low (LQC: defined as three times the LLOQ), medium (MQC: defined as mid-range), and high (HQC: defined as high range) were tested. To prepare the QC samples, blank plasma samples were spiked with OTC standard at four different concentrations to reach the concentrations of 0.1 µg/ml (LLOQ), 0.3 µg/ml (LQC), 80 µg/ml (MQC) and 160 µg/ml (HQC). Five replications of QC samples were analyzed (n=5) in each batch run. All QC samples were freshly prepared for each batch run. The analyzed concentrations were compared with nominal concentrations for the intra-day accuracy. The inter-day accuracy was presented by % accuracy of QCs analyzed in three different days. The acceptance criteria for accuracy were within 85-115% of nominal concentrations, except at LLOQ where the concentration was within 80-120% of nominal concentrations. The precision was determined from QCs and calculated for %CV. The intra-day precision was presented

by %CV of five replications of QCs analyzed within the same day. The inter-day precision was presented by %CV of QCs analyzed in three different days. Percent CV within-day and between days was more than 15%, except at LLOQ which was ±20% CV. Percentage of accuracy and precision (% CV) were calculated by the following equation:

$$\% \text{ Accuracy} = \frac{\text{Mean of measured value}}{\text{Nominal values}} \text{ X } 100$$

$$\text{Precision (\%CV)} = \frac{\text{Standard deviation of measured value}}{\text{Mean of measured value}} \text{ X } 100$$

1.5.5 Recovery

To evaluate the recovery of extraction, the experiments were performed by comparing the detector response of pre-extracted samples with detector response of blanks spiked with the analyte post-extraction. Three QC concentrations (LQC, MQC and HQC) were determined for five replications. For pre-extraction, QC samples (spiked OTC and IS) were added to the mixture for protein precipitation before being centrifuge and collect supernatant for analysis. For post-extraction, blank plasma was added to the mixture for protein precipitation, centrifuge. Then, the supernatant was spiked with OTC and IS before analysis. Recovery (%) was calculated by the following equation:

$$\text{Recovery (\%)} = \frac{\text{Mean of peak area of pre-extracted samples}}{\text{Mean of peak area of post-extracted samples}} \text{ X } 100$$

1.6 Pharmacokinetic analysis

The drug concentration data used for PK analysis was a mean concentration of 5 fish (n=5) at each timepoint. Pharmacokinetics of the drug concentration-time data were analyzed using STATA® software version 15.1 (StataCorp LLC, USA). The data were

applied to non-compartmental model. The graphs of drug concentration (Y-axis) and time (X-axis) were plotted. PK parameter including C_{max} (peak plasma concentration), T_{max} (time of peak concentration), AUC (area under the plasma drug concentration-time curve), K_{el} (elimination rate constant) and $t_{1/2}$ (half-life) were calculated. The AUC in this study was determined using trapezoidal rule with linear-up and log-down. $AUC_{0-\infty}$ was extrapolated from the last three point of concentration. Apparent volume of distribution after non-intravenous administration (V_d/F), apparent total body clearance (CL/F) and mean residence time (MRT) were calculated by following equations (Riviere, 2011).

$$CL/F = \frac{Dose}{AUC_{0-\infty}}$$

$$V_d/F = \frac{Dose}{AUC_{0-\infty} \cdot K_{el}}$$

$$MRT = \frac{\int t \cdot C(t)\, dt}{\int C(t)\, dt} = \frac{AUMC}{AUC}$$

MRT was calculate using the area under the first moment of the plasma drug concentration-time curve (AUMC). The first moment was calculated as concentration times time (C * t). The AUMC was calculated using the trapezoidal rule of the area under the concentration times time versus time curve. The last segment for the $AUMC_{0-\infty}$ curve was calculated by following formula (Riviere, 2011).

$$\frac{C(last) \cdot t(last)}{K_{el}} + \frac{C(last)}{K_{el}^{2}}$$

1.7 Data presentation and statistics

The plasma drug concentrations were reported as the mean ± standard error of the mean (SEM) (n=5). Graphs of plasma drug concentration-time were constructed using GraphPad Prism version 5 (GraphPad Software, USA)

2. Pharmacodynamics of OTC against *Streptococcus agalactiae*

2.1 Bacterial isolates

Fifty-six *S. agalactiae* isolates used in this study were obtained from diseased tilapia in different farming areas of central Thailand from 2018 to 2019. All isolates were previously identified using API microorganism identification test kits (Biomerieux, Marcy L' Etoile, France) and genetically confirmed using polymerase chain reaction (PCR) (Zlotkin et al., 1998; Martinez et al., 2001) by Aquatic Animal Medicine Division, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University.

All samples were stored in freezing broth containing 20% glycerol at -80°C. Before each experiment, stocked bacterial isolates were transferred onto tryptic soy agar (TSA) supplemented with 5% sheep blood and incubated at 30°C for 24 h. Colonies from the pure culture were randomly selected for the procedure thereafter. All procedures were approved by the Biosafety Committee of Faculty of Veterinary Science, Chulalongkorn University (approval number: 2031057).

2.2 OTC stock solution

OTC stock solution at 10 mg/ml was prepared by dissolving oxytetracycline hydrochloride (Sigma Chemical Co., USA) in sterile distilled water. The stock solution was protected from light, stored at -20°C and used within 2 months. OTC stock solution was further diluted and added to Mueller–Hinton agar (MHA) supplemented with 5% sheep blood at different concentrations to be used in MIC and MPC assay.

2.3 Minimum inhibitory concentration (MIC) determination

2.3.1 Preparation of OTC-containing agar plates

OTC stock solution was diluted and added to MHA supplemented with 5% sheep blood to achieve concentrations of OTC at 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/ml, respectively. Five percent sheep blood agar plates without OTC were also prepared as growth control plates. Both OTC-containing agar plates and growth control plates were protected from light and stored at 4 °C and used within 7 days.

2.3.2 Preparation of bacterial isolates

Stock bacterial isolates were transferred onto tryptic soy agar (TSA) supplemented with 5% sheep blood and incubated at 30°C for 24 h. Colonies from the pure culture were randomly selected for MIC procedure.

2.3.3 MIC procedure

MIC of OTC was determined with agar dilution technique as described by the CLSI (2018). Bacterial suspensions were prepared in 0.85% saline solution and adjusted the turbidity to 0.5 McFarland standard (approximately $1x10^8$ cfu/ml) using densitometer. The bacterial suspension was then 10-fold diluted in a sterile 96-well microplate by pipetting 10 µl of bacterial suspension into a well containing 90 ul of sterile Mueller–Hinton broth (MHB). The inoculum was transferred to the blood MHA plates containing a range of OTC concentrations using a 48-pin replicator. The sterile replicator was placed into the microplate to soak the pins and transfer around 2 µl of bacterial suspension onto the blood agar plates. The final inoculum on the agar contains approximately $2x10^4$ cfu/spot (Wiegand et al., 2008). Inoculation was done with a growth control plate without antibiotic first, followed by the plates containing OTC from the lowest concentration to the highest concentration. A second control agar plate was the last plate to be inoculated to ensure that no contamination or antimicrobial agent were carried over during the inoculation.

2.3.4 Quality control procedure

To verify that the inoculum size was appropriate, viable count of the bacterial suspension used for preparing the initial inoculum was performed by spread plate technique using 10-fold serial dilution of bacterial suspension. All agar plates were incubated at 30 °C for 24-48 h. All isolates were performed in triplicates with *E. coli* ATCC 25922 as a quality control isolate (Chideroli et al., 2017; de Oliveira et al., 2018).

2.3.5 MIC detection and Interpretation

The lowest concentration of OTC that completely inhibits colony formation of each isolate was recorded as MIC. MIC range, MIC_{50} and MIC_{90} were calculated. MICs of OTC were interpreted based on MIC breakpoints. Due to the lack of specific breakpoints of OTC for *S. agalactiae* in fish, the breakpoints were extrapolated from terrestrial animal species and humans. MIC breakpoints for Streptococcus group B isolates are susceptible (S) at MIC $\leq$ 2, intermediate (I) at 4 and resistance (R) at MIC $\geq$ 8 µg/ml (CLSI, 2018).

2.4 Mutant prevention concentration (MPC) determination

2.4.1 Preparation of OTC-containing agar plates

OTC stock solution was diluted and added to MHA supplemented with 5% sheep blood to achieve of OTC at concentrations at 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/ml, respectively. Growth control plates without OTC were prepared. All agar plates were stored at 4 °C and used within 7 days.

2.4.2 Preparation of bacterial isolates

Each stock of *S. agalactiae* isolates was cultured onto 3 plates of TSA supplemented with 5% sheep blood and incubated at 30°C for 24 h. All pure bacterial

colonies from 3 plates were transferred and cultured in MHB for 24 hours to prepare very large inoculum (10^{10} cfu/ml) of bacterial suspension for MPC procedure.

2.4.3 MPC procedure

MPC was determined by methods described by Blondeau (2009) with modification. The bacterial suspension was centrifuged at 5000 x *g* for 30 minutes at 4 °C. The pellets were resuspended in small volume of fresh cold MHB and adjusted cell density to approximately 10^{10} cfu/ml using spectrophotometry (Thermo Scientific, Canada) with absorbance reading ≥ 1 at 600 nm. One hundred microlitres of bacterial suspension, containing more than 10^{10} cfu/ml, were plated onto MHA containing 5% sheep blood plates supplemented with OTC at concentrations equal to 1x, 2x, 4x, 8x, 16x, 32x, 64x 128x 256x and 512x MIC. Inoculated plates were incubated at 30°C for 48 h and then screened for growth. All plates were re-incubated for an additional 24 h and reexamined. All MPC determinations were made in duplicates.

2.4.4 Quality control procedure

Viable count was performed in each inoculum using serial dilution and spread plate method to confirm bacterial culture concentration more than 10^{10} cfu/ml.

2.4.5 MPC detection and interpretation

MPC of each isolate was recorded as the lowest OTC concentrations that allow no bacterial growth. MPC values form all isolates were reported as MPC range, MPC_{50} and MPC_{90}. The ratios of MPC/MIC were calculated by dividing MPC_{50} and MPC_{90} with MIC_{50} and MIC_{90}, respectively.

2.5. Data analysis

Descriptive statistics were used to describe percentages and frequencies of the results including MIC_{50}, MIC_{90}, MPC_{50} and MPC_{90}. The ratios of MPC/MIC were calculated

and MSW results were presented as the range of 50th and 90th percentile of MIC and MPC. Correlation between MIC and MPC was analyzed using a scattered plot and linear regression analysis by SPSS Statistics 22 (IBM Co, USA). Graphs were plotted using software GraphPad Prism version 5 (GraphPad Software, USA).

