Studies on Pharmacokinetic and Pharmacodynamic Parameters of Orbifloxacin in Canine Urinary Tract Infections

(犬の尿路感染症におけるオルビフロキサシンの薬物動態学 - 薬力学パラメーターに関する研究)

Takae　SHIMIZU

2018

Contents

General Introduction

Urinary tract infections (UTIs) are the infections of kidney, urinary tracts, bladder and urethra, and can cause various urological symptoms, including inappropriate urination, dysuria, hematuria, stranguria, pollakiuria, and malodorous urine (Smee et al., 2013b). In dogs, as well as humans, UTIs are caused mainly by bacteria, although the other pathogens including fungi, viruses and parasites can also cause the infections (Smee et al., 2013a). Although UTIs may arise from ascending or descending, the most UTIs proceed in an ascending manner by invasion of bacterial flora from gastrointestinal tract and surrounding skin via the urethra (Smee et al., 2013a). *Escherichia coli* is the most common bacterial pathogen associated with UTIs in dogs, followed by *Staphylococcus*, *Proteus*, *Klebsiella*, and *Pseudomonas* species (Ling et al., 2001; Kroemer et al., 2014; Sycamore et al., 2014; Windahl et al., 2014). The UTIs occur in approximately 14% of dogs in their lifetimes, with variable age of onset (Thompson et al., 2011). Canine UTIs often become refractory because of persistent infection, relapse and reinfection (Seguin et al., 2003). Therefore, the UTIs are one of the most commonly encountered bacterial infections in dogs.

Antimicrobial treatment is required for companion animals with bacterial UTIs (Weese et al., 2011). Amoxicillin and trimethoprim-sulfadiazine are good first-line options for canine UTIs (Weese et al., 2011). On the other hand, fluoroquinolones, as well as third-generation cephalosporins, are representative second-line drugs (Thompson et al., 2011). The fluoroquinolone drugs exhibit an excellent bactericidal activity against numerous Gram-negative and Gram-positive bacteria by preventing the DNA replication through inhibition of DNA gyrase (topoisomerase II) and

topoisomerase IV (Walker, 2000; Davis et al., 2006). Thus, the usage of fluoroquinolones is usually considered in antimicrobial treatments for cases with refractory UTIs (Weese et al., 2011). However, a recent study revealed that the rate of fluoroquinolone susceptibility in canine and feline UTI pathogens was lower than that in the pathogens from human (Tsuyuki et al., 2017), because of the spread of fluoroquinolone-resistant bacteria in companion animals. Such the emergence and increase of fluoroquinolone-resistant bacteria in companion animals are a matter of great concern to not only veterinary medicine but also public health (Guardabassi et al., 2004). Furthermore, biofilm formation, defined as communities of bacteria encased in an extracellular matrix (Richards et al., 2009), contributes to shield bacteria from both antimicrobials and the host immune response (Anderson et al., 2004). Biofilm-producing pathogens, in addition to antimicrobial-resistant bacteria, are recognized as one factor of refractory UTIs in dogs, as well as humans (Thompson et al., 2011; Oliveira et al., 2014). Therefore, evidence-based and reasonable use of antimicrobials, including fluoroquinolones, is required to combat refractory cases of canine UTI.

In clinical practices, the efficacy of antimicrobial drugs, including fluoroquinolones, is predicted based on *in vitro* results of the antimicrobial susceptibility test using isolated bacteria (Turnidge et al., 2007). In recent years, however, emphasis has been placed on the importance of combining pharmacokinetic (PK) and pharmacodynamic (PD) data as a basis for predicting potential antimicrobial efficacy in a patient (Boothe et al., 2006). The representative PK parameters of antimicrobial drugs are the maximum plasma drug concentration (C_{max}) and the area under the plasma drug concentration versus time curve (AUC) (Mckellar et al., 2004). On the other hand, the major PD

parameter is the minimum inhibitory concentration (MIC) (Mckellar et al., 2003). The PK/PD indices obtained by integrating PK and PD parameters are useful to connect dosage regimens to clinical effects and vice versa (Ahmad et al., 2016). Among PK/PD indices, most commonly associated with fluoroquinolone efficacy are the ratio of C_{max} to MIC (C_{max}/MIC) and the ratio of area under the plasma drug concentration versus time curve to the MIC (AUC/MIC) (Boothe et al., 2006; Ahmad et al., 2016).

To predict the antimicrobial efficacy for UTIs, further PK and PD parameters are required to be taken into account. One reason why is that the drug concentration in urine is not necessarily correlated with that in serum (Turnidge et al., 2007). Another reason is that components in urine are greatly different from those in serum and interstitial fluid (Drobot et al., 1996). These reasons evoke the need to investigate urinary PK and PD for optimization of antimicrobial treatment for UTIs. In human medicine, urinary bactericidal titer (UBT) is developed to evaluate urinary PK/PD of antimicrobial drugs. The UBTs are an indicator of bactericidal activity in urine obtained after administration of antimicrobial drugs, and are believed to predict antimicrobial efficacy for UTIs (Hofbauer et al., 1997; Well et al., 1998; Wagenlehner et al., 2009). However, the UBT model has not yet been applied to animals including dogs.

In the fluoroquinolone treatment for UTIs, the following PD parameters are also emphasized: minimum biofilm eradication concentration (MBEC) and mutant prevention concentration (MPC). The MBEC is the parameter indicating antimicrobial efficacy against biofilm-producing pathogens (Richards et al., 2009). The MBECs are particularly significant for the second-line drugs, such as fluoroquinolones, that used for refractory UTI, including biofilm-related UTIs (Thompson et al., 2011; Weese et al., 2011). On the other hand, the MPC is the minimum antimicrobial agent concentration

that prevents selection of resistant mutants (Blondeau, 2009). Fluoroquinolone resistance is mainly due to the acquisition of point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV (Piddock, 1999). Such QRDR mutations are facilitated within the mutant selection window (MSW), defined between MICs and MPCs (Awji et al., 2012; Gebru et al., 2012). The MSW theory is emphasized in fluoroquinolone treatment for several infections including UTIs (Hansen et al., 2005; Intorre et al., 2007). However, little is known about MPCs and MBECs of veterinary fluoroquinolones for canine UTI pathogens.

Orbifloxacin (OBFX) is a fluoroquinolone that was developed for use in veterinary medicine, and has achieved a relatively large sales volume (approximately 137 kg in 2011) among fluoroquinolones for companion animals in Japan (National Veterinary Assay Laboratory, 2015). Compared with the other fluoroquinolones, OBFX has high tissue transferability and insusceptibility to metabolism by introducing a cis-3, 5-dimethylpiperazinyl at the 7 position of the quinolone structure (Yamaguchi et al., 1991). Thus, this drugs transfers to high concentrations in the major organs including liver, kidney, lung, muscle, and small intestine, and then is excreted from the kidney without largely metabolized in the body (Hooper et al., 1985; Matsumoto et al., 1997). In Japan, OBFX is indicated for the treatment of various bacterial infections including UTIs, skin infection, and otitis external in companion animals.

The present study was conducted to investigate the new PK/PD parameters (i.e. UBTs, MBECs, and MPCs) of OBFX to establish evidence-based treatment of this drug for canine UTI. In chapter 1, the author evaluated urinary concentration and UBTs of OBFX in urine obtained from dogs administered with the drug to evaluate urinary PK/PD of OBFX in dogs. In chapter 2, the author determined the MBECs of OBFX in

canine UTI pathogens to assess the effect of biofilm formation on efficacy of the drug.

In the last chapter, the author clarified differences in the likelihood of emergence of fluoroquinolone-resistant mutants among major bacterial pathogens.

Chapter 1

Assessment of urinary pharmacokinetics and pharmacodynamics of orbifloxacin in healthy dogs with *ex vivo* modeling

1. Introduction

Antimicrobial treatment is required for dogs with UTI (Weese et al., 2011). Successful antimicrobial treatment is based on site-specific PK/PD principles (Wagenlehner et al., 2004). In dogs with UTIs, most bacteria are present in the urine within the urinary tract, including the kidneys, ureters, urinary bladder and urethra (Weese et al., 2011). Therefore, drug concentrations and antimicrobial activity in the urine (urinary PK/PD) can indicate the treatment efficacy of antimicrobial drugs (Wagenlehner et al., 2004; Wagenlehner et al., 2009). An *ex vivo* model has been established in humans to determine UBTs, which can serve as a PK/PD assessment of antimicrobial agents in the urine (Wagenlehner et al., 2009). However, the UBT model has not yet been applied to dogs.

The aim of the present study was to use liquid chromatography-tandem mass spectrometry (LC-MS/MS) to investigate the urinary PK of OBFX in dogs. A further aim was to measure UBTs and related parameters of OBFX against the common UTI pathogens of dogs.

2. Materials and Methods

1) Sampling of urine from dogs administered OBFX

Animal experiments were conducted under an ethics committee-approved protocol

in accordance with the Tottori University Animal Use Committee (approval number: 14–T–18). Six beagle dogs (5 males and 1 female; mean weight, 11.0 ± 1.4 kg) were purchased from Kitayama Labes Co. Ltd., Nagano, Japan. Three dogs were 10–13 years of age; the remaining three dogs were 2 years old (Table 1). Prior to this study, all dogs were confirmed to be clinically healthy based on physical examination, complete blood count, biochemical blood test and urinalysis. A balloon catheter was placed in the urinary bladder of each dog to allow urine collection. Dogs were orally administered OBFX (Victas®, provided by DS Pharma Animal Health Co. Ltd., Osaka, Japan) at a dose of 5 mg/kg body weight, which is the approved dose in the treatment of canine UTIs in Japan. Whole urine was obtained via the catheter at 4, 8, 12, 16, 20 and 24 h after administration; samples were sterilized by filtration and stored at −80°C until analysis.

2) Measurement of urine OBFX concentration with LC-MS/MS

Reference standard OBFX and lomefloxacin (LMFX) as the internal standard were separately dissolved in acetonitrile and then diluted with ultrapure water. LC-MS/MS was carried out with a Nexera ultra-high-performance liquid chromatograph equipped with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Co. Ltd., Kyoto, Japan). An electro-spray ionization source interface operating in positive-ion mode was applied for multiple reaction monitoring. The precursor ion of OBFX and LMFX were represented by peaks at m/z 396.10 and m/z 352.10, respectively. The product ion mass spectrums of OBFX were m/z 352.10 and m/z 295.15, whereas those of LMFX were

m/z 265.10 and m/z 307.90. The interface settings were as follows: nebulizing gas flow, 3 L/min; desolvation line temperature, 250°C; heat block temperature, 400°C; and drying gas flow, 10 L/min. The compounds were separated on a 2-mm internal diameter × 50-mm length, 3-μm analytical column operated at 40°C (Mastro C18; Shimadzu GLC Ltd., Tokyo, Japan). The mobile phase comprised 0.1% formic acid aqueous solution and acetonitrile, and the flow rate was 0.3 mL/min. The injection volume was 0.1 μL. Standard samples for creation of a calibration curve were prepared with blank urine matrix spiked with four concentrations of OBFX (1, 10, 50 and 500 μg/mL). Standard and dog urine samples (50 μL) were mixed with 100 μg/mL of LMFX (50 μL) for the internal standard and methanol (400 μL). After centrifugation at 13,000 rpm for 5 min, the supernatants were harvested, and then diluted 100-fold with ultrapure water for analysis. The validity of the LS-MS/MS assay was verified, according to the guideline by the US Food and Drug Administration (2001). The area under the time-urine concentration curve during the first 24 h after administration (urinary $AUC_{0\text{-}24}$) was calculated with the trapezoidal rule. The half-life time ($T_{1/2}$) in urine was calculated by linear regression of the semi-logarithmic plot of urine concentration versus the midpoint of the urine collection time.

3) Test organisms

The following 14 bacterial strains from the urine of dogs with UTI were used in this study: *E. coli* (strains EC1-EC6), *P. aeruginosa* (strains PA1 and PA2), *K. pneumoniae* (strains KP1 and KP2), *P. mirabilis* (strains PM1 and PM2) and *S. pseudintermedius*

(strains SP1 and SP2). Strains of *K. pneumoniae* and *S. pseudintermedius* were kindly provided by Mr. Y. Tsuyuki (Sanritsu Zelkova Veterinary Laboratory); the other strains were selected from the collected isolates (Harada et al., 2012a, 2012b & 2014).