3. Pharmacokinetic/Pharmacodynamic (PK/PD) analysis of OTC-LA against *S. agalactiae*

3.1 MSW integrated with plasma OTC concentration-time curve

To plot MSW graphs, plasma OTC concentration-time curves and PD data as MIC and MPC values were applied (Blondeau, 2009). In this study, MIC and MPC were drawn onto an OTC plasma concentration-time curves of OTC-LA at both 50 mg/kg and 100 mg/kg dosages. MSW was the range of OTC concentrations between MIC and MPC.

3.2 PK/PD ratios

For the PK-PD approach of OTC, it is well established that tetracyclines display a time-dependent action with considerable post antibiotic effect (PAE). Thus, important PK-PD indices are ratio of area under the plasma drug concentration-time curve to minimum inhibitory concentration (AUC/MIC) and time that plasma drug concentration above MIC (T>MIC) (van Ogtrop et al., 2000). From general PK/PD target ratios of tetracyclines, AUC/MIC ratio should be greater than 30-50 whereas T>MIC ratio should be greater than 50% (Hesje et al., 2007). Due to the intention of the use of OTC-LA formulation is to minimize animal handling by using less frequency of drug administration so duration of treatment should be longer than 24 h interval. Thus, AUC_{0-168} was used instead of AUC_{0-24} according to the suggestion by Papich (2014). In this study, MIC_{50} and MIC_{90} of OTC against *S. agalactiae* isolates were used to calculate the AUC/MIC and T>MIC ratios by using the pharmacokinetic data of OTC-LA. These PK-PD parameters were used to determine the OTC-LA dosage in tilapia. Moreover, MPC_{50} and MPC_{90} were integrated

along with PK values, yielding AUC/MPC and T>MPC to evaluate the probability of OTC-LA dosing regimen to prevent the resistant mutant bacteria.

CHAPTER IV

RESULTS

1. Pharmacokinetics of OTC-LA after intraperitoneal injection in tilapia

1.1 Animals

One hundred and twenty healthy male tilapia used in this study weighed 450.00±37.47 g. Water quality parameters that monitored throughout the study were shown in Table 1. All fish did not show any adverse effects after OTC-LA administration either at a dosage of 50 or 100 mg/kg bodyweight.

Table 1: Water quality parameters

Parameters	Temperature (°C)	Dissolved oxygen (mg/l)	Nitrite (ppm)	Ammonia (ppm)	pH
Range	28.1 - 29.3	5.90 - 7.6	0	0	7 - 7.5
Average	28.71	6.40	0	0	7.36
SD	0.26	0.55	0	0	0.23
Reference values	28 – 32	>5	0	<0.1	7 - 7.5

1.2 OTC analytical procedure

1.2.1 HPLC analytical protocol

In our HPLC system, the retention time of OTC and CTC were 10.39 min and 13.05 min, respectively. Typical chromatograms of blank tilapia plasma, tilapia plasma spiked with OTC and tilapia plasma spiked with CTC were shown in Figures 1-3.

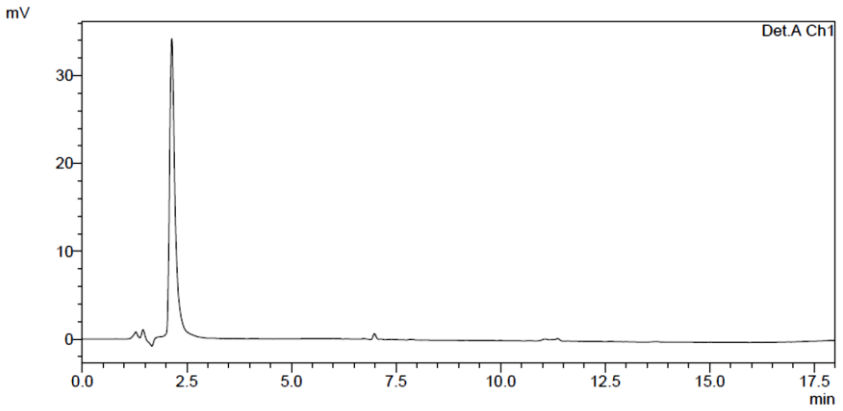


Figure 1: Chromatogram of blank tilapia plasma

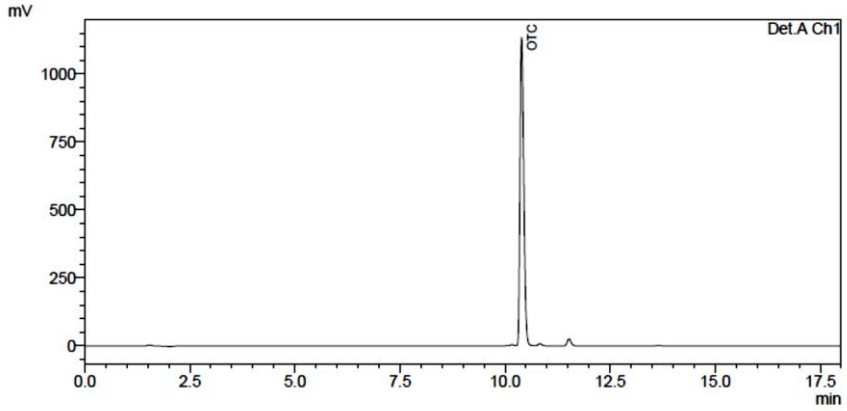


Figure 2: Chromatogram of tilapia plasma spiked with OTC, retention time = 10.39 min

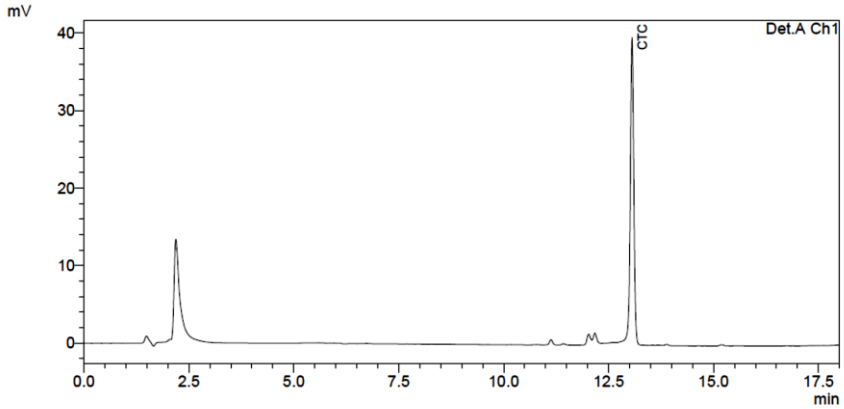


Figure 3: Chromatogram of tilapia plasma spiked with CTC, retention time = 13.05 min

1.2.2 Calibration curve

In this study, 8 OTC standard concentrations ranging from 0.1 - 320 µg/ml were used to conduct the calibration curve. Peak area ratio and the concentration of OTC showed a linear relationship over the tested concentrations. The standard calibration curve equation of OTC was y = (0.086542) X + 0.001965139 (R^2 = 0.99997) as showed in Figure 4.

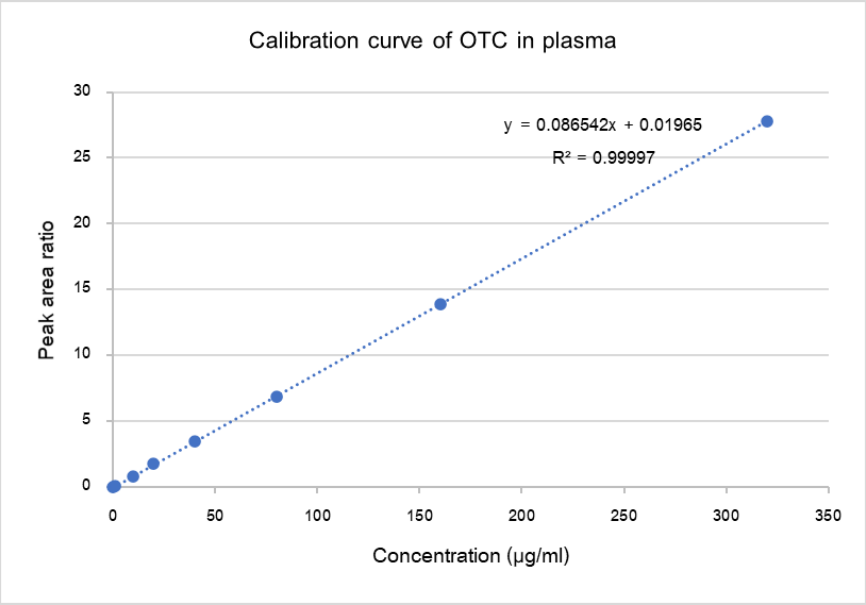


Figure 4: Calibration curve of OTC in plasma

1.3 Method validation

1.3.1 Selectivity

The selectivity analysis of blank plasma samples from six individual sources demonstrated that no significant interferences from endogenous components were observed at the retention time of OTC and CTC.

1.3.2 Linearity and reproducibility of calibration curve

In this study, eight OTC standard concentrations were evaluated for linearity. Linear equation was judged to produce the best fit for the concentration versus area response relationship. The weighing factor was 1/C. The method was successfully

validated over a range of 0.1 to 320 µg/ml for OTC in tilapia plasma. The reproducibility for calibration curve determined on three different days, demonstrated that the values were within the acceptance range (Table 2).

1.3.3 Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was 0.1 µg/ml. From the evaluation of five replications of LLOQ, the results showed that the values were within the acceptance criteria (80-120% of nominal concentration) and the precisions (% coefficient of variance or %CV) were ≤ 20% (Table 3).

1.3.4 Accuracy and precision

Accuracy and precision were determined at LLOQ, low, medium and high concentrations of QC samples, based on the expected range. The results were expressed in % accuracy and %CV as presented in Tables 3 and 4. Intra-day accuracies ranged from 85.718% to 105.620% and Inter-day accuracies ranged from 97.222% to 99.938%. Intra-day precisions ranged from 2.145% to 5.822% and Inter-day precisions ranged from 3.630% to 11.381%.

Table 2: Calibration curve summary with back-calculated concentrations of OTC in plasma

Run date	Parameter	Back-calculated concentrations of OTC in plasma (µg/ml)								Equation of calibration curve	R^2
		CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8		
Day 1	Back cal.	0.109	0.950	9.994	20.105	40.098	80.868	158.798	320.268	Y = 0.081611X -	0.99996
	% Accuracy	108.702	94.982	99.945	100.523	100.246	101.085	99.249	100.084	0.00134162	
Day 2	Back cal.	0.111	0.914	9.587	21.042	38.885	79.149	161.246	320.166	Y = 0.091905X +	0.99979
	% Accuracy	110.555	91.441	95.868	105.208	97.213	98.936	100.779	100.052	0.00450608	
Day 3	Back cal.	0.100	1.010	10.258	19.037	39.685	80.448	162.406	318.154	Y = 0.085550X +	0.99983
	% Accuracy	100.042	101.043	102.581	95.185	99.212	100.559	101.504	99.423	0.00292195	
Average of % accuracy		106.433	95.822	99.465	100.305	98.890	100.193	100.511	99.853	-	-
SD		5.612	4.856	3.382	5.015	1.542	1.120	1.151	0.373	-	-
%CV		5.273	5.068	3.400	5.000	1.559	1.118	1.145	0.373	6.01450*	-
Nominal value (µg/ml)		0.1	1	10	20	40	80	160	320	-	-

Acceptance range: % Accuracy should be within 85-115% for all standards except CC1 (LLOQ) within 80-120%, $R^2 \geq 0.99$, %CV $\leq$ 15%

*Calculated from the slope of equation

Table 3: The intra-day accuracies and precisions

Run date	Concentration of OTC in plasma (µg/ml)			
	LLOQ	LQC	MQC	HQC
Day 1	0.087	0.281	118.155	249.421
	0.086	0.274	133.292	259.146
	0.081	0.284	131.380	258.560
	0.083	0.289	127.647	242.131
	0.092	0.288	134.517	242.214
Mean	0.086	0.283	128.998	250.294
SD	0.004	0.006	6.595	8.357
Precision (%CV)	4.754	2.146	5.112	3.339
Nominal value (µg/ml)	0.100	0.300	130.000	260.000
% Accuracy	85.718	94.394	99.229	96.267
Day 2	0.100	0.280	127.522	261.302
	0.108	0.288	123.935	263.211
	0.106	0.273	134.002	250.837
	0.105	0.277	123.646	256.464
	0.109	0.285	121.297	266.828
Mean	0.106	0.281	126.081	259.728
SD	0.03	0.06	4.956	6.220
Precision (%CV)	3.080	2.145	3.931	2.395
Nominal value (µg/ml)	0.100	0.300	130.000	260.000
% Accuracy	105.620	93.559	96.985	99.896
Day 3	0.105	0.331	128.014	286.729
	0.111	0.323	134.887	271.549
	0.100	0.294	135.218	278.208
	0.104	0.305	140.011	263.671

Run date	Concentration of OTC in plasma (μg/ml)			
	LLOQ	LQC	MQC	HQC
	0.105	0.304	134.960	245.587
Mean	0.105	0.311	134.681	269.149
SD	0.004	0.015	4.279	15.669
Precision (%CV)	3.832	4.864	3.179	5.822
Nominal value (μg/ml)	0.100	0.300	130.000	260.000
% Accuracy	105.083	103.762	103.552	103.519

LLOQ: Lower limit of quantification, LQC: low concentration quality control sample, MQC: medium concentration quality control sample, HQC: high concentration quality control sample

Table :4 The inter-day accuracies and precisions

Run date	Concentration of OTC in plasma (μg/ml)			
	LLOQ	LQC	MQC	HQC
Mean of day 1	0.086	0.283	128.998	250.294
Mean of day 2	0.106	0.281	126.081	259.728
Mean of day 3	0.105	0.311	134.681	269.149
Mean	0.099	0.291	129.920	259.724
SD	0.0113	0.017	4.3735	9.428
Precision (%CV)	11.383	5.751	3.366	3.630
Nominal value (μg/ml)	0.100	0.300	130.000	260.000
% Accuracy	99.000	97.222	99.938	99.894

LLOQ: Lower limit of quantification, LQC: low concentration quality control sample, MQC: medium concentration quality control sample, HQC: high concentration quality control sample

1.3.5 Recovery

The recovery was determined by comparing the detector response of pre- and post-extracted QC samples. In this study, the mean recoveries of the low, medium and high QC samples of OTC in plasma were 84.527 %, 87.943 % and 94.885 %, respectively. For CTC (IS), average recovery was 85.115%. The results demonstrated high % recoveries in plasma. (Table 5).

Table 5: The recoveries of OTC and CTC in tilapia plasma

QC sample	Area of pre-extracted sample		Area of post-extracted sample		% Recovery	
	TS	IS	TS	IS	TS	IS
LQC1	3753	174009	3531	192010	84.527	85.115
LQC2	3683	175176	4323	205033		
LQC3	3753	171897	4214	194428		
LQC4	3500	157373	4779	189451		
LQC5	3584	161642	4771	196450		
Mean	3654.600	-	4323.600	-		
SD	110.744	-	511.806	-		
%CV	3.030	-	11.838	-		
MQC1	1638750	169993	1907274	200537	87.943	
MQC2	1534824	141130	1744151	185813		
MQC3	1743208	162624	1722167	180665		
MQC4	1536610	147543	1959301	188215		
MQC5	1697693	154684	1935738	176029		
Mean	1630217.000	-	1853726.200	-		

QC sample	Area of pre-extracted sample		Area of post-extracted sample		% Recovery	
	TS	IS	TS	IS	TS	IS
SD	93881.419	-	111863.631	-		
%CV	5.759	-	6.035	-		
HQC1	3342103	164220	3295353	190841	94.885	
HQC2	3246662	153543	3455538	200770		
HQC3	3107336	147287	3670320	183248		
HQC4	3342103	169164	3646002	205273		
HQC5	3640804	184220	3510964	171484		
Mean	3335801.600	162300.333	3515635.400	190683.133		
SD	195734.692	12060.247	152545.592	10131.335		
%CV	5.868	7.431	4.339	5.313		

TS: Test standard (Oxytetracycline), IS: Internal standard (Chlortetracycline)

LLOQ: Lower limit of quantification, LQC: low concentration quality control sample, MQC: medium concentration quality control sample, HQC: high concentration quality control sample

1.4 OTC concentrations in tilapia plasma

The plasma OTC concentration at each time point was the average plasma OTC concentration from 5 fish. At 50 mg/kg, the peak OTC concentration (C_{max}) was 110.698 ± 5.614 µg/ml found at 2 h after administration (Table 7). Plasma OTC concentration-time curve showed rapid absorption since drug concentrations were detected within 15 minutes after administration as showed in Figure 5. The plasma OTC depleted rapidly in the first 24 h, followed by moderated depletion until 48 h post-administration. Thereafter, OTC in plasma slowly declined and remained detectable at 168 h (7 day) after administration.

For the dosage of 100 mg/kg, the peak OTC concentration of 287.848 ± 8.028 µg/ml was reached at 4 h after administration. Plasma OTC concentration-time curve pattern appeared to be similar to that of the lower dosage. OTC in plasma was still detectable at 168 h (7 day) after administration, at the higher level of that detected from the lower dosage.

Table 6: OTC plasma concentrations (μg/ml) following OTC-LA IP administration at the dosages of 50 and 100 mg/kg bodyweight at different time points in Nile tilapia (mean ± SEM, n = 5).

Time (h)	OTC plasma concentrations (μg/ml)	
	Dose 50 mg/kg	Dose OTC 100 mg/kg
0	0	0
0.25	59.729 ± 5.228	131.576 ± 10.381
0.5	74.060 ± 6.215	217.772 ± 10.367
1	92.080 ± 5.491	222.704 ± 11.466
2	110.698 ± 5.614	244.166 ± 22.988
4	76.148 ± 4.472	287.848 ± 8.028
8	58.199 ± 5.042	242.492 ± 16.458
12	44.210 ± 3.217	197.537 ± 15.330
24	28.088 ± 2.545	133.019 ± 4.170
48	17.601 ± 1.318	62.082 ± 3.359
72	15.588 ± 1.165	50.760 ± 3.678
120	7.743 ± 0.726	28.291 ± 2.220
168	3.998 ± 0.476	23.004 ± 2.507

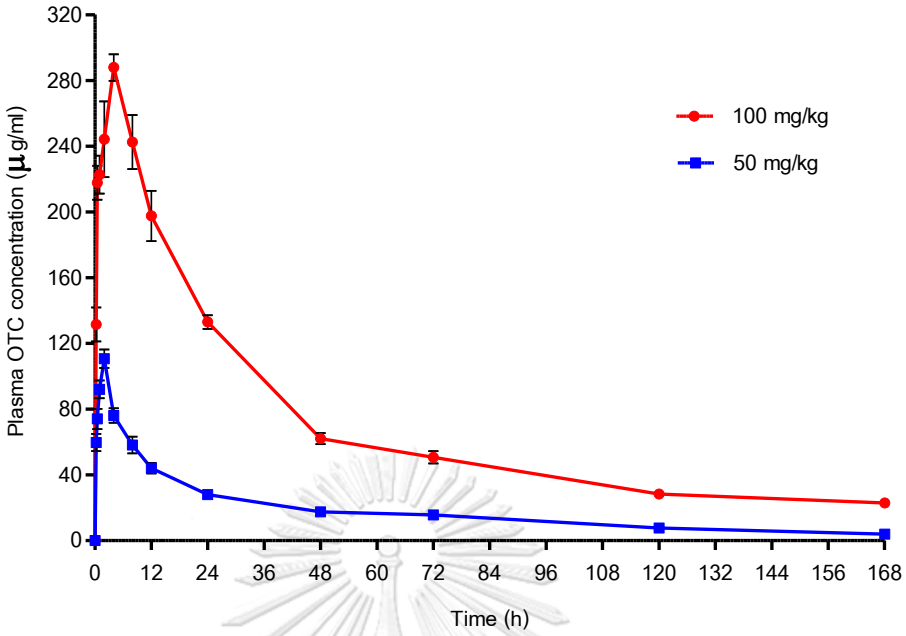


Figure 5: Oxytetracycline concentrations in tilapia plasma after IP administration with OTC-LA at 50 and 100 mg/kg bodyweight.

1.5 Pharmacokinetics of OTC-LA after single IP administration in tilapia

Pharmacokinetic parameters of the drug concentration-time data were analyzed using STATA® software program version 15.1 (StataCorp LLC, USA). The data were applied to non-compartmental model. PK parameters of OTC-LA after IP administration at the dosages of 50 and 100 mg/kg bodyweight were reported in Table 7.