4) Determination of MIC and minimum bactericidal concentration

MICs of OBFX in cation-adjusted Mueller-Hinton broth (CAMHB) against all strains were determined using the broth dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2013a). Based on MICs, all strains were categorized as susceptible, intermediate or resistant, according to the MIC breakpoint established by the CLSI (2013b). For quality control, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were used. The minimum bactericidal concentration (MBC) were also determined after MIC determination, and were defined as the minimum concentration of drug needed to kill ≥99.9% of viable organisms after incubation for 24 h, according to CLSI guidelines (1999).

5) Determination of UBT, the area under the UBT-versus-time curve and minimum urinary bactericidal concentration

UBTs are corresponding to the maximal dilution titer of urine allowing bactericidal activity, and were determined as described previously (Well et al., 1998). A logarithmic serial two-fold dilution was prepared using a 1:1 mixture of the urine sample obtained at every 4 hours after administration (see section ‘Sampling of urine from dogs

administered OBFX'), and the subject's individual antimicrobial-free urine obtained prior to drug administration. UBTs were determined using a microdilution test system. Each well of the microplates contained 100 μL of the prepared dilution. The final inoculum was about 5×10^5 colony-forming units (CFU)/mL. The plates were incubated at 35°C for 18 h. Then the subcultured urine was transferred to antimicrobial-free agar. The plates were incubated at 35°C overnight. The number of colonies subsequently grown was used to determine the bactericidal endpoint. The UBT was defined as a ≥99.9% reduction of the initially inoculated colony counts. A UBT of 0 was defined as no bactericidal activity and a UBT of 1 was assigned when only undiluted urine displayed bactericidal activity. UBTs were transformed into ordinal data and described with reciprocal numbers (Wagenlehner et al., 2009).

The UBT-versus-time curve (AUBT) was calculated as the sum of the products of the reciprocal UBT values and the respective time (h) intervals for each test organism, to easily compare UBT data among the tested strains. The calculation of AUBT values is an approximation considering the time intervals of 4 h and the nonlinear kinetics in urine (Wagenlehner et al., 2009).

The minimum urinary bactericidal concentration (MUBC) for each strain was determined by dividing the antimicrobial concentration in a urine sample by the corresponding UBT (Well et al., 1998).

6) Statistical analysis

The median values of UBT, AUBT and MUBC between six dogs were calculated

from the average value of the two middle elements. Spearman's rank correlation coefficient (ρ) was calculated between parameters (i.e. urine concentration vs. median UBT, MIC vs. median AUBT, and MBC vs. median MUBC). A *P*-value of < 0.05 was considered significant for all analyses.

3. Results

1) Urine concentration

In this study, the LC-MS/MS assay showed lower limit of quantitation at 1 ng/mL for OBFX in dog urine. The temporal changes in urine OBFX concentration in each dog are shown in Table 1. The maximum concentration periods were 0–4 h (n = 2), 4–8 h (n = 3) and 8–12 h (n = 1). The maximum urinary concentration (U_{max}) and urinary $AUC_{0\text{-}24}$ ranged from 118.82 to 611.33 μg/mL (383 ± 171 μg/mL) and from 1982.20 to 5758.58 h·μg/mL (4461 ± 1509 h·μg/mL), respectively. The $T_{1/2}$ in urine ranged from 6.04 to 9.06 h (6.82 ± 1.14 h).

2) MICs and MBCs

Table 2 shows the MICs determined with the broth microdilution method, ranging from 0.03 to 128 μg/mL. Three strains of *E. coli* were categorized as susceptible, one as intermediate and two as resistant. One strain of *P. aeruginosa* was categorized as susceptible and one as intermediate. Two strains of *K. pneumoniae* and two strains of *P.*

mirabilis were categorized as susceptible. In addition, one strain of *S. pseudintermedius* was categorized as susceptible and one as intermediate. The MBCs ranged from 0.06 to 128 μg/mL, one to four times the corresponding MIC for each strain.

3) UBTs and AUBTs

The temporal changes of median UBTs for each strain are shown in Fig. 1. The median UBTs of OBFX peaked at 0–4 or 4–8 h after administration and then gradually decreased for all strains, except for two resistant strains of *E. coli*, in which most median UBTs were consistently 0 during the 24 h after administration. The highest median UBTs (range) of OBFX for strains EC1, EC2, EC3 and EC4 were 1536 (256–2048), 768 (4–1024), 16 (8–64) and 24 (1–64), respectively. In the strains other than *E. coli*, the highest median UBTs (range) for strains PA1, PA2, KP1, KP2, PM1, PM2, SP1 and SP2 were 64 (16–256), 12 (4–32), 160 (4–1024), 96 (16–256), 48 (16–128), 64 (16–64), 80 (1–128) and 3 (1–16), respectively.

Of the tested strains, the highly susceptible (MIC of less than 1 μg/mL) strains (n = 5) had the highest median AUBTs (1045–16,768), followed by less susceptible (MIC of 1 μg/mL) strains (n = 4, 225–652), intermediate (MIC of 2–4 μg/mL) strains (n = 3, 53–229) and resistant (MIC of more than 4 μg/mL) strains (n = 2, 2–5). The Spearman's rank correlation coefficient between MICs and median AUBTs was −0.968 ($P < 0.01$).

4) MUBCs

The median values (ranges) of MUBCs in tested strains are shown in Table 2. In OBFX-susceptible and intermediate strains, the median MUBCs ranged from 0.25 to 59.8 μg/mL. Ratios of median MUBC to corresponding MBC ranged from 2.2 to 15.0, and median MUBCs significantly correlated with MBCs ($\rho = 0.925$, $P < 0.01$). In contrast, median MUBCs of OBFX-resistant strains could not be determined because of extremely low UBTs.

4. Discussion

To date, many antimicrobials, including OBFX, have been approved for treating UTIs in dogs. However, little is known about the urinary PK/PD of veterinary antimicrobials. Accumulation of knowledge and better understanding of PK/PD principles is essential to promote evidence-based veterinary medicine. This is the first report to investigate the urinary PK/PD of veterinary drugs in dogs by using LC-MS/MS and an *ex vivo* model established in humans.

Unlike the maximum drug concentration time (T_{max}) in blood, there has been little investigation of the urinary T_{max} of veterinary antimicrobials in dogs. This study showed that the urine concentration of OBFX peaked at 0–4, 4–8, or 8–12 h after oral administration, whereas the serum T_{max} value was previously reported to be 1.7 ± 0.2 h after oral administration (Matsumoto et al., 1997). In addition, $T_{1/2}$ in urine was comparable with those in serum after oral administration at dose of 2.5 and 5.0 mg/kg body weight (7.1 and 6.51 h, respectively) (Matsumoto et al., 1997; Heinen, 2002). This study also clarified the extremely high U_{max} of OBFX, which was over 100 times higher

than the maximum drug concentration in plasma (C_{max}: 3.29 μg/mL) after oral administration at the same dose (Matsumoto et al., 1997). Similarly in the dogs orally administered with enrofloxacin, the U_{max} of enrofloxacin plus ciprofloxacin reached over 100 times C_{max} (Monlouis et al., 1997). These findings are likely explained by the fact that both drugs are eliminated mainly by renal excretion (Brown, 1996).Such urinary PK may elucidate that once daily administration of OBFX, as well as enrofloxacin, maintains high concentrations in dog urine up to 24 h.

In the present study, temporal UBTs and AUBTs of OBFX for 12 bacterial strains were determined for six dogs. In all strains except the EC6 strain, the period of maximum UBTs were 0–4 and/or 4–8 h after administration, similar to the urinary T_{max}. In addition, the median UBTs in susceptible and intermediate strains fluctuated closely with the urine concentration during the same period. Thus, it is likely that the temporal UBTs of OBFX strongly reflect its urinary PK in dogs. In contrast, AUBTs, which reflect the overall UBT values, greatly depended on the respective MIC of each strain. A similar finding was confirmed in a study on the urinary PK/PD of fluoroquinolone drugs in humans (Wagenlehner et al., 2006). Therefore, the UBTs and AUBTs can be parameters to estimate urinary PK/PD of antimicrobials in dogs, as well as in humans.

Breakpoints are usually established on the basis that they are relevant at all sites of infection. However, this assumption vastly increases the complexity of breakpoint setting, especially in infections where drug concentrations are substantially different, such as urinary tract infections (Turnidge et al., 2007). The CLSI (2013b) has defined the MIC breakpoint of OBFX for UTI in dogs as ≥8 μg/mL. The reasonableness of the breakpoint is supported by these results that OBFX concentration in dog urine was

maintained to have bactericidal activity against the susceptible and intermediate strains up to 24 h after oral administration, but not against the resistant strains.

The author found broad interindividual variability in the median UBTs and AUBTs for the same strain, although significant differences in these parameters between ages were not confirmed. Likewise, an approximately 22-fold variation of UBT and AUBT values was confirmed among seven different human patients with UTIs (Wagenlehner et al., 2009). In this study, the interindividual variation in these values might be explained by the differences in maximum urinary concentration and peak concentration period among individuals. Further studies would be needed to clarify whether such variable urinary PK of OBFX can cause interindividual differences in clinical efficacy.

It is known that fluoroquinolones exhibit lower antimicrobial activity in human urine than in standard microbiological media such as CAMHB (Miyazaki et al., 1996; Well et al., 1998). In the present study, MUBCs were calculated in each test strain based on urine concentration and UBTs to assess the activity of OBFX in dog urine. As a result, the median MUBCs were approximately 2–15-fold higher than the corresponding MBCs. These findings indicate that the antimicrobial activity of OBFX against bacterial species tested in this study decreases in dog urine, and can explain the reason why OBFX in dog urine had no antibacterial activity against the resistant strains in spite of the high drug concentration in urine. In humans, the activity of fluoroquinolones in urine depends on the urine pH, osmotic pressure and the concentrations of various solutes, mainly cations (Naber, 2001; Hofbauer et al., 1997; Drobot et al., 1996). These factors might similarly affect the activity of fluoroquinolones in dog urine; however, verification of this speculation needs further study. At least, such a decrease in

antimicrobial activity in dog urine implies that the urine concentration of antimicrobials does not fully indicate the PD of the drug in urine.

In conclusion, the urinary PK/PD was assessed of OBFX in dogs with LC-MS/MS and an *ex vivo* model. The fluctuation of UBTs closely correlated with that of urine concentration, and UBT values depended on the susceptibility of the bacterial strains to OBFX. The present data support the reasonableness of the CLSI breakpoint for OBFX for UTIs in dogs when administered at 5 mg/kg once daily. The author strongly believes that the UBTs and the related parameters are important indicators of urinary PK/PD of antimicrobials indicated for UTIs in dogs as well as humans.

Table 1. Urinary $AUC_{0–24}$ of OBFX after a single oral dose of 5 mg/kg in six healthy dogs.

			Urine concentration (μg/mL) for the following collection period (h)*						Urinary AUC_{0-24}	$T_{1/2}$ (h)
Animal	Age	Gender	0–4	4–8	8–12	12–16	16–20	20–24	(h·μg/mL)	in urine
Dog 1	11	Male	**278.58**	271.34	107.81	88.25	73.79	34.94	3348.92	6.95
Dog 2	13	Male	357.07	**363.62**	206.91	128.77	92.87	24.64	4646.29	6.29
Dog 3	10	Male	431.7	**461.39**	246.37	144.19	104.76	66.61	5686.88	6.26
Dog 4	2	Female	**611.33**	215.21	193.18	177.83	97.37	83.00	5345.67	6.04
Dog 5	2	Male	107.45	**118.82**	93.33	78.66	64.57	65.44	1982.20	9.06
Dog 6	2	Male	16.18	370.76	**465.86**	322.75	240.15	47.87	5758.58	6.31
Average			300.39	300.19	218.91	156.74	112.25	53.75	4461.42	6.82

* The maximum urine concentration is indicated by boldface.

Table 2. The MIC, MUBC and area under the AUBT of OBFX for the 14 bacterial strains tested in this study.