Peak plasma OTC concentrations of OTC-LA at 100 mg/kg showed more than two times higher than that of 50 mg/kg. Time of peak concentration (T_{max}) of OTC-LA at the dosage of 100 mg/kg IP was twice of that of 50 mg/kg. The area under the plasma drug concentration-time curve (AUC) including AUC_{0-24} AUC_{0-168} and $AUC_{0-\infty}$ of the dosage of 100 mg/kg were ≥ 3 times higher than those of 50 mg/kg. On the contrary, the apparent volume of distribution after non-intravenous administration (V_d/F), elimination rate constant (*Kel*), and apparent total body clearance (CL/F) of OTC at the dosage of 100 mg/kg were lower than those of 50 mg/kg. However, the elimination half-life ($t_{1/2}$) of OTC at the dosage

of 100 mg/kg was almost twice of those of 50 mg/kg. The mean residence time from 0 to 168 h (MRT_{0-168}) of two OTC-LA doses were similar, while the mean residence time from 0 to infinity ($MRT_{0-\infty}$) was higher with OTC-LA at 100 mg/kg.

Table 7: Pharmacokinetic parameters of OTC-LA after IP injection in Nile tilapia

PK parameters	Unit	Dosages	
		50 mg/kg	100 mg/kg
C_{max}	µg/ml	110.699 ± 5.614	287.848 ± 8.028
T_{max}	h	2	4
AUC_{0-24}	µg·h/ml	1248.934	4828.652
AUC_{0-168}	µg·h/ml	2995.304	11483.991
$AUC_{0-\infty}$	µg·h/ml	3277.303	14274.239
V_d/F	ml/kg	1074.398	849.755
CL/F	ml/h/kg	15.256	7.006
$t_{1/2}$	h	48.897	84.076
Kel	1/h	0.0142	0.0082
MRT 0-168	h	46.785	47.473
MRT 0-inf	h	63.240	94.743

AUC_{0-24}: area under the plasma drug concentration-time curve from 0 to 24 h, AUC_{0-168}: area under the plasma drug concentration-time curve from 0 to 168 h, $AUC_{0-\infty}$: area under the plasma drug concentration-time curve from 0 to infinity, C_{max}: peak plasma concentration, t_{max}: time of peak concentration, $t_{1/2}$: elimination half- life, *Kel*: elimination rate constant, V_d/F: Apparent volume of distribution after non-intravenous administration, CL/F: apparent total body clearance, MRT_{0-168}: mean residence time from 0 to 168 h, $MRT_{0-\infty}$: mean residence time from 0 to infinity

2. Pharmacodynamics of OTC against *Streptococcus agalactiae* (*S. agalactiae*)

2.1 Bacterial isolates

Fifty-six *S. agalactiae* isolates obtained from diseased tilapia in different farming areas of central Thailand during 2018 to 2019 were distributed geographically as presented in Table 8.

Table 8: Geographic distribution of the *S. agalactiae* sample sources

Province of sample sources	Number of isolates
Samutprakarn	18
Prachinburi	16
Chachoengsao	15
Nakhon Nayok	3
Phetchaburi	2
Ratchaburi	2

2.2 Minimum inhibitory concentration (MIC) of OTC against *S. agalactiae*

MICs of OTC in all 56 clinical *S. agalactiae* isolates ranged from 0.5 to 2 µg/ml. MIC distribution of OTC against *S. agalactiae* is presented in Figure 6. The most frequent MIC of OTC was 0.5 µg/ml (n= 47/56), while MIC_{50} and MIC_{90} were 0.5 µg/ml and 1 µg/ml, respectively.

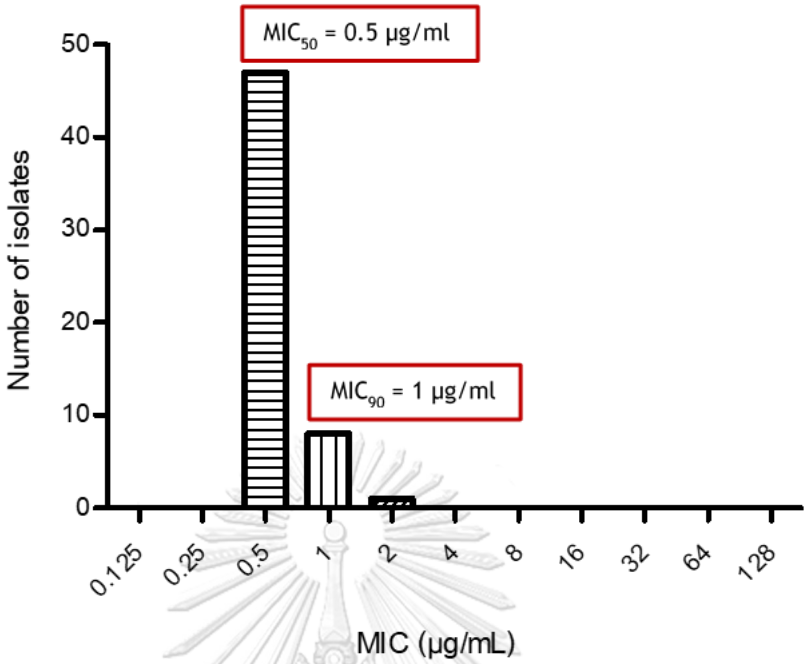


Figure 6: Distribution of MIC

MICs of OTC were interpreted based on MIC breakpoints. Due to the lack of specific breakpoints of OTC for *S. agalactiae* in fish, the MIC breakpoints in this study were extrapolated from terrestrial animal species and human as follows: susceptible (S) at MIC ≤ 2, intermediate (I) at 4 and resistance (R) at MIC ≥ 8 µg/ml (CLSI, 2018). Therefore, based on these MIC breakpoints, all 56 *S. agalactiae* isolates in our study were susceptible to OTC. MIC results are summarized and presented in Table 9.

2.3 Mutant prevention concentration (MPC) of OTC against *S. agalactiae*

MPC of OTC in all 56 clinical *S. agalactiae* isolates were in the ranges from 4 to 512 µg/ml. MPC distribution was presented in Figure 7. MPC of OTC at 32 µg/ml was the most frequency (n=17/56), with MPC_{50} and MPC_{90} at 32 µg/ml and 128 µg/ml, respectively.

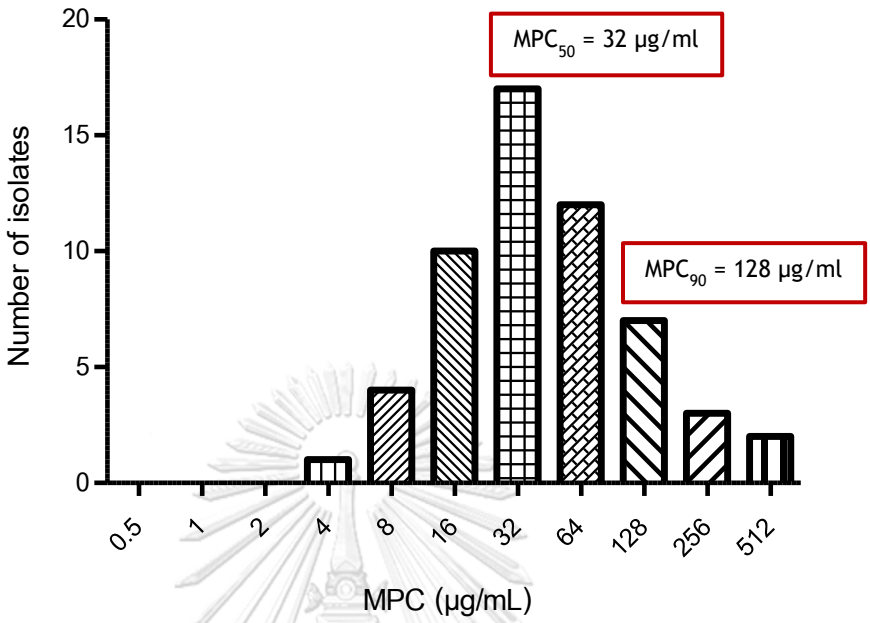


Figure 7: Distribution of MPC

Currently, MPC breakpoints have not been established, therefore susceptible and resistant MPC breakpoints were applied based on MIC breakpoints. In this study, almost all *S. agalactiae* isolates (n=55/56) showed MPC ≥ 8 µg/ml, accounted for 98.21% OTC resistance, with none exhibited susceptible MPC. (Table 9).

2.4 MPC/MIC ratio and MSW

From all clinical *S. agalactiae* isolates, MPC_{50}/MIC_{50} of OTC was 64 (32/0.5), while MPC_{90}/MIC_{90} was 128 (128/1) consecutively. MSW_{50} (MSW from 50^{th} percentile of MIC and MPC) ranged from 0.5 to 32 µg/ml. While at 90^{th} percentile, MSW_{90} ranged from 1 -128 µg/ml (Table 9).

Table 9: MICs, MPCs and MPC/MIC ratios and MSW of OTC against clinical *S. agalactiae* (n=56)

PD parameters		Values
MIC (μg/ml)	Range	0.5 - 2
	MIC_{50}	0.5
	MIC_{90}	1
MPC (μg/ml)	Range	4 - 512
	MPC_{50}	32
	MPC_{90}	128
MPC/MIC	MPC_{50}/MIC_{50}	64
	MPC_{90}/MIC_{90}	128
MSW	MSW_{50}	0.5 - 32
	MSW_{90}	1 - 128

2.5 Correlation of MIC and MPC of OTC against *S. agalactiae*

Correlation between MICs and MPCs of OTC against *S. agalactiae* isolates evaluated using a scattered plot and linear regression analysis was showed in Figure 8. The correlation coefficient (R^2) value was very low (0.229), indicating poor correlation between MICs and MPCs in this study.

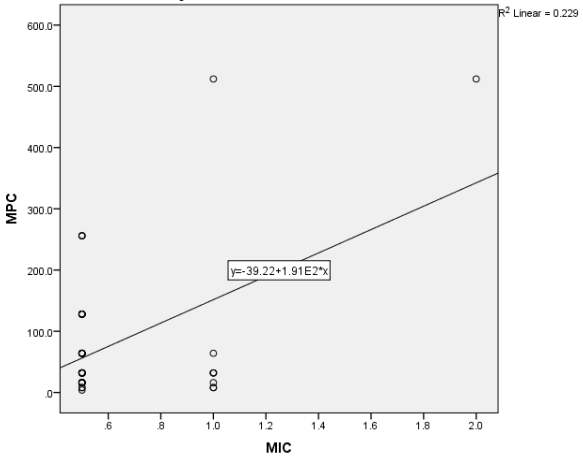


Figure 8: Scatter plot and linear regression analysis of relationship between MIC and MPC

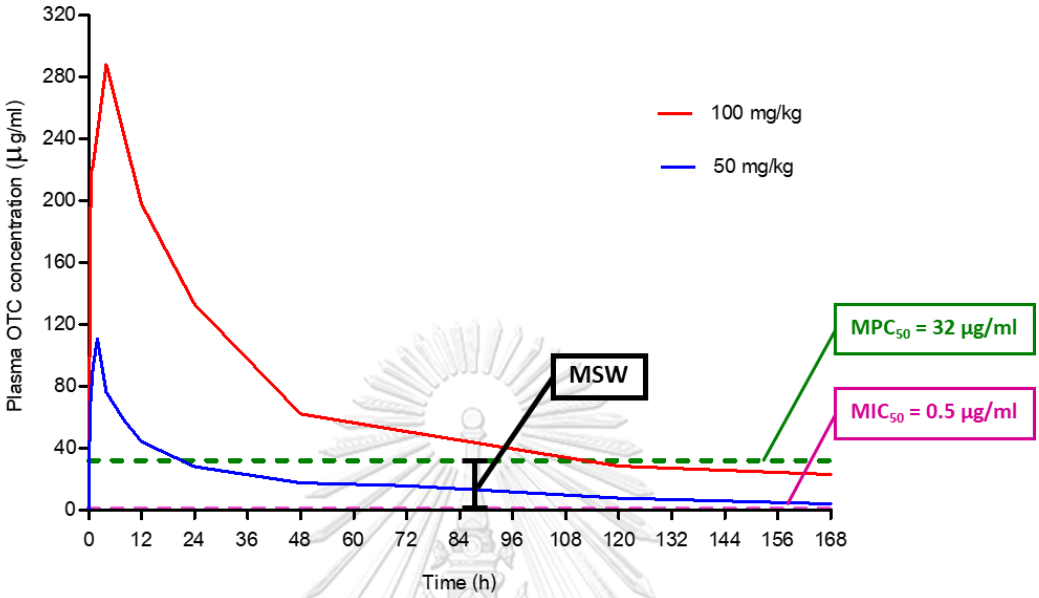
3. Pharmacokinetic/Pharmacodynamic (PK/PD) analysis of OTC-LA against *S. agalactiae*

3.1 MSW integrated with plasma OTC concentration-time curve

MSW of MIC_{50} and MPC_{50} drawing on plasma OTC concentration-time curves were presented in Figure 9 (A). MSW of MIC_{50} and MPC_{50} ranged from 0.5 to 32 µg/ml. At the dosage of 50 mg/kg, plasma OTC concentrations were above MSW for 21 h. While at 100 mg/kg dosage, plasma OTC concentrations were above MSW for 112 h then the rest fell in MSW.

MSW of MIC_{90} and MPC_{90} was in the range from 1 to 128 µg/ml. Figure 9 (B) showed MSW of MIC_{90} and MPC_{90} plotting on plasma OTC concentration-time curves. For the dosage of 50 mg/kg, plasma OTC concentrations fell in MSW for all experimental period since all OTC concentrations from the dosage of 50 mg/kg were below MPC_{90}. At 100 mg/kg dosage, plasma OTC concentrations were above MSW for 26 h. OTC concentrations were within MSW thereafter.

A) MSW of MIC_{50} and MPC_{50}



B) MSW of MIC_{90} and MPC_{90}

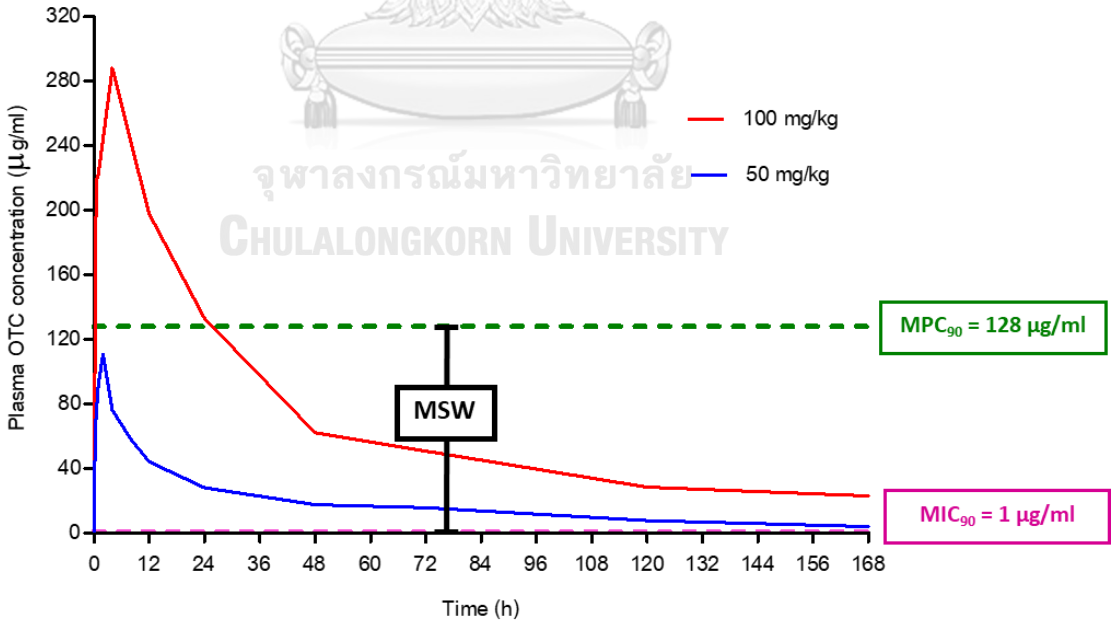


Figure 9: Mutant selection window (MSW) of OTC against *S. agalactiae* (A) MSW of MIC_{50} and MPC_{50}, (B) MSW of MIC_{90} and MPC_{90}

3.2 PK/PD ratios

PK/PD ratios based on MIC and MPC data of *S. agalactiae* isolates in this study were presented in Table 11. The results showed that high AUC/ MIC ratios were observed for both 50 mg/kg and 100 mg/kg doses. T>MIC_{50} and T>MIC_{90} were more than 168 h. For the results of PK/PD ratios evaluated using MPC data, AUC/ MPC ratios were much lower than AUC/MIC. The $AUC_{0\text{-}168}/MPC_{50}$ and $AUC_{0\text{-}168}/MPC_{90}$ ratios of the dosage of 100 mg/kg were almost four times higher than those of the lower dosage. The T>MPC_{50} of OTC-LA at 50 mg/kg was more than 3 times shorter than those of 100 mg/kg. The T>MPC_{50} were more than four times longer than T>MPC_{90}.

Table 11: PK/PD ratios evaluated based on MIC and MPC data of *S. agalactiae* isolates

PK/PD	Parameters	Dosages	
		50 mg/kg	100 mg/kg
PK	$AUC_{0\text{-}168}$ (μg·h/ml)	2995.304	11483.991
PD	MIC_{50} (μg/ml)	0.5	
	MIC_{90} (μg/ml)	1	
	MPC_{50} (μg/ml)	32	
	MPC_{90} (μg/ml)	128	
PK/PD: MIC_{50}	$AUC_{0\text{-}168}$ / MIC_{50}	5990.608	22967.982
	T > MIC_{50} (h)	>168	>168
PK/PD: MIC_{90}	$AUC_{0\text{-}168}$ / MIC_{90}	2995.304	11483.991
	T > MIC_{90} (h)	>168	>168
PK/PD: MPC_{50}	$AUC_{0\text{-}168}$ / MPC_{50}	93.603	358.875
	T > MPC_{50} (h)	21	112
PK/PD: MPC_{90}	$AUC_{0\text{-}168}$ / MPC_{90}	23.401	89.719
	T > MPC_{90} (h)	0	26

CHAPTER V
DISCUSSION

1. Pharmacokinetics of OTC-LA following intraperitoneal injection in tilapia

1.1 Animals procedure

In this study, 120 healthy male tilapia were administered with OTC-LA at either 50 or 100 mg/kg bodyweight, despite the extra-label dose of OTC injection in fish at 25-50 mg/kg bodyweight. (Stoffregen et al., 1996; Noga, 2010; Stoskopf, 2011). Since OTC has a generally wide margin of safety (del Castillo, 2013). Thus, we decided to use the dose at 100 mg/kg bodyweight (twice of the extra-label OTC dose) as the higher OTC-LA dosage in this study, because the higher dose may be required to block the mutant sub-population. In this study, none of the fish showed any adverse effects or die during the study period indicating that tilapia was well tolerated to OTC. This result was consistent with the previous study of OTC-LA in other fish species such as grouper (Rigos et al., 2010) and white sea bream (Ali et al., 2019).

1.2 HPLC analytical method for determining OTC in plasma samples

Various assays such as microbiological assay, fluorometry and chromatography have been developed to determine the concentrations of OTC in blood and tissues. However, because of its specificity, reliability and sensitivity, high performance liquid chromatography (HPLC) is now considered as the present standard method. This technique has been used for the determination of OTC concentrations in various biological matrices. Pharmacokinetic studies utilizing HPLC to determine concentrations of OTC in blood and tissues have been conducted in many fish species including Atlantic salmon (Elema et al., 1996; Meinertz et al., 1998), rainbow trout (Meinertz et al., 1998; Dagoglu et al., 2004), pacu (Doi et al., 1998), grass carp (Zhang and Li, 2007) and grouper (Rigos et al., 2010).

The present HPLC procedure is simple and reliable for the detection and quantification of OTC in tilapia plasma. For the sample extraction, protein precipitation was performed by adding trichloroacetic acid and acetonitrile followed by centrifugation and supernatant collection, before being injected to the HPLC system. This process can produce high percentages of recovery. Many studies required complex pretreatment of the plasma sample before being injected onto the HPLC column, our simple protein precipitation could produce good recovery of OTC in tilapia plasma samples. It may be due to the lower of plasma protein in fish comparing to mammals (Davies and Morris, 1993; Mlay et al., 2007). Use of Symmetry® C18 column (150 x 4.6 mm i.d., 5 µm) for chromatographic separation at ambient temperature and binary gradient of mobile phase consisting of 0.01M oxalic acid and acetonitrile: methanol (80:20 v/v) provided the optimal peak shape and good resolution for both OTC and the internal standard. However, the total run time of this method was quite longer than the previous method using similar column and mobile phase (Zhang and Li, 2007; Poapolathep et al., 2017). This might be from the different calibration method either the use of external standard calibration or the use of other compounds such as the internal standard which can be separated faster. In this study, the quantitative determination was performed by the internal standard calibration. CTC as internal standard was completely separated from the target drug. For the calibration curves, OTC standard concentrations ranging from 0.1 - 320 µg/ml, exhibited good linearity with R^2 of plasma at 0.99997. This calibration range covered all the OTC levels in plasma. Thus, it can be applicable for OTC determination in tilapia plasma in this experiment without the further dilution of samples. The selectivity results demonstrated that no significant interferences from endogenous components were observed. This indicated that this method is selective for OTC analysis. The intra-day and inter-day accuracy and precision results were within acceptable limits. The recovery exhibited high percentage for both OTC (85- 95 %) and CTC (85%). In summary, the

method used in this study achieved the standard requirements of bioanalysis method validation (USFDA, 2018).