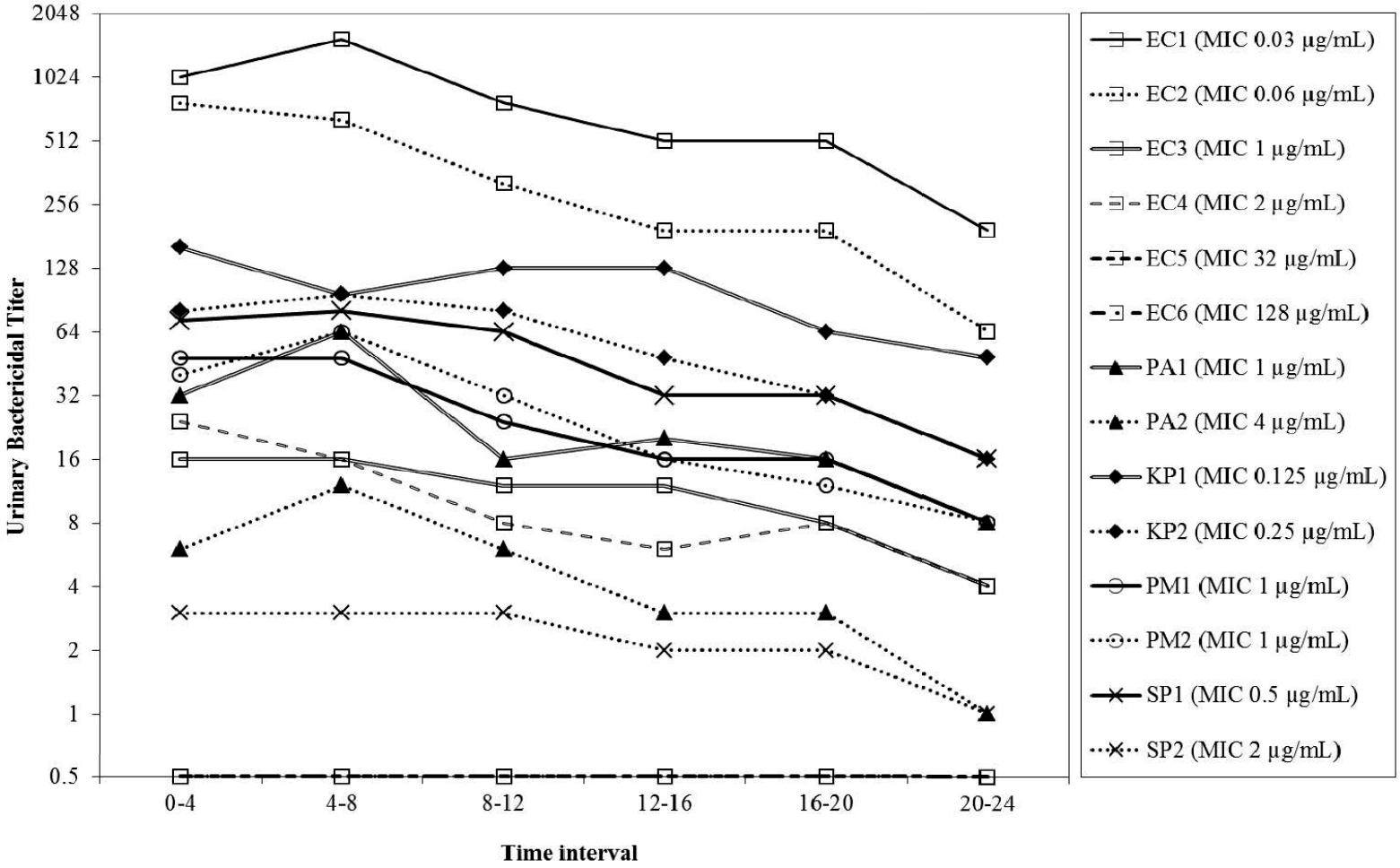
Species	Strain	MIC (μg/mL)	Category*	MBC (μg/mL)	MUBC (μg/mL)		MUBC/MBC	AUBT	
					Median	Range		Median	Range
E. coli	EC1	0.03	S	0.06	0.25	0.1–0.7	4	16768	3584–28672
	EC2	0.06	S	0.06	0.6	0.1–2.7	10	7328	2184–30720
	EC3	1	S	2	15.2	0.1–23.7	7.6	225	136–2144
	EC4	2	I	2	14.4	2.2–26.2	7.2	229	136–2816
	EC5	32	R	32	ND†	ND†	ND†	5	0–14
	EC6	128	R	128	ND†	ND†	ND†	2	0–10
P. aeruginosa	PA1	1	S	4	8.7	1.3–10.9	2.2	536	296–2024
	PA2	4	I	8	38.6	22.1–80.7	4.8	109	34–276
K. pneumoniae	KP1	0.125	S	0.125	1.4	0.50–3.3	10.9	2140	576–8448
	KP2	0.25	S	0.25	2.9	1.0–11.8	11.4	1304	416–4224
P. mirabilis	PM1	1	S	2	8.0	3.5–11.8	4.0	548	242–1040

	PM2	1	S	2	6.8	3.2–11.8	3.4	652	272–928
S. pseudintermedius	SP1	0.5	S	1	3.4	2.1–29.6	3.4	1045	172–1568
	SP2	2	I	4	59.8	27.7–133.7	15.0	53	26–32

[*] Strains were categorized as susceptible (S), intermediate (I) or resistant (R), based on CLSI breakpoints for OBFX for UTI in dogs (CLSI, 2013b).

[†] Not determined because of extremely low UBT (median 0).

Figure 1. Reciprocal UBTs of OBFX (5mg/kg) for the 14 bacterial strains tested in this study.



Chapter 2

Determination of minimum biofilm eradication concentrations of orbifloxacin for canine bacterial uropathogens over different treatment periods

1. Introduction

To select antimicrobials that effectively eliminate bacterial biofilms, the susceptibility of biofilms to various antimicrobial drugs must be measured (Morck et al., 1994). The Calgary biofilm method is a newly developed assay for rapid and reproducible determination of the MBEC of a drug (Ceri et al., 1999). In general, MBECs are determined following 24 hr of exposure to the antimicrobials, as previously reported (Ceri et al., 1999; Antunes et al., 2010; Naparstek et al, 2014). However, MBECs for extended exposure to antimicrobials need to be investigated to estimate clinical efficacy against canine biofilm-related UTIs because several guidelines recommend at least a 7-day course of antibiotic treatment for this type of infection (Weese et al., 2011; Smee et al., 2013b).

Previous *in vitro* studies have demonstrated that almost all clinical *E. coli* and *S. pseudintermedius* isolates from dogs can produce biofilms (Oliveira et al., 2014; Singh et al., 2013). Likewise, most *P. mirabilis*, *K. pneumoniae*, and *P. aeruginosa* isolates from human patients have been identified as biofilm producers (Shikh-Bardsiri et al., 2013; Seifi et al., 2016; Azimi et al., 2016). It is likely that these bacteria can induce biofilm-related UTIs in dogs; however, MBECs of veterinary antimicrobials against these pathogens remain to be investigated.

The aim of this study was to investigate the MBECs of veterinary OBFX (Cazedey et al., 2013), trimethoprim-sulfamethoxazole (TMS) and amoxicillin/clavulanate (ACV) against uropathogenic *E. coli* over several different treatment periods. Further, the

author aimed to compare the MBECs of OBFX for common UTI pathogens of dogs.

2. Materials and Methods

1) Tested strains

Thirty bacterial strains were used in this study, including 10 *E. coli*, five *S. pseudintermedius*, five *P. aeruginosa*, five *K. pneumoniae* and five *P. mirabilis* strains. *S. pseudintermedius* strains obtained from different dogs with UTIs were kindly provided by Mr. Y. Tsuyuki (Sanritsu Zelkova Veterinary Laboratory, Kanagawa, Japan). Several other strains were randomly selected from the collected isolates (Harada et al., 2012a, 2012b, 2014 & 2016) on the basis of the following criteria: i) isolates obtained from different dogs with UTIs, and ii) fluoroquinolone-susceptible isolates (in the case of *E. coli*, isolates susceptible to TMS and ACV, in addition to fluoroquinolones).

2) Determination of MICs

The MICs of OBFX, TMS and ACV for *E. coli* were determined using a broth microdilution method, according to the guidelines of the CLSI (2013a). The MIC of OBFX was also determined for all other bacteria using the same method.

3) MBEC determination

The MBECs of OBFX, TMS and ACV were determined for *E. coli* according to a previously described protocol (Melchior et al., 2007), whereas the MBEC of OBFX was determined for all other species. Briefly, a 1.0 McFarland standard suspension of each strain was prepared in tryptic soy broth and then diluted 30-fold in tryptic soy broth. An aliquot (150mL) of each dilution was dispensed into an individual well of a 96-well plate, after which a peg lid (Immuno TSP; Nunc, Roskilde, Denmark) was placed into each well. The plates were incubated for 24 hr at 37°C with shaking (150 rpm) to allow bacterial biofilms to grow on the pegs. Following incubation, several pegs were removed from the lid and the number of bacteria within biofilms on them determined by 10-fold serial dilutions (i.e. 10^6–10^7 CFU/peg). The remaining pegs were dipped for 1 min into the wells of a 96-well plate containing 200 mL of 0.85% saline solution (rinse plate) to remove any loosely adherent planktonic cells from the biofilms. The peg lids were then transferred into the wells of a 96-well plate, each containing 200mL of Mueller-Hinton broth (MHB) supplemented with two-fold-diluted antimicrobial solution (challenge plate). The plates were then incubated at 37°C for 24, 72 or 168 hr. During the incubation period, challenge plates were freshly prepared every 24 hr. Following challenge, the peg lids were washed twice in fresh rinse plates for 1 min each wash, and then placed into a 96-well plate containing 200 mL of MHB per well (recovery plate). The pegs were then sonicated for 1 hr to disrupt the biofilms and the peg lids discarded. The original microtiter plate lids were replaced, and the plates incubated at 37°C for 24 hr. Following incubation, the lowest concentration of antimicrobial that prevented visible growth of the bacterium was deemed to be the MBEC. Additionally, the MBECs of OBFX for *E. coli* were determined in dog urine, in addition to MHB. Each experiment was performed three times; the results are shown

as median values.

4) Statistical analysis

The Wilcoxon matched-pairs signed-rank test was used to compare MICs and MBECs, as well as the 24 hr MBECs with the 72 or 168 hr MBECs for each drug and bacterial species. $P < 0.05$ was considered to denote significance for all analyses.

3. Results

1) OBFX, TMS, and ACV MBECs for canine uropathogenic *E. coli*

The MICs and MBECs determined following 24, 72, or 168 hr exposure of the 10 *E. coli* strains to the three tested drugs are presented in Table 3. For OBFX, TMS, and ACV, the 24 hr MBECs (medians: 64–2048, 16/304–>256/4864, and >512/256 μg/mL, respectively) were significantly higher than the MICs ($P < 0.05$), while the 72 hr MBECs (medians: 0.25–64, 4/76–256/4864, and 8/4–128/64 μg/mL, respectively) and 168 hr MBECs (medians: 0.25–2, 0.25/4.75–2/38, and 4/2–512/256 μg/mL, respectively) were significantly lower than the 24 hr MBECs ($P < 0.05$). The median MBECs of OBFX, TMS, and ACV were assessed according to the CLSI guidelines on susceptibility breakpoints for canine UTIs (1, 2/38, and 8/4 μg/mL, respectively) (CLSI, 2013b). Most of *E. coli* strains had susceptible MBECs for all three drugs after 72 and/or 168 hr exposure, with the exception of several strains. For example, after both 72

and 168 hr exposure, the EC1 and EC2 strains had non-susceptible MBECs for ACV and the EC9 strain had non-susceptible MBECs for OBFX. After 72 hr exposure, the majority (8/10) of the strains had MBECs classed as susceptible for OBFX, whereas 0/10 and 3/10 of the strains had susceptible MBECs for TMS and ACV, respectively. After 168 hr exposure, most or all of the strains had susceptible MBECs for all three drugs.