1.3 PK parameters of OTC-LA

The empirical (extra-label) dose suggestion of OTC injection in fish is 25-50 mg/kg bodyweight single injection (Stoffregen et al., 1996; Noga, 2010; Stoskopf, 2011). However, the precise dosage of OTC-LA injection in tilapia has never been established. Due to the inter-species differences, it is valuable to specify the dosing regimen for the prudent use of antibiotics in each animal species. Therefore, in this study we aimed to study the PK of OTC-LA after single IP administration with 2 different dosages: 50 and 100 mg/kg bodyweight.

From the plasma OTC concentration results in our study, peak plasma concentration of OTC after IP administration is much higher than the previous reports in other fish species (Table 12). In our study, C_{max} achieved from OTC-LA at 50 mg/kg IP was 110.70 ± 5.61 µg/ml, while C_{max} of OTC from the conventional injectable formulation at the same dose were 41.54 µg/ml (IM) in rainbow trout (Dagoglu et al., 2004), 29.00 ± 2.60 µg/ml (IM) and 32.00 ± 8.00 µg/ml (IP) in yellow perch (Bowden, 2001). Peak serum OTC level at 39.70 ± 10.1 µg/ml was also observed in grouper receiving OTC-LA at the similar dosage intramuscularly (Rigos et al., 2010). For OTC-LA at the dosage of 100 mg/kg IP in this study, the C_{max} was 287.85 ± 8.03 µg / ml, more than two times greater that of 50 mg/kg. Although there was a study of OTC-LA at the dosage of 100 mg/kg IP administration in white sea bream (Ali et al., 2019), C_{max} and other PK parameters have not been determined. That study reported OTC level at 24 h until 168 h post-administration. However, when comparing the OTC plasma concentrations at each time point, for example OTC level in our study at 24 h post-administration (133.02 ± 4.17 µg/ml) was also higher than that in white sea bream (34.57±1.09 µg/ml). These variations could

be due to the different OTC formulation, rearing environment (e.g., salinity and temperature), physiological variation among species as well as the analytical method. The previous PK data of OTC in tilapia have been reported only from oral administration. Peak plasma concentration of OTC after IP administration in this study was extremely higher than the previous reports of oral OTC administration either via medicated feed (C_{max}: 1.4 – 1.76 μg/ml) (Chen et al., 2004) or oral solution (C_{max}: 1.22 – 1.34 μg/ml) (Sidhu et al., 2018). These may reflect the varied low oral bioavailability of OTC in tilapia, leading to insufficiency of drug levels in fish circulation, which may result in the treatment failure. Moreover, the resistance sup-population of organisms might be selected by the low level of OTC.

The T_{max} values obtained in this study was similar to the previous reports of conventional injectable OTC in freshwater fish species (Bowden, 2001; Dagoglu et al., 2004), but was quite longer than T_{max} in grouper which is marine fish (Rigos et al., 2010) (Table 13). Thus, the absorption rate of OTC might be increased by the effect of salinity. This suggestion supports the previous report of Sidhu et al. (2018), who studied the PK of OTC after single oral administration in tilapia maintained in three different salinities. They found that the rising in water salinity level increases rates of absorption and elimination of OTC.

In this study, OTC concentrations were detected at quite high level in the first 15 minutes after OTC-LA administration. This suggested that the absorption of the drug was rapid. These results are, however, inconsistent with previous reports in terrestrial animals including pigs (El Korchi et al., 2001), calves (Kumar and Malik, 1998) and buffaloes (Poapolathep et al., 2017) which slow absorption occurred after OTC-LA treatment. This might be due to different routes of administration. In terrestrial animals, OTC-LA is recommended to be administered by deep IM injection, thus the drug is released slowly from the injection site into the blood circulation.

The AUC represents the total drug exposure across time. In this study, AUC_{0-24} achieved from OTC-LA at 50 mg/kg (1243.93 μg·h/ml) was more than that reported in grouper (363.5 μg·h/ml) receiving OTC-LA at the same dosage intramuscularly (Rigos et al., 2010). The results of $AUC_{0-\infty}$ obtained from OTC-LA at 50 mg/kg in our study was 3277.30 μg·h/ml, which was also more than $AUC_{0-\infty}$ reported in other fish species receiving conventional OTC injectable formulation at similar dosage including rainbow trout and yellow perch. In our study, high AUC results were corresponding to the high plasma OTC concentration and high C_{max}. Our results demonstrated that AUC_{0-24}, AUC_{0-168}, and $AUC_{0-\infty}$ of OTC at the dosage of 100 mg/kg were ≥ 3 times higher than those of 50 mg/kg. This high AUC may yield a positive outcome since AUC is one of the PK parameters used as a reference value in PK/PD and the AUC/MIC ratio is the index mostly correlated with efficacy of tetracyclines (Craig, 2002; Craig, 2003; Hesje et al., 2007). Therefore, the high AUC could provide a better chance to achieve antimicrobial efficacy.

The volume of distribution or V_d is the volume of fluid that the drug needs to be dissolved until it reaches the same concentration as in plasma (Riviere, 2011). Drug with high V_d indicates that the drug has good distribution to extravascular fluid and tissues. (Toutain and Bousquet-MÉLou, 2004). OTC is already known to exhibit good distribution and penetration into body fluids and tissues due to its lipophilic property (Riviere and Spoo, 1995). From previous report, the apparent volume of distribution after non-intravenous administration (V_d/F) of OTC-LA after IM injection were 8.6 L/kg in Kilis goat (Aktas and Yarsan, 2017) and 2.4-3.4 L/kg in Thai swamp buffaloes (Poapolathep et al., 2017), but lack of distribution data (V_d/F) of OTC in fish. In this study, V_d/F at 0.87-1.08 L/kg was reported in tilapia after IP injection, indicating that OTC-LA injection in tilapia also presented good volume of distribution although the value was lower than those found in terrestrial animal. This might be due to the different doses and route of administration,

and species variations. In addition, there are many factors affecting and limiting drug distribution such as blood flow, plasma protein or tissue binding as well as membrane barrier (Riviere, 2011).

Elimination half-life ($t_{1/2}$) defines the time required to reduce 50% of plasma drug concentration. From our results, $t_{1/2}$ of OTC obtained in tilapia was long, suggesting that the overall rate of elimination of OTC-LA injection in tilapia was slow. The long $t_{1/2}$ reported here was consistent with the low elimination rate constant (*Kel*) and the slow apparent total body clearance (CL/F). The half-life values in this study were longer than the previous report in rainbow trout (Dagoglu et al., 2004), but shorter than those reported in yellow perch (Bowden, 2001) (Table 13). However, no data of $t_{1/2}$, clearance and MRT after OTC-LA injection in fish have been reported. The mean residence time or MRT represents the average time a molecule stays in the body. The MRT of OTC-LA in this study was long (46.8 - 47.5 h) and longer than the previous reports of OTC-LA in claves (35.3 h) (Kumar and Malik, 1998) and goats (25.6 h) (Aktas and Yarsan, 2017) but shorter than those reported in pigs (85 h) (El Korchi et al., 2001) and buffaloes (70 h) (Poapolathep et al., 2017). These differences, again, might be resulted from the different doses and route of administration and the variation among species.

From the PK parameters, it was obvious that C_{max} and AUC increased when OTC dosage was increased. However, delayed T_{max} was also observed with increased dose. These results are similar with the OTC-LA injection at different doses in buffaloes (Poapolathep et al., 2017). Since absorption and excretion of OTC occur by passive diffusion (Pindell et al., 1958). The higher drug dosage provides the higher drug concentration to be absorbed, thus the time to reach the peak plasma concentration could be delayed. For the elimination, the elimination half-life of OTC at the dosage of 100 mg/kg was longer than those of 50 mg/kg, suggesting that the elimination half-life increased with

increased dose. This might be explained by the effect of long-acting formulation which retained the drug at the injection site and slow released to the circulation. Thus, delayed absorption at the higher dose could be prolonged the elimination of the drug.

Table 12: PK studies of OTC following IP or IM injection of various fish species

Species	OTC formulation	Temp. (°C)	Salinity	Dose (mg/kg)	Route	C_{ma} (μg/ml)	AUC (μg·h/ml)	T_{max} (h)	$t_{1/2}$ (h)	*K*el (1/h)	CL/F (ml/kg)	References
Rainbow trout	Conventional	10.5	Freshwater	50	IM	41.54	$AUC_{0-\infty}$:1116.18	2	24.3	-	-	(Dagoglu et al., 2004)
Yellow perch	Conventional	16.5	Freshwater	50	IP	32.00 ± 8.00	$AUC_{0-\infty}$: 1718.3	2	112.4	-	-	(Bowden, 2001)
				50	IM	29.00 ± 2.60	$AUC_{0-\infty}$: 2658.5	4	123.8	-	-	
Grouper	Long acting	20	Seawater	50	IM	39.70 ± 10.1	AUC_{0-24}: 363.5 AUC_{0-48}: 668.7	1	-	-	-	(Rigos et al., 2010)
Tilapia	Long acting	28.7	Freshwater	50	IP	110.70 ± 5.61	AUC_{0-24}: 1248.93 AUC_{0-168}: 2995.30 $AUC_{0-\infty}$: 3277.30	2	49	0.014	15.32	This study
				100	IP	287.85 ± 8.03	AUC_{0-24}: 4828.65 AUC_{0-168}: 11483.99 $AUC_{0-\infty}$: 14274.24	4	89	0.0084	6.93	

From the plasma concentration-time curve of both OTC-LA dosages showed similar patterns. OTC in plasma was slowly depleted and maintained at around 4 μg/ml and 23 μg/ml at 168 h or 7 day after administration for the dosages of 50 and 100 mg/kg, respectively. Form these results, plasma OTC concentrations after IP administration of OTC-LA were high and long-lasting. These results are consistent with the previous study of OTC-LA IP injection at 100 mg/kg in white sea bream broodstock that the OTC concentration remained more than 5 μg/ml until seven days (Ali et al., 2019). Nearly similar result was reported by Bowden (2001), who found that OTC remains over 4 μg/ml after 168 h post-IM or IP administration in yellow perch. Dissimilar pattern of OTC depletion was observed by Rigos et al. (2010), which an unexpected non-gradual pattern of OTC depletion found in grouper. After OTC rapidly reached the peak concentration, the sudden drop was observed, followed by a second peak at 12 h post-administration which may be resulted from drug reabsorption from intestine after biliary excretion.