2) OBFX MBECs for other canine uropathogenic bacteria

The MIC and MBEC values of OBFX for the four non- *E. coli* bacterial species (*S. pseudintermedius*, *P. aeruginosa*, *K. pneumoniae*, and *P. mirabilis*) are presented in Table 4. Like *E. coli*, the 24 hr OBFX median MBECs were significantly higher than the MICs ($P < 0.05$) for each bacterial species. In the three species other than *P. aeruginosa*, all strains had low OBFX median MBECs (4 μg/mL) after 168 hr exposure, whereas between strain variations were observed in OBFX median MBECs after 72 hr exposure: SP5 strain had a relatively high concentration (128 μg/mL) in *S. pseudintermedius*, whereas KP3 and PM4 strains had relatively low concentrations (1 and 4 μg/mL, respectively) in *K. pneumoniae* and *P. mirabilis*, respectively. The 72 and 168 hr OBFX median MBECs for *S. pseudintermedius* and *K. pneumoniae* and the 168 hr MBEC for *P. mirabilis* were significantly lower than the 24 hr MBECs ($P < 0.05$). In *P. aeruginosa*, no significant difference was observed between 24 hr MBECs (medians: 32–>2048 μg/mL) and 72 hr MBECs (medians: 2048–>2048 μg/mL) or 168 hr MBECs (medians: 64–>2048 μg/mL; $P < 0.05$).

3) Comparison between OBFX MBECs for *E. coli* in MHB and dog urine

The MBECs of OBFX for *E. coli* strains in MHB and dog urine were shown in Table 5. In dog urine, as well, as MHB, the 72 and 168 hr MBECs were significantly lower than the 24 hr MBECs ($P < 0.05$). There were no significant differences in the 72 and 168 hr MBECs between dog urine and MHB, although the 168 hr MBECs in urine were significantly higher than that in MHB ($P < 0.05$).

4. Discussion

To estimate the clinical efficacy of OBFX, a second-line drug for the treatment of canine UTIs, the author investigated the MBECs of OBFX and other major first-line drugs (TMS and ACV) for uropathogenic canine *E. coli* strains. The author determined that the 24 hr MBECs of all three drugs were significantly higher than the respective MICs, as previously reported for other veterinary pathogens (Melchior et al., 2007; Olson et al., 2002). Antunes et al. (Antunes et al., 2010) have reported that all strong, moderate and weak biofilm-producing isolates have higher 24 hr MBECs than MICs, whereas the MICs and MBECs of non-biofilm producers were identical in their study. These findings imply that all *E. coli* strains used in this study are biofilm producers and that the demonstrated resistance to the tested drugs was attributable to biofilm formation. Therefore, 24 hr of treatment with either the first- or second-line drugs tested is unlikely to eradicate biofilm-forming *E. coli* uropathogens.

The present data also demonstrate that 72 and 168 hr challenge with the tested drugs significantly decreases the MBECs, indicating that longer courses of antibiotics should be used to eradicate biofilm-forming *E. coli* strains. When compared with CLSI susceptibility breakpoints for canine UTIs (CLSI, 2013b), the 168 hr MBECs of all three drugs were classed as susceptible for the majority of the *E. coli* strains tested. In contrast, most of the isolates had susceptible MBECs for OBFX after 72 hr, but had resistant MBECs for both TMS and ACV. These data suggest that a 3-day course of OBFX, as well as the standard 7-day course, may be sufficient to eradicate biofilm-forming *E. coli* strains. Shorter courses of antibiotics are also preferable in regard to development of resistance (Olofsson et al., 2007). Further clinical trials are needed to determine the appropriate duration of OBFX treatment for biofilm-related UTIs in dogs.

The author determined the MBECs of OBFX for *E. coli* in dog urine, in addition to MHB. As a result, although the MBECs tended to be higher than the MBECs challenged with OBFX in MHB, there was significantly difference at only 168 hr MBEC. Therefore the MBEC in MHB may be helpful in evaluating the efficacy of OBFX against biofilm formed by *E. coli* in dog urine.

The 24 hr MBECs against *E. coli* in urine were higher than the U_{max} of OBFX. However the urine MBECs decreased due to prolongation of exposure time and 72 and/or 168 hr MBECs were lower than the urinary concentration similarly the MBECs in MHB. Therefore when biofilm forms in urine, it is not able to eradicate biofilm by 1 day treatment of OBFX. It is considered that 3 or 7 days treatment of OBFX is able to eradicate the biofilm in urine of dogs because the 72 and/or 168 hr MBECs in dog urine

were lower than the urine concentration although the activity to eradicate biofilm in urine is lower than that in MHB.

In this study, the author also investigated the MBECs of OBFX for several other canine uropathogens over different treatment periods. Like *E. coli*, all *S. pseudintermedius*, *K. pneumoniae*, *P. mirabilis* and *P. aeruginosa* strains tested had high 24 hr MBECs for OBFX, implying that all strains are biofilm producers. Exposure to OBFX for 72 and/or 168 hr induced a decrease in the MBECs for *S. pseudintermedius*, *K. pneumoniae* and *P. mirabilis*, whereas the OBFX MBECs for *P. aeruginosa* were consistently extremely high for at least 168 hr. The author found that the number of bacteria within biofilm were mostly the same for the various tested bacterial species; thus, it is likely that between-species differences in MBECs are independent of the quantity of biofilm. *P. aeruginosa* has a remarkable ability to form biofilms, which renders most antimicrobial treatments ineffective (Rasamiravaka et al., 2015). This trait explains these findings, which imply that administration of OBFX alone will have negligible effects on biofilm-forming strains of *P. aeruginosa*. In recent years, several studies have identified anti-biofilm effects associated with some antimicrobials (e.g., azithromycin) and other substances (e.g., N-acetylcysteine, cranberry proanthocyanidin and lactoferrin) (El-Feky et al., 2009; Ulrey et al., 2014; Moreau-Marquis et al., 2015; Saini et al., 2015). Further studies are required to establish the efficacy of combination therapies with OBFX and antibiofilm agents against canine UTIs caused by biofilm-forming strains of *P. aeruginosa*.

In conclusion, the author investigated the MBECs by extended exposure to OBFX, together with TMS and ACV, for *E. coli* strains, and compared OBFX MBECs among

common bacterial species from dogs with UTI. These data suggest that OBFX is likely to eradicate biofilm-forming *E. coli* by shorter-term administration course, compared with TMS and ACV, although the three drugs have the similar efficacy under long-term condition. The present study also found the clear differences in OBFX MBECs among common pathogens: 72 and/or 168 h exposure of OBFX can eradicate biofilm-forming isolates of *E. coli*, *S. pseudintermedius*, *K. pneumoniae* and *P. mirabilis*, but not the strains of *P. aeruginosa*. In addition, the author demonstrated that biofilm-forming *E. coli* can be eradicated by long-term exposure of OBFX in dog urine, as well as MHB.

These data indicate that the administration period and bacterial species of uropathogens are important factors affecting the OBFX MBECs, and thus should be considered in the treatment against biofilm-related UTIs in dogs.

Table 3. MBECs of OBFX, TMS, and ACV for 10 uropathogenic *E. coli* strains from dogs after challenge for 24, 72, or 168h.

Strains	Anitimicrobials[†]	MIC (μg/mL)	MBEC (μg/mL)					
			24 h		72 h		168 h	
			Median	Range	Median	Range	Median	Range
EC1	OBFX	≤0.03	256	256	**≤0.25**	≤0.25	**≤0.25**	≤0.25
	TMS	≤0.03/0.57	256/4864	256/4864	64/1216	64/1216	**2/38**	2/38
	ACV	4/2	>512/256	>512/256	64/32	32/16–>512/256	16/8	8/4–>512/256
EC2	OBFX	0.125	256	128–256	**1**	0.5–2	**0.5**	≤0.25–0.5
	TMS	≤0.03/0.57	>256/4864	>256/4864	4/76	4/76–8/152	**0.25/4.75**	0.25/4.75–0.5/9.5
	ACV	4/2	>512/256	512–>512	32/16	16/8–64/32	16/8	8/4–256/128
EC3	OBFX	0.06	512	256–1024	**≤0.25**	≤0.25–0.5	**≤0.25**	≤0.25
	TMS	≤0.03/0.57	>256/4864	>256/4864	4/76	4/76–8/152	**1/19**	0.5/9.5–1/19
	ACV	4/2	>512/256	128–>512	**8/4**	8/4	**8/4**	4/2–16/8
EC4	OBFX	0.06	512	512	**≤0.25**	≤0.25	**≤0.25**	≤0.25
	TMS	≤0.03/0.57	>256/4864	>256/4864	8/152	4/76–16/304	**0.25/4.75**	0.25–0.5
	ACV	4/2	>512/256	>512/256	**8/4**	4/2–8/4	**8/4**	4/2–8/4
EC5	OBFX	0.125	1024	512–1024	**0.5**	≤0.25–0.5	**0.5**	≤0.25–0.5
	TMS	0.06/1.14	>256/4864	>256/4864	4/76	2/38–4/76	**0.25/4.75**	0.25/4.75

	ACV	4/2	>512/256	>512/256	32/16	32/16	**8/4**	8/4–16/8
EC6	OBFX	≤0.03	1024	512–1024	**1**	0.5–1	**0.5**	≤0.25–0.5
	TMS	≤0.03/0.57	256/4864	64/1216–256/4864	32/608	32/608–1282432	**0.5/9.5**	0.5/9.5
	ACV	4/2	>512/256	256–>512	128/64	32/16–256/128	**4/2**	4/2–8/4
EC7	OBFX	0.125	64	32–64	64	64–128	**≤0.25**	≤0.25
	TMS	0.06/1.14	16/304	16/304–32/608	8/152	4/76–16/304	**0.5/9.5**	0.5/9.5
	ACV	4/2	>512/256	>512/256	16/8	16/8–32/16	**8/4**	4/2–8/4
EC8	OBFX	0.06	256	128–256	**0.5**	≤0.25–0.5	**≤0.25**	≤0.25
	TMS	0.06/1.14	128/2432	128/2432	32/608	8/72–64/1216	**0.5/9.5**	0.5/9.5
	ACV	4/2	>512/256	>512/256	16/8	16/8–32/16	**8/4**	8/4
EC9	OBFX	≤0.03	>2048	2048–>2048	16	8–32	2	2
	TMS	≤0.03/0.57	64/1216	32/608–128/2432	16/304	16/304	**1/19**	0.5/9.5–4/76
	ACV	4/2	>512/256	>512/256	16/8	16/8–32/16	**8/4**	4/2–16/8
EC10	OBFX	≤0.03	64	64–128	**0.5**	≤0.25–2	2	1–2
	TMS	≤0.03/0.57	128/2432	64/1216–128/2432	256/4864	256/4864–>256/4864	**0.25/4.75**	≤0.03/0.57–1/19
	ACV	2/1	>512/256	>512/256	**8/4**	4/2–8/4	512/256	512/256–>512/256

Bold faces mean susceptible-level MBECs (median) based on CLSI susceptibility breakpoints for canine urinary tract infections (OBFX, ≤1 μg/mL; TMS, ≤2/38 μg/mL; ACV, ≤8/4 μg/mL).

Table 4. MBECs of OBFX for 20 bacterial uropathogens other than *E. coli* from dogs after challenge for 24, 72, or 168 h.

Species	Strains	MIC (μg/mL)	MBEC (μg/mL)					
			24 h		72 h		168 h	
			Median	Range	Median	Range	Median	Range
S. pseudintermedius	SP1	0.5	2048	2048–>2048	2	2–4	1	1–2
	SP2	0.125	128	64–512	2	0.5–4	1	0.5–2
	SP3	0.5	64	64–128	1	1–2	2	1–2
	SP4	0.5	1024	512–1024	1	1–2	2	1–2
	SP5	0.5	1024	1024–>2048	128	128	4	4
P. aeruginosa	PA1	4	>2048	>2048	2048	2048–>2048	2048	2048–>2048
	PA2	4	2048	2048	>2048	1024–>2048	>2048	2048–>2048
	PA3	2	>2048	>2048	>2048	>2048	1024	512–2048
	PA4	4	2048	1024->2048	>2048	>2048	64	64
	PA5	8	2048	256->2048	>2048	>2048	>2048	>2048
K. pneumoniae	KP1	0.25	2048	512–2048	128	16–256	0.5	≤0.25–0.5
	KP2	0.5	256	256	16	16	1	1–2
	KP3	0.25	512	512	1	1–2	1	1–2
	KP4	0.5	256	256–512	128	128–256	2	2
	KP5	0.25	2048	64–2048	64	32–64	2	1–2

P. mirabilis	PM1	1	2048	2048	2048	2048	2	2
	PM2	2	>2048	1024–>2048	2048	2048–>2048	4	2–4
	PM3	0.5	>2048	2048–>2048	2048	1024–2048	2	2
	PM4	0.5	>2048	2048–>2048	4	4	4	2–16
	PM5	0.5	>2048	>2048	1024	512->2048	4	4

Table 5. MBECs of OBFX for 10 uropathogenic *E. coli* strains in MHB and dog urine.

Strains	MIC (μg/mL)	Median MBEC (μg/mL)								
		24 hr			72 hr			168 hr		
		MHB	Urine	Ratios*	MHB	Urine	Ratios*	MHB	Urine	Ratios*
EC1	≤0.03	256	256	1	≤0.25	2	≥8	≤0.25	2	8
EC2	0.125	256	1024	4	1	4	4	0.5	2	4
EC3	0.06	512	128	0.25	≤0.25	2	≥8	≤0.25	1	4
EC4	0.06	512	16	0.031	≤0.25	2	≥8	≤0.25	1	4
EC5	0.125	1024	64	0.063	0.5	2	4	0.5	2	4
EC6	≤0.03	1024	1024	1	1	1	1	0.5	2	4
EC7	0.125	64	1024	16	64	4	0.063	≤0.25	8	32
EC8	6	256	4	0.015	0.5	2	1	≤0.25	4	8
EC9	≤0.03	>2048	1024	0.5	16	32	2	2	32	16
EC10	≤0.03	64	1024	16	0.5	64	128	2	4	2

*The MBECs in urine/those in MHB.

Chapter 3

Mutant prevention concentration of orbifloxacin: Comparison between *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus pseudintermedius* of canine origin.

1. Introduction

The incidence of canine pathogens showing decreased susceptibility or increased resistance to fluoroquinolones was reported in Japan (Harada et al., 2012a & 2012b; Onuma et al., 2012) as well as worldwide. Determination of the MPC, the antimicrobial concentration that prevents selection of resistant mutants, is important for reduction of the incidence of fluoroquinolone resistance (Blondeau, 2009). It is hypothesised that drug exposure below the MPC may promote selection of resistant strains and the concentration range between the MIC and MPC, which is referred to as the MSW, may enrich and amplify resistant mutants. Thus, MPC and MSW are important parameters for evaluation of the potential for emergence of fluoroquinolone-resistant mutants for antimicrobial-pathogen combinations (Blondeau, 2009). MPCs of fluoroquinolone in canines have been sporadically reported for single pathogens (Gebru et al., 2011 & 2012; Awji et al., 2012). However, a consistent comparison of fluoroquinolone MPCs among different pathogens has not been performed. Gebru et al. (2012) and Awji et al. (2012) found that OBFX MPCs were relatively high compared to those of other veterinary fluoroquinolones, which may be helpful in establishing a comprehensive understanding of the variation of MPCs among different pathogens.

The purpose of the present study is to investigate differences in the likelihood of emergence of fluoroquinolone-resistant mutants among major bacterial pathogens based on MPC determination and to characterize the resistance mechanism of mutants. The author compared the MPCs of OBFX among fluoroquinolone-susceptible *E. coli*, *P. aeruginosa*, and *S. pseudintermedius* strains. Mutants arising after exposure to sub-MPC

concentrations were screened for QRDR mutations and the effects of efflux pump inhibitors (EPIs) on the MICs of OBFX were determined.

2. Materials and Methods

1) Bacterial isolates

Ten fluoroquinolone-susceptible strains each of the following three bacterial species were used in this study: *E. coli* (strains E1–E10), *P. aeruginosa* (strains P1–P10), and *S. pseudintermedius* (strains S1–S10). *E. coli* and *P. aeruginosa* strains were selected from the collected isolates of urine and ear/skin samples, respectively, obtained from domestic dogs (Harada et al., 2012a & 2012b). *S. pseudintermedius* strains were isolated from swabs obtained from dogs with canine pyoderma at the Veterinary Medical Teaching Hospital, Nippon Veterinary and Life Sciences University, and at three veterinary hospitals located in Tokyo, Japan. Swabs were streaked onto mannitol salt agar (Eiken Chemical, Japan) and typical colonies were collected. Bacterial identification was carried out by Gram staining, catalase and coagulase tests, and multiplex-polymerase chain reaction (PCR) (Sasaki et al., 2010). All confirmed *S. pseudintermedius* isolates were stored at −80°C in 10% skimmed milk.

2) Determination of MPCs and mutant recovery

MPCs were determined using a previously described protocol (Pasquali et al., 2007) with slight modifications. A concentrated cellular suspension of each bacterial

strain (200 μl) containing >10^{10} CFU/mL was plated onto each of three Mueller-Hinton agar (Becton Dickinson, France) plates, which were supplemented with OBFX at a concentration equal to the MIC and six doubling dilutions higher than the MIC (i.e. 2 ×, 4 ×, 8 ×, 16 ×, 32 ×, and 64 × MIC). Plates were incubated at 37°C for 5 days because preliminary tests showed no significant differences in MPCs between incubations for 2 and 5 days, similarly with the previous report (Pasquali et al., 2007). The lowest drug concentration that prevented the emergence of mutants after the 5-day incubation period was recorded as the MPC. Each experiment was performed twice.

A mutant of each original strain (EM1–EM10, PM1–PM10, and SM1–SM10) was randomly selected from plates with a concentration of OBFX that was one dilution (i.e. twofold) lower than the MPC (sub-MPC). Each mutant was cultured on antimicrobial-free agar plates for three serial passages and then stored at −80°C until further analysis.

3) Susceptibility testing for OBFX

MICs of OBFX against the original strains and mutants were determined using the agar dilution method, according to the guidelines of the CLSI (CLSI, 2008). MICs of OBFX were also determined in the presence of EPIs: 80 μg/mL of Phe-Arg-β-naphthylamide (PAβN, Sigma-Aldrich, MO, USA) for *E. coli* and *P. aeruginosa*, and 20 μg/mL reserpine (Sigma-Aldrich) for *S. pseudintermedius*. All inoculated agar plates were incubated at 35°C for 16–20 h. *E. coli* ATCC 25922, *P. aeruginosa* ATCC27853, *S. aureus* ATCC29213, and *Enterococcus faecalis* ATCC29212 were used as quality control strains.

4) PCR amplification and DNA sequencing of QRDRs

The QRDRs of the *gyrA* and *parC* genes for *E. coli* and *P. aeruginosa* or of the *grlA* and *gyrA* genes for *S. pseudintermedius* in the original strains and in representative mutants of each original strain were amplified by PCR using previously described primers (Akasaka et al., 2001; Lee et al., 2005; Intorre at al., 2007). The amplicons were bidirectionally sequenced using the PCR primers.

5) Statistical analysis

One-way analysis of variance (ANOVA) was used to compare MPCs and MPC/MIC, C_{max}/MPC, and AUC/MPC ratios among the three bacterial species, based on the results for ten original isolates per species. A Tukey test was used to evaluate differences among the geometric means of these parameters. A Welch test was used for pairwise comparison of MICs. The threshold for significance was set at a value of $P < 0.05$ in all analyses.

3. Results

1) MICs of original strains and mutants in the presence or absence of EPIs

The results of the study are summarised in Table 6. The MICs of OBFX against the original strains and mutants were 0.063–2 μg/mL and 1–8 μg/mL, respectively, for *E.*

coli, and 1–4 μg/mL and 16–128 μg/mL, respectively, for *P. aeruginosa*. Thus, the OBFX MICs against the original strains of *P. aeruginosa* were significantly increased by drug exposure compared with those of the *E. coli* original strains (4- to 32-fold vs. 2- to 16-fold, $P < 0.05$).

For *S. pseudintermedius*, the MICs of OBFX against the original strains were 0.25–1 μg/mL. After drug exposure, the MICs of the high-susceptible strains (S1–S6) increased 1- to 4-fold, whereas those of the low-susceptible strains (S7–S10) increased 8- to 64-fold. The MICs of OBFX against the mutants of this species were widely distributed (0.5–64 μg/mL).

Addition of PAβN, an EPI, resulted in a decrease in the MICs of OBFX against the mutants and original strains of *E. coli* and *P. aeruginosa* ($P < 0.05$) by 8- to 32-fold and 32- to 512-fold, respectively. In contrast, the MICs of OBFX against the original strains and mutants of *S. pseudintermedius* were unaffected by addition of reserpine ($P > 0.05$). By comparison of the MICs of mutants for all three bacterial species, the decrease in the MIC of OBFX against *P. aeruginosa* was more pronounced, compared with those for *E. coli* and *S. pseudintermedius* ($P < 0.05$).

2) QRDR mutations in original strains and mutants

Sequence analysis of QRDRs revealed that four low-susceptible original strains (E7–E10; MIC: 0.5–2 μg/mL) and all the mutants of *E. coli* harboured one point mutation (Ser-83 to Leu or Asp-87 to Asn) in *gyrA*. In *P. aeruginosa*, four strains (PM4, PM7–PM9) harboured one point mutation (Thr-83 to Ile). No mutations were found in the *parC* gene of *E. coli* or *P. aeruginosa*.

In *S. pseudintermedius*, four low-susceptible original strains (S7–S10; MIC: 1 μg/mL) and four mutants (SM6–SM10) harboured one point mutation (i.e. Ser-80 to Ile) in *grlA*. Of these mutants, three high-level resistant mutants (strains SM7, SM8, and SM10) harboured an additional mutation (i.e. Ser84 to Trp or Leu) in *gyrA*.

Some original strains (i.e. E10, S2 and S5) gave atypical mutants without significant increases in MICs and an additional QRDR mutation emerged after drug exposure, indicating that these original strains have lower incidence of mutations.

3) MPCs and MPC/MIC ratios

The MPCs and MPC/MIC ratios for the original strains of *E. coli* were 0.5–32 μg/mL and 4–32, respectively. In this species, low-susceptible strains with one QRDR mutation (strains E7–E10) had relatively higher MPCs (8–32 μg/mL), compared with high-susceptible strains (0.5–2 μg/mL). *P. aeruginosa* exhibited similar MPCs (16–128 μg/ml) and MPC/MIC ratios (16–64) for all original strains. In *S. pseudintermedius*, the MPCs (16–128 μg/mL) and MPC/MIC ratios (16–128) in low-susceptible strains (S7–S10) with one QRDR mutation were higher than those in high-susceptible strains without QRDR mutations (S1–S6; MPC: 2–8 μg/mL and MPC/MIC: 4–16).

According to the published PK data of OBFX, the C_{max} and AUC of OBFX at a dose of 7.5 mg/kg are 6.9 mg/L and 42.9 mg.h/L, respectively, were obtained (Walker 2000). Using these data, the C_{max}/MPC and AUC/MPC ratios were calculated for *E. coli*, *P. aeruginosa*, and *S. pseudintermedius* as 0.22–13.8 (C_{max}/MPC) and 1.34–85.8 (AUC/MPC), 0.05–0.43 and 0.34–2.68, and 0.05–3.45 and 0.34–21.45, respectively. A comparison among the bacterial species showed that MPC was significantly higher, but

the AUC/MPC and C_{max}/MPC ratios were significantly lower, for *P. aeruginosa* compared to *E. coli* ($P < 0.05$). There were no significant differences in these values between *S. pseudintermedius* and the other two bacterial species. There were also no significant differences in the MPC/MIC ratios among the three bacterial species.

4. Discussion

Since introduction of the concept of the MPC, there have been numerous reports of MPCs for fluoroquinolones against Gram-positive and Gram-negative bacteria, but no comparisons of MPCs of fluoroquinolones against different bacterial species under the same experimental conditions. Thus, this is the first comparison of the MPCs of fluoroquinolones against canine pathogens, and the first determination of the MPC of OBFX against *P. aeruginosa*.