In summary, PK parameters of OTC-LA after single IP administration in tilapia demonstrated high plasma level. OTC has rapid absorption, high distribution, long elimination half-life and slow clearance in Nile tilapia. Plasma OTC levels were long-lasting and still detectable beyond 7-day post-administration. It is difficult to compare the PK parameters with other studies. Inter-study variations in the PK parameters may be resulted from the differences in several biological factors such as species, age/size and health status, or nonbiological factors including the route of administration, drug formulation, temperature, mode of sampling, sample preparation as well as analytical methods (Rigos and Smith, 2015).

2. Pharmacodynamics of OTC against *Streptococcus agalactiae* (*S. agalactiae*)

In this study, pharmacodynamics of OTC was determined as MIC and MPC in 56 *S. agalactiae* isolates from diseased tilapia in different farming areas of central Thailand during 2018 - 2019.

2.1 Minimum inhibitory concentration (MIC) of OTC against *S. agalactiae*

From our results, MICs of 56 clinical *S. agalactiae* isolates ranged from 0.5 to 2 µg/ml. MIC_{50} and MIC_{90} were 0.5 µg/ml and 1 µg/ml, respectively. MIC breakpoints for *Streptococcus* group B were as follows, susceptible (S) at MIC $\leq$ 2, intermediate (I) at MIC = 4 and resistance (R) at MIC $\geq$ 8 µg/ml (CLSI, 2018). Therefore, based on MIC breakpoint, all 56 *S. agalactiae* isolates were susceptible to OTC or 100% susceptibility.

The OTC susceptibility studies of *S. agalactiae* isolates from disease tilapia in different years and aquaculture area of Thailand have been reported. Dangwetngam et al. (2016) studied antimicrobial susceptibility of 144 *S. agalactiae* isolates from tilapia in Thailand from 2003 to 2011 using disk diffusion method. The results showed that most of the *S. agalactiae* isolates were susceptible to OTC (86.1%). Antimicrobial susceptibility testing of 100 streptococcal isolates from diseased tilapia during 2003 to 2012 were reported by Lukkana et al. (2015). The results showed that the susceptibility pattern of OTC was varied between years. Eighty-nine percent of OTC susceptibility was found during 2003–2008. Thereafter, during 2009-2012, 100% (48 isolates) susceptible to OTC was observed. Recently report was on antimicrobial susceptibility testing of 124 *S. agalactiae* isolates from diseased tilapia across Thailand during 2012 to 2014. The results showed that more than 70% susceptibility to OTC were observed in clinical *S. agalactiae* isolates from the north, northeastern and central areas. While all *S. agalactiae* isolates from the southern area were susceptible to OTC (100% susceptibility) (Kannika et al.,

2017). From all these previous reports indicated that *S. agalactiae* isolates in Thailand were highly susceptible to OTC which are consistent with our results.

However, multidrug-resistant, and highly virulent serotype of *S. agalactiae* in tilapia were reported in Brazil. These isolates were resistant to multiple groups of antimicrobials including tetracyclines (Chideroli et al., 2017). It was suggested that the differences in susceptibility pattern of *S. agalactiae* to antimicrobials could be due to environmental variability, serotype variety and different practices in antimicrobial uses in aquaculture (Abuseliana et al., 2010).

For the MIC values, MICs of OTC against *S. agalactiae* in Thailand were reported by Lukkana et al. (2015). The distribution of OTC MIC was ranging from 0.5 to 32 µg/ml. MIC_{50} and MIC_{90} were 0.5 µg/ml and 4 µg/ml, respectively. Comparing to the results from our study, MIC range was narrower than the previously reports, with similar MIC_{50}. However, MIC_{90} in this study was lower (decreased from 4 to 1 µg/ml). From these results, it seems that *S. agalactiae* population increase susceptibility to OTC. However, it should be noted that the number of *S. agalactiae* isolates in our study was rather small, thus, it might not be enough to represent the whole population.

2.2 Mutant prevention concentration (MPC) of OTC against *S. agalactiae*

MPC of 56 clinical *S. agalactiae* isolates were in the range from 4 to 512 µg/ml. MPC_{50} and MPC_{90} were 32 µg/ml and 128 µg/ml, respectively. Due to the lack of MPC breakpoints, therefore OTC susceptibility and resistance were based on OTC MIC breakpoints. From our study, none of the *S. agalactiae* isolates had MPC less than susceptible MIC breakpoint (2 µg/ml) and more than 98% of *S. agalactiae* isolates had MPC more than resistance MIC breakpoint (8 µg/ml), which means that almost all of *S. agalactiae* isolates turn to be resistant with increased bacterial density.

MPC was used to calculate MPC to MIC ratio. The results of this study, MPC_{50}/MIC_{50} ratio was 64 and MPC_{90}/MIC_{90} ratio was 128. The ratios of MPC/MIC of tetracyclines against gram-positive bacteria have been reported by Hesje et al. (2015). They found that MPC_{90}/MIC_{90} ratio of tigecycline against *Streptococcus pneumoniae* was greater than 500. The study of tetracyclines against some gram-negative bacteria showed lower ratio results. MPC_{90}/MIC_{90} ratio of tetracycline from 15 isolates of *Brucellae melitensis* were 8 (Coban et al., 2007). MPC_{90}/MIC_{90} ratio of tigecycline from 80 isolates of *Acinetobacter baumannii* were 16 (Cui et al., 2010). From these reports along with our results, MPC/MPC ratios of gram-negative bacteria seems to be lower than that of gram-positive bacteria. Therefore, one should be careful when using tetracyclines based on only MIC values for the treatment of gram-positive bacteria. MPC should be considered and applied for obtaining therapeutic outcome and preventing selection of resistant mutants.

Mutant selection window (MSW) defines the concentration range between MIC (lower boundary) and MPC (upper boundary). The window is considered as dangerous zone because the antibacterial concentrations above MIC but below MPC can inhibit only susceptible bacteria, but not the resistant subpopulation. Antimicrobial concentration that falls inside window may raise the antimicrobial resistant mutants through selective pressure and amplification (Drlica and Zhao, 2007). In general, MSW is estimated from the median (50^{th} percentile) of MIC and MPC. The results of this study, MSW ranged from 0.5 - 32 µg/ml. While at the 90^{th} percentile, MSW ranged from 1 -128 µg/ml. This is the first report of MPC and MSW of OTC against clinical isolates of *S. agalactiae* from tilapia. The width of MSW is varied among different drugs and pathogens. The wide gap or window between MPC and MIC indicates that many drug-resistant mutants are hidden in the bacterial population. This may lead to a high risk of resistance. On the other hand, the

narrow window indicates that the hidden resistant mutants in population are less, leading to a low risk of resistance (Drlica and Zhao, 2007).

Traditionally, pharmacodynamic parameters based on MIC have been used to determine appropriate dosing regimens for antimicrobial agents. However, many of data supports the use of MPC and MSW instead of MIC to optimize dose and dosing intervals (Epstein et al., 2004; Drlica and Zhao, 2007). Narrowing the MSW or lowering MPC/MIC ratio produces the low chance of drug concentrations falling inside the window. Thus, the new antimicrobial drugs should have very narrow selection window to be effective with less chance to be resistant (Drlica and Zhao, 2007). For the existing antimicrobial drugs, minimizing the length of time that the drug concentrations remain in the MSW may reduce the likelihood for development of resistance during therapy (Berghaus et al., 2013). Moreover, there was suggested that MPC can be used instead of the MIC in pharmacodynamic considerations of antimicrobial agents with higher values for area under the 24 h plasma drug concentration versus time curve to MPC ratio (AUC_{24}/MPC) that are less likely to selectively enrich for resistant mutants (Drlica and Zhao, 2007; Blondeau, 2009).

However, there are many factors affecting MPC such as bacterial strains, antibacterial drugs, bacterial density, and mutation types (Gianvecchio et al., 2019). The isolates as mutant subpopulation is varied with various multistep of mutants that may or may not present at the determination of MPC (Campion et al., 2004; Drlica et al., 2006). Thus, high variability should be concerned when MPC are applied.

2.3 Correlation of MIC and MPC of OTC against *S. agalactiae*

In this study, the correlation between MIC and MPC for OTC against *S. agalactiae* isolates was evaluated by using a scattered plot and linear regression model. The correlation coefficient was very low which indicates poor correlation between MIC and MPC. This result is in accordance with previous report that a low correlation between MIC and MPC were observed when *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* were tested against several fluoroquinolones (Drlica et al., 2006). Moreover, Poor correlation between MIC and MPC of tetracycline was observed with *Acinetobacter baumannii* isolates (Cui et al., 2010). Both studies confirm the poor correlation between MIC and MPC, which is independent of the antimicrobial agents and bacterial species. It implies that MIC cannot be used to reliably predict MPC. Thus, these results emphasize the importance of MPC determination to be used in PK/PD analysis for selecting the optimal dosing regimens of OTC to maximize the antibacterial efficacy and minimize the antimicrobial resistance of *S. agalactiae* in tilapia.

3. Pharmacokinetic/pharmacodynamic (PK/PD) ratio

Inappropriate used of antimicrobial drug is one of the factors contributing to emergence of antimicrobial resistance. Inadequate treatment including sub-therapeutic doses, infrequent administrations as well as improper selection of active drug can result in a failure to attain the appropriate PK/PD target, which can lead to drug resistance (Papich, 2014). Exposure with sub-optimal dose is the most important factor in emergence of antibacterial resistance (Lees et al., 2008). Therefore, antimicrobial administration using dosing regimens that attain appropriate PK/PD target values have been developed. Different antimicrobial drugs or microorganisms require different target ratio values. For example, the ratios for fluoroquinolones against gram negative bacteria were AUC/MIC ≥

125 and C_{max}/MIC ≥ 8 (Forrest et al., 1993; Zelenitsky et al., 2003), the T>MIC for penicillins was more than 50% (Craig, 2002). The AUC_{0-24}/MIC ratios>400 was established for vancomycin against *Staphylococcus aureus* (Holmes et al., 2013). However, PK/PD target values have not been established for all antimicrobial agents and/or antibiotic classes. For the drugs in tetracyclines group, the studies of PK/PD integration were limited. Burgess et al. (2007) studied the PK/PD modeling to develop susceptibility breakpoints of many antimicrobials for *Neisseria meningitidis*. AUC/MIC ratio of ≥ 25 were chosen as the PK/PD target value for antibacterial activity of tetracyclines. Prats et al. (2005) studied the PK/PD of doxycycline administration via drinking water for the main porcine bacterial pathogens of the respiratory tract (*Pasteurella multocida, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica* and *Mycoplasma hyopneumoniae*). The PK/PD indices and values were AUC/MIC ratio of ≥ 125 and 70% T>MIC. However, PK/PD target values for OTC, and all tetracyclines, have not been developed specifically for *Streptococcus agalactiae* in animal or human studies. Thus, it was necessary to apply PK/PD targets form other organisms. The surrogate PK/PD values have been established for gram-positive bacteria, the AUC/MIC value has been suggested to be at least 30-50 and T> MIC is 40-50% of dosing interval (Hesje et al., 2007).