These results showed that the MPC of OBFX against *P. aeruginosa* is higher than that against *E. coli*. Pasquali et al. (2007) also found that the MPCs of enrofloxacin and ciprofloxacin are higher against *P. aeruginosa* than against *E. coli*. Collectively, these results indicate that *P. aeruginosa* has a tendency to exhibit higher MPCs for various drugs compared with *E. coli*. In contrast, the OBFX MPCs against *S. pseudintermedius* did not differ significantly from those of *E. coli* and *P. aeruginosa*. This result may be explained by the considerable variation in MPCs among the strains of *S. pseudintermedius*. Awji et al. (2012) also found that *S. pseudintermedius* exhibited a wider range of OBFX MPCs, compared with those for other veterinary fluoroquinolones. Therefore, the variable MPCs among *S. pseudintermedius* strains are likely to be due to the type of bacterial species and the susceptibility of the pathogen to

OBFX.

To examine the basis for the differences in MPCs of OBFX among the three bacterial species, the author determined the MICs and fluoroquinolone-resistance mechanisms in MPC mutants of each species. In this study, high-susceptible strains of *E. coli* lacking a QRDR mutation and low-susceptible strains with one QRDR mutation were used as original strains. The MICs of OBFX against all mutants were categorised as susceptible or intermediate based on the CLSI breakpoint criteria for OBFX (MIC ≥8 μg/mL) (CLSI, 2008), except for one strain (strain E9), which exhibited a MIC of 8 μg/mL. Sequence analysis revealed that all *E. coli* mutants harboured only one QRDR mutation in the *gyrA* gene, as also found by Gebru et al. (2011). The two types of *gyrA* mutations found in the current study (S83L and D87N) are known to cause elevated fluoroquinolone MICs in *E. coli* (Yoshida et al., 1990; Oram et al., 1991). Generally, MICs of fluoroquinolone against *E. coli* increase in correspondence to the number of QRDR mutations (Webber et al., 2001), which is the primary mechanism for fluoroquinolone resistance (Chang et al., 2007). The emergence of only one QRDR mutation in *E. coli* in this study may be mainly responsible for the failure to acquire OBFX resistance. Similarly, several studies have shown that most *E. coli* mutants from MPC plates had one or none of QRDR mutations even when parent strains with one *gyrA* mutation were used (Gebru et al., 2011; Gebru et al., 2012; Pasquali et al., 2007). These findings imply that *E. coli* rarely acquires two or more QRDR mutations in MPC experiments performed under static conditions. In contrast, all strains of *P. aeruginosa* exhibited higher OBFX MICs than the CLSI breakpoint and the MICs were significantly higher than those against *E. coli*. However, a T83I mutation, which elevates fluoroquinolone MICs (Harada et al., 2012a; Akasaka et al., 2001) was detected

in fewer mutants of *P. aeruginosa*, compared with *E. coli*, and there were no differences in MICs between *P. aeruginosa* mutants with and without QRDR mutation. These findings suggest that QRDR mutations in *P. aeruginosa* play an insignificant role in the increased MICs of OBFX against the mutants and increased MPC of OBFX.

MICs of OBFX for *P. aeruginosa* and *E. coli* mutants were significantly decreased by addition of an EPI (PAβN) but the effect of this EPI was greater on *P. aeruginosa* strains than on *E. coli* strains. Pasquali and Manfreda (2007) similarly found that the decreases in the MICs of enrofloxacin and ciprofloxacin in the presence of PAβN were more pronounced for *P. aeruginosa* than for *E. coli*. The author previously showed that efflux pumps, rather than QRDR mutations, play an important role in the development of fluoroquinolone resistance in *P. aeruginosa* (Harada et al., 2012a). Differential expression of efflux pumps in *E. coli* and *P. aeruginosa* is likely to be the main factor in the variable increases in OBFX MIC values against mutants of *E. coli* and *P. aeruginosa* and in OBFX MPCs against these two species.

In *S. pseudintermedius*, unlike *E. coli* and *P. aeruginosa*, OBFX MICs against the mutants and MPCs against the original strains differed markedly based on the susceptibility of the original strain. Sequence analysis revealed three types of QRDR mutations at codon 80 of *grlA* and codon 84 of *gyrA*, which are hotspots for mutations that decrease fluoroquinolone susceptibility in *Staphylococcus* spp., including *S. pseudintermedius* (Onuma et al., 2012; Piddock, 1999). High-susceptible original strains lacking the QRDR mutation yielded relatively low OBFX MICs for mutants, which resulted in relatively low MPCs for original strains. In contrast, low-susceptible strains with one QRDR mutation mostly exhibited an additional QRDR mutation after drug exposure and yielded relatively high MICs for mutants, which resulted in relatively high

MPCs for original strains. For *S. pseudintermedius*, the relationship between fluoroquinolone susceptibility of the original strain and the MPC value of OBFX has not been investigated previously. These results imply that fluoroquinolone susceptibility and the status of QRDR mutations in the original strains can greatly affect the MICs of OBFX against mutants and MPC values for original strains. Further studies are needed to explore these findings. Addition of an EPI did not significantly affect the MICs of OBFX against *S. pseudintermedius* mutants, consistent with the results of Awji et al. (2012). These findings suggest that efflux pumps are not responsible for conferring fluoroquinolone resistance in *S. pseudintermedius*.

Conversion of *in vitro* MPCs into clinically useful data requires use of PK/PD parameters of a drug. The C_{max}/MPC and AUC/MPC ratios are important predictors for prevention of the emergence of resistant bacteria. This study obtained these parameters based on published C_{max} and AUC values for OBFX in dogs (Walker, 2000) and found that both C_{max}/MPC and AUC/MPC for OBFX were lower in *P. aeruginosa* than in *E. coli*, although the values in these two species did not differ significantly from those of *S. pseudintermedius*. Olofsson et al. (Olfsson et al., 2006) suggested that an AUC/MPC ratio ≥22 is predictive of prevention of emergence of a fluoroquinolone-resistant mutant. Thus, these data may imply that appropriate OBFX AUC/MPC ratios cannot be achieved, especially in low-susceptible strains of *E. coli* and *S. pseudintermedius*, and in *P. aeruginosa* strains. However, the OBFX concentration may be higher at infection sites of these bacteria (i.e. urine and skin) than in serum (Matsumoto et al., 1997; Kay-Mugford et al., 2002). Thus, determination of the *in vivo* AUC/MPC ratio at each infection site is required to evaluate the practical likelihood of the emergence of fluoroquinolone-resistant mutants.

Based on urinary $AUC_{0–24}$ of OBFX in dog (Chapter 1), the urinary $AUC_{0–24}$/MPC ratios of OBFX for *E. coli* was 139.42–8922.84, which is higher than the ratio to prevent emergence of fluoroquinolone-resistant mutant (Olfsson et al., 2006). The similar result was also shown in the previous study demonstrating urinary concentration of ciprofloxacin in dogs administrated with enrofloxacin (Daniels et al., 2014). However, fluoroquinolone-resistant mutants in UTI pathogens from companion animals demonstrated an upward trend (Tsuyuki et al., 2017). One reason why is that urine concentration of veterinary fluoroquinolones increase more slowly, compared with the blood concentrations (Chapter 1), which can contribute to the prolonged transit time in MSW.

In conclusion, the results of this study showed that the MPCs and MPC/MIC ratios of orbifloxacin against *E. coli*, *P. aeruginosa*, and *S. pseudintermedius* are mainly determined by the primary resistance mechanism of each bacterial species. Notably, *E. coli* and *P. aeruginosa*, which are representative Gram-negative bacteria frequently encountered in companion animal medicine, yielded markedly different MPCs of orbifloxacin. MPCs were also affected by the susceptibility (high or low) of the original isolate, especially in *S. pseudintermedius*. Therefore, the type of bacterial species and the fluoroquinolone susceptibility of the pathogen should be taken into consideration when using fluoroquinolone drugs such as orbifloxacin in canines.

Table6. MICs and MPCs of OBFX and QRDR mutations in the *gyrA*, *parC*, and *grlA* genes of the original stains and mutants used in the study.

Parent strains and mutants[a]	MIC (μg/mL)	MIC (+EPI)[b] (μg/mL)	QRDR mutation[c]		MPC (μg/mL)	MPC /MIC	C_{max} /MPC[d]	AUC /MPC[d]
			gyrA	*parC* (*grlA*)				
E. coli								
E1	0.063	<0.015	wt	wt	1	16	6.9	42.9
E2	0.063	<0.015	wt	wt	0.5	8	13.8	85.8
E3	0.063	<0.015	wt	wt	1	16	6.9	42.9
E4	0.125	<0.015	wt	wt	2	16	3.45	21.45
E5	0.125	<0.015	wt	wt	2	16	3.45	21.45
E6	0.25	<0.015	wt	wt	1	4	6.9	42.9
E7	0.5	0.063	S83L	wt	16	32	0.43	2.68
E8	1	0.063	D87N	wt	8	8	0.86	5.36
E9	2	0.125	D87N	wt	8	4	0.86	5.36
E10	2	0.125	S83L	wt	32	16	0.22	1.34
EM1 (0.5)	1	0.125	S83L	wt	-	-		
EM2 (0.25)	1	0.125	S83L	wt	-	-		
EM3 (0.5)	1	0.125	S83L	wt	-	-		

EM4 (1)	2	0.125	S83L	wt	-	-		
EM5 (1)	2	0.25	S83L	wt	-	-		
EM6 (0.5)	2	0.125	S83L	wt	-	-		
EM7 (8)	4	0.125	S83L	wt	-	-		
EM8 (4)	4	0.25	D87N	wt	-	-		
EM9 (4)	8	0.25	D87N	wt	-	-		
EM10 (16)	4	0.125	S83L	wt	-	-		
P. aeruginosa								
P1	1	0.015	wt	wt	32	32	0.22	1.34
P2	1	0.031	wt	wt	16	16	0.43	2.68
P3	1	0.063	wt	wt	32	32	0.22	1.34
P4	2	0.125	wt	wt	64	32	0.11	0.67
P5	2	0.063	wt	wt	32	16	0.22	1.34
P6	2	0.125	wt	wt	32	16	0.22	1.34
P7	2	0.031	wt	wt	64	16	0.11	0.67
P8	2	0.063	wt	wt	128	64	0.05	0.34
P9	4	0.25	wt	wt	64	16	0.11	0.67
P10	4	0.125	wt	wt	64	16	0.11	0.67
PM1 (16)	32	0.063	wt	wt	-	-		
PM2 (8)	16	0.25	wt	wt	-	-		
PM3 (16)	16	0.5	wt	wt	-	-		

PM4 (32)	32	1	T83I	wt	-	-		
PM5 (16)	32	0.25	wt	wt	-	-		
PM6 (16)	64	0.125	wt	wt	-	-		
PM7 (32)	64	1	T83I	wt	-	-		
PM8 (64)	64	0.125	T83I	wt	-	-		
PM9 (32)	128	2	T83I	wt	-	-		
PM10 (32)	16	0.25	wt	wt	-	-		
S. pseudintermedius								
S1	0.25	0.25	wt	wt	2	8	3.45	21.45
S2	0.25	0.5	wt	wt	2	4	3.45	21.45
S3	0.25	0.5	wt	wt	8	16	0.86	5.36
S4	0.5	0.5	wt	wt	2	8	3.45	21.45
S5	0.5	0.5	wt	wt	4	8	1.73	10.73
S6	0.5	0.5	wt	wt	4	4	1.73	10.73
S7	1	1	wt	S80I	128	128	0.05	0.34
S8	1	1	wt	S80I	128	128	0.05	0.34
S9	1	1	wt	S80I	64	64	0.11	0.67
S10	1	1	wt	S80I	16	16	0.43	2.68
SM1 (1)	1	1	wt	wt	-	-		
SM2 (1)	0.5	0.5	wt	wt	-	-		
SM3 (4)	1	1	wt	wt	-	-		

SM4 (1)	2	1	wt	wt	-	-
SM5 (2)	0.5	0.5	wt	wt	-	-
SM6 (2)	1	2	wt	S80I	-	-
SM7 (64)	64	128	S84W	S80I	-	-
SM8 (64)	64	128	S84L	S80I	-	-
SM9 (32)	8	8	wt	S80I	-	-
SM10 (8)	32	32	S84L	S80I	-	-

[a] Number in parenthesis indicates the OBFX concentration (μg/mL) supplemented in the agar from which mutants were derived.

[b] MICs in the presence of EPIs

[c] *parC* of *E. coli* and *P. aeruginosa*, and *grlA* of *S. pseudintermedius*; wt, wild type; S83L, Ser-83 to Leu; D87N, Asp-87 to Asn; T83I, Thr-83 to Ile; S80I, Ser-80 to Ile; S84W, Ser84 to Trp; S84L, Ser84 to Leu.