One of the aims of using OTC-LA formulation in tilapia broodstock was to minimize animal handling by single administration. In this study plasma OTC levels were measured until 168 h or 7-day post-administration based on the hypothesis that single administration could provide plasma OTC at least 7 days. Therefore, AUC_{0-168} was used to calculate AUC/MIC and AUC/MPC ratios and percentage of time that above MIC and MPC were evaluated using 168 h as dosing interval.

For the results of PK/PD integration based on MIC values of OTC-LA at both 50 mg/kg and 100 mg/kg, AUC_{0-168}/MIC ratios (MIC_{50} and MIC_{90}) were very high and achieved the target values (AUC/MIC ratio ≥ 50). It is suggested that OTC level in plasma reached optimal concentration to inhibit bacterial growth after OTC-LA administered at either 50 mg/kg or 100 mg/kg when MIC was ≤ 1 μg/ml (MIC_{90}). T>MIC_{50} and T>MIC_{90} were greater than 168 hours, indicating that a single IP injection of OTC-LA was sufficient to produce plasma OTC levels greater than MIC for at least 7 days. Based on proposed 168 h dosing interval, T>MIC_{50} and T>MIC_{90} were 100% (168/168), exceeding the target value of T>MIC (at least 50% of the dosing interval).

However, applying MPC as PD parameter for PK/PD analysis of antimicrobials is a recent concept to overcome the antimicrobial resistant-mutant subpopulation. PK/PD target values based on MPC have not been established yet. Therefore, PK/PD target values based on MIC were applied in this study. For PK/PD integration based on MPC values of the OTC-LA at 50 mg/kg dosage, AUC_{0-168}/MPC_{50} ratios exceeded the target value, indicating that plasma OTC level achieved concentration to prevent the development of resistant-mutant subpopulation when MPC was ≤ 32 μg/ml (MPC_{50}). While AUC_{0-168}/MPC_{90} ratios did not attain the target value suggesting that OTC-LA treatment at 50 mg/kg dosage could not reach the concentration required for preventing the emergence of resistant-mutant subpopulation with high MPC (MPC_{90}= 128 μg/ml).

For OTC-LA at 100 mg/kg dosage, AUC_{0-168}/MPC ratios (both MPC_{50} and MPC_{90}) attained the target values, indicating that this high dose provided enough plasma OTC level to prevent development of resistant-mutant subpopulation even when MPC was up to 128 μg/ml. From these results, OTC-LA at 100 mg/kg showed more beneficial for the prevention of resistant-mutant subpopulation.

From T> MPC_{50} and T> MPC_{90}, our results indicated that single IP injection of OTC-LA at 100 mg/kg provided plasma OTC level greater than MPC_{50} and MPC_{90} for 112 h and 26 h, respectively. T> MPC_{50} was 66.67% (112/168), attaining the target values. It indicated that OTC-LA at 100 mg/kg single administration would be effective to prevent development of resistant-mutant subpopulation of 50% of organism (MPC_{50} $\leq$32 µg/ml) for 4 - 7 days. While T> MPC_{90} was 15.48% (26/168) which did not attain the target values. This result implied that single administration was insufficient. The dosing interval should be adjusted to be every 1-2 day to prevent development of resistant-mutant subpopulation of 90% of organism (MPC_{90}= 128 µg/ml). For the application of OTC-LA injection in aquaculture, it should be noted that drug administration by multiple injections is impractical for fish. Therefore, OTC-LA injection might not be suitable for treatment of infection caused by *S. agalactiae* with MPC more than 32 µg/ml.

In this study, PK/PD ratios based on MIC and MPC were much different because the MPC values were much higher than the MICs, consistent with the study of PK/PD integration of OTC-LA against the porcine pneumonia pathogens (Dorey et al., 2017). They found that PK/PD ratios based on MPC of *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* were much lower than the ratios based on MIC. Moreover, they studied PK/PD modelling using Monte Carlo simulations to evaluate PK/PD breakpoints for specific pathogen and to predict specific dosing regimens. Their results showed that the predicted OTC-LA dosages exceeded the recommended dosing regimen of OTC-LA in pigs (20 mg/kg bodyweight) against both bacterial species.

Unfortunately, the PK/PD approach cannot be fully applied in aquatic animals due to a lack of recommended values of PK/PD ratios, large variation of PK and PD within species and effect of environmental factors on PK and PD of antimicrobials (Rigos and Smith, 2015). In fish, only one study of PK/PD integration to establish dosing regimens

using MPC values has been reported by Xu et al. (2013). They studied PK/PD integration of enrofloxacin against *Aeromonas hydrophila* in grass carp. The results showed that enrofloxacin at 10-30 mg/kg bodyweight achieved the target values based on MIC, but only 20 and 30 mg/kg dosages attain the target values based on MPC. In addition, once-daily dosage of 30 mg/kg predicted a positive clinical outcome and minimized the selection of drug-resistant mutants. Like our study, their study used surrogate PK/PD values of fluoroquinolones for gram negative bacteria in PK/PD analysis.

4. Dosing regimens

According to our PK/PD findings based on MIC and MPC data, optimal dosing regimen of OTC-LA injection could be predicted as follow:

OTC-LA IP injection either at the dosage of 50 mg/kg or 100 mg/kg bodyweight provided sufficient OTC level in plasma to inhibit bacterial growth in susceptible *S. agalactiae* with MIC ≤ 1 µg/ml for at least 7 days. Thus, single administration would be possible in case of acute infection. This dosing regimen supports the empirical dose suggestion of conventional-OTC injection in fish which is 25-50 mg/kg bodyweight single administration (Stoffregen et al., 1996; Noga, 2010; Stoskopf, 2011).

Nevertheless, the dosing regimens for preventing the emergence of resistant-mutant subpopulation of *S. agalactiae* depends on MPC values. OTC-LA IP administration at 100 mg/kg bodyweight single administration would be proposed for *S. agalactiae* with MPC ≤ 32 µg/ml. For higher MPC (>32 µg/ml), single administration would be unsuitable.

Therefore, the use of antimicrobials should be based on antimicrobial susceptibility data, not only MIC but also MPC. We recommend that the optimal dosing regimens for achieving therapeutic efficacy, minimizing of antimicrobial resistance and possibly being single administration is OTC-LA IP administration at 100 mg/kg bodyweight

for *S. agalactiae* population with high susceptibility and low risk of resistant-mutant subpopulation (MPC ≤ 32 μg/ml).

However, it is important to remember that OTC exhibits bacteriostatic activity that the host immune system is ultimately responsible for success in combating pathogens. Thus, the outcome may differ when using OTC-LA in clinical treatment due to various factors such as host immunity, bacterial burden, and environment. The studies in clinically sick tilapia might be required. Moreover, it should be noted that PK/PD target ratios from our study are surrogate values estimated from the previous reports with the limited number of *S. agalactiae* isolates. Using the full power of our PK data, PK/PD modelling using Monte Carlo simulations and time-kill study for predicting more specific dosing regimens should be performed in the future.

CHAPTER VI
CONCLUSION

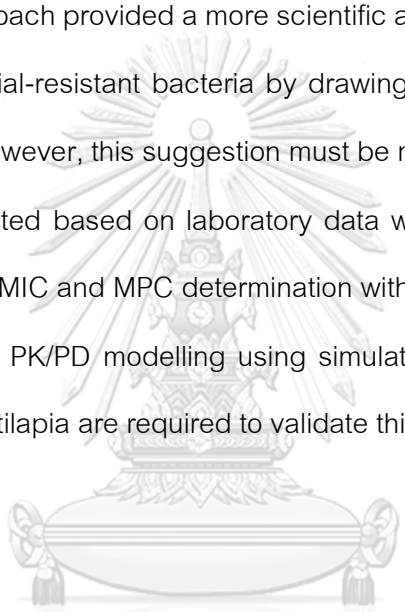
In this study, pharmacokinetics of OTC-LA after single IP administration in tilapia broodstock were established. The results demonstrated that PK parameters of OTC-LA after IP administration showed high plasma level. OTC-LA have rapid absorption, high distribution, long elimination half-life and slow clearance in Nile tilapia. Plasma OTC levels were long-lasting and still detectable until 7 days post administration.

The pharmacodynamic parameters (MIC, MPC and MSW) of *S. agalactiae*, isolated from tilapia were characterized. Clinical *S. agalactiae* isolates were highly susceptible to OTC based on MIC values. While almost all isolates turned to be resistant when increase bacterial density that interpreted from the high MPC values results. MPC_{50}/MIC_{50} ratio was 64 (MSW ranged from 0.5 - 32 µg/ml) and MPC_{90}/MIC_{90} ratio was 128 (MSW ranged from 1 -128 µg/ml). Moreover, MIC cannot be used to reliably predict MPC as the poor correlation between MIC and MPC of OTC against *S. agalactiae* isolates was observed. These emphasize the importance of MPC determination to be used in PK-PD analysis for selecting the most optimal dosing regimen of OTC for the treatment of *S. agalactiae* in tilapia.

PK/PD parameters demonstrated that PK/PD integration based on MIC values of OTC-LA both at 50 mg/kg and 100 mg/kg dosages achieve the target values and provide the plasma OTC level above the MIC for at least 168 h. While PK/PD ratios based on MPCs were much lower than the ratios based on MICs. Only OTC-LA at 100 mg/kg dosage can reach the target values for both MPC_{50} and MPC_{90} that would achieve therapeutic efficacy, prevent the development of resistant-mutant subpopulation, and provide the plasma OTC level above the MPC_{50} and MPC_{90} for 112 h and 26 h, respectively.

In conclusion, for the prudent use of antimicrobials to maximize the therapeutic effect and minimize the development of antimicrobial resistance, the use of antimicrobials should be based on antimicrobial susceptibility data, not only the MIC but also the MPC. Based on our results, we suggested that the optimal dosing regimens of OTC-LA IP administration is 100 mg/kg bodyweight single administration for *S. agalactiae* population with high susceptibility and low risk of resistant-mutant subpopulation (MPC $\leq$ 32 µg/ml).

Our PK/PD approach provided a more scientific and effective strategy to face the challenge of antimicrobial-resistant bacteria by drawing a specific dosing guideline of antimicrobial agents. However, this suggestion must be made with cautions because the PD values are investigated based on laboratory data with limited number of samples. Further studies such as MIC and MPC determination with greater number of isolates, the killing property of OTC, PK/PD modelling using simulation software as well as PK/PD studies in clinically sick tilapia are required to validate this recommendation.



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