[d] Data for maximum concentration (C_{max}: 6.9 mg/l) and area under the concentration time-curve (AUC: 42.9 mg.h/l) of OBFX (dose of 7.5 mg/kg) are from reference (Walker, 2000).

General Conclusion

The use of antimicrobial drugs contributes to the health and welfare of companion animals by treating bacterial infections, including UTIs. However, the improper use of antimicrobial drugs may lead not only to the failure of treatments but also to the development of resistant bacteria. Notably, UTIs are the frequently encountered bacterial infections in companion animal medicine, and thus the improper antimicrobial prescription for UTIs might have a significant negative impact. The PK and PD parameters are utilized to optimize dose against target pathogens and to assess risk of antimicrobial resistance in the pathogens. In the present study, the author investigated urinary PK/PD parameters, including UBTs, MBECs, and MPCs, against canine uropathogens to promote evidence-based medicine with OBFX, as a representative of veterinary antimicrobial drugs.

In the first chapter, the author assessed the urinary PK and bactericidal activity of OBFX in dogs by LC-MS/MS and an *ex vivo* modeling to determine UBTs of antimicrobials. As the result, the author confirmed that OBFX concentration in dog urine is significantly higher than that in dog serum, and closely correlates with the fluctuation of UBTs. On the other hand, the author also found that the bactericidal activity in dog urine depends on OBFX susceptibility of each bacterial strain, and furthermore, is lower than that in standard microbiological media. It is widely believed that the concentration of antimicrobials in infection sites is an indicator of efficacy of antimicrobial treatment. However, the present data indicates that the concentration of OBFX in urine does not directly reflect bactericidal activity of the drug in urine because

of potential factors decreasing the activity in urine. Such effects of property and components of urine on bactericidal activity should be comprehended for antimicrobial treatment for canine UTIs.

In the second chapter, the author investigated the MBECs of OBFX, along with TMS and ACV, for canine uropathogenic *E. coli* strains, and compared MBECs of OBFX among common bacterial species to estimate the impact of biofilm formation on antimicrobial treatment for bacterial UTIs. The results of this study indicate that 24h MBECs were significantly higher than respective MICs, regardless of bacterial species and antimicrobial agents. Among the three antimicrobials, OBFX can eradicate biofilm-forming *E. coli* more promptly, compared with TMS and ACV. The author also found the clear differences in OBFX MBECs between the uropathogens: extended exposure to OBFX can eradicate biofilm-forming isolates of *E. coli*, *S. pseudintermedius*, *K. pneumoniae*, and *P. mirabilis*, but not those of *P. aeruginosa*. These data suggest that the duration of administration and uropathogenic bacterial species are important factors affecting the MBECs of OBFX. Although unfortunately, the incidence of biofilm-related UTIs in companion animals, as well as humans, remains unknown, the impact of biofilm formation should be taken into account in antimicrobial treatment of biofilm-related UTIs in dogs.

In the last chapter, the author determined the MPCs of OBFX against *E. coli*, *P. aeruginosa*, and *S. pseudintermedius* to obtain the knowledge on prevention of fluoroquinolone-resistant mutants in the treatment for UTIs. The results of this study showed that the primary resistance mechanisms of each bacterial species were differed: QRDR mutations in *gyrA* and *parC* in *E. coli* and *S. pseudintermedius*, respectively,

whereas overexpression of efflux pumps in *P. aeruginosa*. Also, the significant differences were found in MPCs and MPC/MIC ratios between the three bacterial species possibly due to the diversity of the main resistance mechanisms. Notably, a considerable difference was found in MPCs of OBFX between *E. coli* and *P. aeruginosa*, which are representative Gram-negative bacteria frequently encountered in companion animal medicine. Furthermore, MPCs were corresponded to the MICs of the respective original strains, especially in *S. pseudintermedius*. These data indicates that bacterial species and fluoroquinolone susceptibility of original isolates might affect the incidence of fluoroquinolone-resistant mutants. Accordingly, prior species identification and susceptibility testing of pathogens are strongly recommended to prevent the development of fluoroquinolone resistance in canine UTI pathogens.

Overall, the author determined multiple important PK and/or PD parameters for the treatment with OBFX for canine UTIs, and then confirmed several significant findings. Firstly, the author would like to emphasize the results that PK parameters of OBFX in urine are different from those in blood, and, furthermore, PD parameters of OBFX in urine are different from those in microbiological media. These findings imply that ordinary concepts based on blood PKs and/or MICs cannot be necessarily applied to antimicrobial treatment with UTIs, and might be a factor causing refractory UTIs. Next, these urinary PK/PD parameters of OBFX (e.g. MBECs and MPCs) are consistently predisposed to depend on bacterial species of uropathogen. Currently, dosage and administration of antimicrobial drugs are established for each infectious disease but not for each bacterial species of pathogen. The present data raised the necessity that proper regimen of antimicrobials, including OBFX, should be designed in consideration of

characteristics of each pathogen, in addition to each infectious disease. Further studies would be needed to clarify relationship between urinary PK/PD and clinical efficacy of OBFX for canine UTIs. The findings in this thesis will contribute to successful treatment with OBFX for canine UTIs, and appeal for the need to investigate urinary PK/PD of the other veterinary antimicrobials for UTIs.

References

Ahmad, I., Huang, L., Hao, H., Sanders, P. and Yuan, Z. (2016) Application of PK/PD modeling in veterinary field: dose optimization and drug resistance prediction. BioMed Res Int. 2016, Article ID 5465678.

Akasaka, T., Tanaka, M., Yamaguchi, A. and Sato, K. (2001) Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. 45, 2263-2268.

Anderson, G.G., Martin, S.M. and Hultgren, S.J. (2004) Host subversion by formation of intracellular bacterial communities in the urinary tract. Microbes Infect. 6, 1094-1101.

Antunes, A.L., Trentin, D.S., Bonfanti, J.W., Pinto, C.C., Perez, L.R., Macedo, A.J. and Barth A.L. (2010) Application of a feasible method for determination of biofilm antimicrobial susceptibility in staphylococci. APMIS. 118, 873-877.

Awji, E.G., Tassew, D.D., Lee, J.S., Lee, S.J., Choi, M.J., Reza, M.A., Rhee, M.H., Kim, T.H. and Park, S.C. (2012) Comparative mutant prevention concentration and mechanism of resistance to veterinary fluoroquinolones in *Staphylococcus pseudintermedius*. Vet Dermatol. 23, 376-380.

Azimi, S., Kafil, H.S., Baghi, H.B., Shokrian, S., Najaf, K., Asgharzadeh, M., Yousefi, M., Shahrivar, F. and Aghazadeh, M. (2016) Presence of *exoY*, *exoU* and *exoT* genes, antibiotic resistance and biofilm production among *Pseudomonas aeruginosa* isolates in Northwest Iran. GMS Hyg Infect Control. 11, Doc04.

Blondeau, J.M. (2009). New concepts in antimicrobial susceptibility testing: the mutant

prevention concentration and mutant selection window approach. Vet Dermatol. 20, 383-396.

Boothe, D.M., Boeckh, A., Simpson, R.B. and Dubose, K. Comparison of pharmacodynamic and pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. J Vet Intern Med. 20, 1297-1306.

Brown, S.A. (1996) Fluoroquinolones in animal health. J Vet Pharmacol Ther. 19, 1-14.

Cazedey, E.C.L., Salgado and H.R.N. (2013) Orbifloxacin: a review of properties, its antibacterial activities, pharmacokinetic/pharmacodynamic characteristics, therapeutic use, and analytical methods. Crit Rev in Anal Chem. 43, 79-99.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. and Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 37, 1771-1776.

Chang, T.M., Lu, P.L., Li, H.H., Chang, C.Y., Chen, T.C. and Chang L.L. (2007) Characterization of fluoroquinolone resistance mechanisms and their correlation with the degree of resistance to clinically used fluoroquinolones among *Escherichia coli* isolates. J Chemother. 19, 488-494.

Clinical and Laboratory Standards Institute. (1999) Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline. CLSI document M26-A. Wayne, PA.

Clinical and Laboratory Standards Institute. (2008) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard - Third Edition. PA: CLSI document M31-A3 Wayne.

Clinical and Laboratory Standards Institute. (2013a) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From

Animals; Approved Standard-Fourth edition. CLSI document VET01-A4. Wayne, PA.

Clinical and Laboratory Standards Institute. (2013b) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Second Informational Supplement. CLSI document VET01-S2. Wayne, PA.

Daniels, J.B., Tracy, G., Irom, S.J. and Lakritz, J. (2014) Fluoroquinolone levels in healthy dog urine following a 20-mg/kg oral dose of enrofloxacin exceed mutant prevention concentration targets against *Escherichia coli* isolated from canine urinary tract infections. Vet Pharmacol Ther. 37, 201-204.

Davis, J.L., Papich, M.G. and Weingarten, A. (2006) The pharmacokinetics of orbifloxacin in the horse following oral and intravenous administration. J Vet Pharmacol Ther, 29, 191-197.

Drobot, G.R., Karlowsky, J.A., Hoban, D.J. and Zhanel, G.G. (1996) Antibiotic activity in microbiological media versus that in human urine: comparison of ampicillin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Antimicrob Agents Chemother. 40, 237-240.

El-Feky, M.A., El-Rehewy, M.S., Hassan, M.A., Abolella, H.A., ABD, El-Baky R.M. and Gad, G.F. (2009) Effect of ciprofloxacin and N-acetylcysteine on bacterial adherence and biofilm formation on ureteral stent surfaces. Pol J Microbiol. 58, 261-267.

Food and Drug Administration. (2001) Guidance for Industry, Bioanalytical Method Validation. URL: http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf#search=%27Bioan

alytical+Method+Validation%2C+Center+for+Drug+Evaluation+and+Research.%27. Accessed on Jan. 15, 2016.

Gebru, E., Choi, M.J., Lee, S.J., Damte, D. and Park, S.C. (2011) Mutant-prevention concentration and mechanism of resistance in clinical isolates and enrofloxacin/marbofloxacin-selected mutants of *Escherichia coli* of canine origin. J Med Microbiol 60, 1512-1522.

Gebru, E., Damte D., Choi, M.J., Lee, S.J., Kim, Y.H. and Park, S.C. (2012) Mutant prevention concentration and phenotypic and molecular basis of fluoroquinolone resistance in clinical isolates and in vitro-selected mutants of *Escherichia coli* from dogs. Vet Microbiol. 154, 384-394.

Guardabassi, L., Schwarz, S. and Lloyd, D.H. (2004) Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother. 54, 321-332

Hansen, G.T. and Blondeau, J.M. (2005) Comparison of the minimum inhibitory, mutant prevention and minimum bactericidal concentrations of ciprofloxacin, levofloxacin and garenoxacin against enteric Gram-negative urinary tract infection pathogens. J Chemother. 17, 484-492.

Harada, K., Arima, S., Niina, A., Kataoka, Y. and Takahashi, T. (2012a) Characterization of *Pseudomonas aeruginosa* isolates from dogs and cats in Japan: current status of antimicrobial resistance and prevailing resistance mechanisms. Microbiol Immunol. 56, 123-127.

Harada, K., Niina, A., Nakai, Y., Kataoka, Y. and Takahashi, T. (2012b) Prevalence of antimicrobial resistance in relation to virulence genes and phylogenetic origins among urogenital *Escherichia coli* isolates from dogs and cats in Japan. Am J Vet Res. 73, 409-417.

Harada, K., Niina, A.., Shimizu, T., Mukai, Y., Kuwajima, K., Miyamoto, T. and Kataoka, Y. (2014) Phenotypic and molecular characterization of antimicrobial resistance in *Proteus mirabilis* isolates from dogs. J Med Microbiol. 63, 1561-1567.

Harada, K., Shimizu, T., Mukai, Y., Kuwajima, K., Sato, T., Usui, M., Tamura, Y., Kimura, Y., Miyamoto, T., Tsuyuki, Y., Ohki, A. and Kataoka, Y. (2016) Phenotypic and molecular characterization of antimicrobial resistance in *Klebsiella* spp. isolates from companion animals in Japan: clonal dissemination of multidrug-resistant extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae*. Front Microbiol. 7, 1021.

Heinen, E. (2002) Comparative serum pharmacokinetics of the fluoroquinolones enrofloxacin difloxacin, marbofloxacin, and orbifloxacin in dogs after single oral administration. J Vet Pharmacol Ther. 25, 1-5.

Hofbauer, H., Naber, K. G., Kinzig-Schippers, M., Sörgel, F., Rustige-Wiedemann, C., Wiedemann, B., Reiz, A. and Kresken, M. (1997) Urine bactericidal activity of pefloxacin versus norfloxacin in healthy female volunteers after a single 800-mg oral dose. Infection. *2*5, 121-126.

Hooper, D.C. and Wolfson, J.S. (1985) The fluoroquinolones: pharmacology, clinical uses, and toxicities in humans. Antimicrob Agents Chemother. 28, 716-721.

Intorre, L., Vanni, M., Di, Bello, D., Pretti, C., Meucci, V., Tognetti, R., Soldani, G., Cardini, G. and Jousson, O. (2007) Antimicrobial susceptibility and mechanism of resistance to fluoroquinolones in *Staphylococcus intermedius* and *Staphylococcus schleiferi*. J Vet Pharmacol Ther. 30, 464-469.

Kay-Mugford, P.A, Weingarten, A.J., Ngoh, M., Zolynas, R., White, A., Katz, T., Simmons, R. and Varma, K.J. (2002) Determination of plasma and skin

concentrations of orbifloxacin in dogs with clinically normal skin and dogs with pyoderma. Vet Ther. 3, 402-408.

Kroemer, S., El, Garch, F., Galland, D., Petit, J.L., Woehrle, F. and Boulouis, H.J. (2014) Antibiotic susceptibility of bacteria isolated from infections in cats and dogs throughout Europe (2002-2009). Comp Immunol Microbiol Infect Dis. 37, 97-108.

Lee, Y.J., Cho, J.K., Kim, K.S., Tak, R.B., Kim, A.R., Kim, J.W., Im, S.K. and Kim, B.H. (2005). Fluoroquinolone resistance and *gyrA* and *parC* mutations of *Escherichia coli* isolated from chicken. J Microbiol. 43, 391-397.

Ling, G.V., Norris, C.R., Franti, C.E., Eisele, P.H., Johnson, D.L., Ruby, A.L. and Jang, S.S. (2001) Interrelations of organism prevalence, specimen collection method, and host age, sex, and breed among 8,354 canine urinary tract infections (1969-1995). J Vet Intern Med. 15, 341-347.

Matsumoto, S., Takahashi, M., Yoshida, M., Komatsu, T., Kitadai, Y., Horii, Y. and Katae, H. (1997) Absorption, distribution and excretion of orbifloxacin in dogs and cats. J Jap Vet Med Assoc. 50, 470-474. in Japanese with English summary.

McKellar, Q.A., Sanchez, Bruni, S.F. and Jones, D.G. (2004) Pharmacokinetic/pharmacodynamic relationships of antimicrobial drugs used in veterinary medicine. J Vet Pharmacol Ther. 27, 503-514.

Melchior, M.B., Fink-Gremmels, J. and Gaastra, W. (2007) Extended antimicrobial susceptibility assay for *Staphylococcus aureus* isolates from bovine mastitis growing in biofilms. Vet Microbiol. 125, 141-149.

Miyazaki, S., Matsui, T., Arakawa, S., Kamidono, S., Maeda, Y. and Mizuyama, K. (1996) Antibacterial activity of pazufloxacin in urine after administration to healthy volunteers. Jap J Chemother. 44, 90-95.

Monlouis, J.D., De, Jong, A., Limet, A. and Richez, P. (1997) Plasma pharmacokinetics and urine concentrations of enrofloxacin after oral administration of enrofloxacin in dogs. J Vet Pharmacol Ther. 20 (Suppl 1), 61-63.

Morck, D.W., Lam, K., McKay, S.G., Olson, M.E., Prosser, B., Ellis, B.D., Cleeland, R. and Costerton, J.W. (1994) Comparative evaluation of fleroxacin, ampicillin, trimethoprimsulfamethoxazole, and gentamicin as treatments of catheter-associated urinary tract infection in a rabbit model. Int J Antimicrob Agents. 2, 21-27.

Moreau-Marquis, S., Coutermarsh, B. and Stanton, B.A. (2015) Combination of hypothiocyanite and lactoferrin (ALX-109) enhances the ability of tobramycin and aztreonam to eliminate *Pseudomonas aeruginosa* biofilms growing on cystic fibrosis airway epithelial cells. J Antimicrob Chemother. 70, 160-166.

Naber, K.G. (2001) Which fluoroquinolones are suitable for the treatment of urinary tract infections? Int J Antimicrob Agents. 17, 331-341.

Naparstek, L., Carmeli, Y., Navon-Venezia, S. and Banin, E. (2014) Biofilm formation and susceptibility to gentamicin and colistin of extremely drug-resistant KPC-producing *Klebsiella pneumoniae*. J Antimicrob Chemother. 69, 1027-1034.

National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries (2015): Sales Amounts and Sales Volumes (Active Substance) of Antibiotics, Synthetic Antibacterials, Antihelmintics and Antiprotozoals.

Oliveira, M., Dias, F.R. and Pomba, C. (2014) Biofilm and fluoroquinolone resistance of canine *Escherichia coli* uropathogenic isolates. BMC Res Notes. 7, 499.

Olofsson, S.K. and Cars, O. (2007) Optimizing drug exposure to minimize selection of antibiotic resistance. Clinical Infectious Diseases. 45, S129-136.

Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G. and Read, R.R. (2002) Biofilm

bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res. 66, 86-92.

Onuma, K., Tanabe, T. and Sato, H. (2012) Antimicrobial resistance of *Staphylococcus pseudintermedius* isolates from healthy dogs and dogs affected with pyoderma in Japan. Vet Dermatol. 23, 17-22.

Oram, M. and Fisher, L.M. (1991) 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrob Agents Chemother. 35, 387-389.

Pasquali, F. and Manfreda, G. (2007) Mutant prevention concentration of ciprofloxacin and enrofloxacin against *Escherichia coli*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa*. Vet Microbiol. 119, 304-310.

Piddock, L.J. (1999) Mechanisms of fluoroquinolone resistance: an update 1994-1998. Drugs. 58(Suppl 2), 11-18.

Rasamiravaka, T., Lavtani, Q., Duez, P. and ElJaziri, M. (2015) The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. BioMed Res Int . 759348.

Richards, J.J. and Melander, C. (2009) Controlling bacterial biofilms. Chembiochem. 10, 2287-2294.

Sasaki, T., Tsubakishita, S., Tanaka, Y., Sakusabe, A., Ohtsuka, M., Hirotaki, S., Kawakami, T., Fukata, T. and Hiramatsu, K. (2010) Multiplex-PCR method for species identification of coagulase-positive staphylococci. J Clin Microbiol. 48, 765-769.

Saini, H., Chhibber, S. and Harjai, K. (2015) Azithromycin and ciprofloxacin: a possible synergistic combination against *Pseudomonas aeruginosa* biofilm-associated

urinary tract infections. Int j Antimicrob Agents. 45, 3593-3567.

Seguin, M.A., Vaden, S.L., Altier, C, Stone, E. and Levine, J.F. (2003) Persistent urinary tract infections and reinfections in 100 dogs (1989-1999). J Vet Intern Med. 17, 622-631.

Seifi, K., Kazemian, H., Heidari, H., Rezagholizadeh, F., Saee, Y., Shirvani, F. and Houri, H. (2016) Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR. Jundishapur J Microbiol. 9, e30682.

Shikh-Bardsiri, H. and Shakibaie, MR. (2013) Antibiotic resistance pattern among biofilm producing and non producing *Proteus* strains isolated from hospitalized patients; matter of hospital hygiene and antimicrobial stewardship. Pak J Biol Sci. 16, 1496-1502.

Singh, A., Walker, M., Rousseau, J. and Weese, J.S. (2013) Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. BMC Vet Res. 9, 93.

Smee, N., Loyd, K. and Grauer, G. (2013a) UTIs in small animal patients: part 1: etiology and pathogenesis. J Am Anim Hosp Assoc. 49, 1-7.

Smee, N., Loyd, K., and Grauer, G. (2013b) UTIs in small animal patients: part 2: Diagnosis, Treatment, and Complications. J Am Anim Hosp Assoc. 49, 83-94.

Sycamore, K.F., Poorbaugh, V.R., Pullin, S.S. and Ward, C.R. (2014) Comparison of urine and bladder or urethral mucosal biopsy culture obtained by transurethral cystoscopy in dogs with chronic lower urinary tract disease: 41 cases (2002 to 2011). J Small Anim Pract. 55, 364-368.

Thompson, M. F., Litster, A. L., Platell, J. L. and Trott, D. J. (2011) Canine bacterial

urinary tract infections: new developments in old pathogens. Vet J. 190, 22-27.

Tsuyuki, Y. and Takahashi, T. (2017) Isolated bacteria and their antimicrobial susceptibility through urine culture obtained from companion animals in Japan. J. J. A. Inf. D. 91, 392-398.

Turnidge, J. and Paterson, D.L. (2007) Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev. 20, 391-408.

Ulrey, R.K., Barksdale, S.M., Zhou, W. and van, Hoek, M.L. (2014) Cranberry proanthocyanidins have anti-biofilm properties against *Pseudomonas aeruginosa*. BMC Complement Altern Med. 14, 499.

Wagenlehner, F.M., Kinzig-Schippers, M., Tischmeyer, U., Wagenlehner, C., Sörgel, F. and Naber, K.G. (2006) Urinary bactericidal activity of extended-release ciprofloxacin (1,000 milligrams) versus levofloxacin (500 milligrams) in healthy volunteers receiving a single oral dose. Antimicrob Agents Chemother. 50, 3947-3949.

Wagenlehner, F.M. and Naber, K.G. (2004) Antibiotic treatment for urinary tract infections: pharmacokinetic/pharmacodynamic principles. Expert Rev Anti Infect Ther. 2, 923-931.

Wagenlehner, F.M., Wagenlehner, C., Redman, R., Weidner, W. and Naber, K. G. (2009) Urinary bactericidal activity of doripenem versus that of levofloxacin in patients with complicated urinary tract infections or pyelonephritis. Antimicrob Agents Chemother. 53, 1567-1573.

Walker, R.D. (2000) The use of fluoroquinolones for companion animal antimicrobial therapy. Aust Vet J. 78, 82-90.

Webber, M. and Piddock, L.J. (2001) Quinolone resistance in *Escherichia coli*. Vet Res.

32, 275-284.

Weese, J.S., Blondeau, J.M., Boothe, D., Breitschwerdt, E.B., Guardabassi, L., Hillier, A., Lloyd, D.H., Papich, M.G., Rankin, S.C., Turnidge, J.D. and other authors. (2011) Antimicrobial use guidelines for treatment of urinary tract disease in dogs and cats: antimicrobial guidelines working group of the international society for companion animal infectious diseases. Vet Med Int. Article ID 263768.

Well, M., Naber, K.G., Kinzig-Schippers, M. and Sörgel, F. (1998) Urinary bactericidal activity and pharmacokinetics of enoxacin versus norfloxacin and ciprofloxacin in healthy volunteers after a single oral dose. Int J Antimicrob Agents. 10, 31-38.

Windahl, U., Holst, B.S., Nyman, A., Grönlund, U. and Bengtsson, B. (2014) Characterisation of bacterial growth and antimicrobial susceptibility patterns in canine urinary tract infections. BMC Vet Res. 10, 217.

Yamaguchi, T., Yokogawa, M., Sakashita, M., Itokawa, A., Kurono, M. and Sekine, Y. (1991) Metabolism od Sparfloxacin in Rats, Dogs, Monkeys and Man. Drug Metab Dispos. 6, 21-32.

Yoshida, H., Bogaki, M., Nakamura, M. and Nakamura, S. (1990) Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob Agents Chemother. 34, 1271-1272.

Acknowledgements

The author is extremely grateful to supervisor Associate Professor Kazuki Harada, Tottori University, for his excellent guidance, criticisms in the preparation of this manuscript, invaluable advices, suggestions, and escorting the first step as a researcher.

The author would like to express the deepest appreciation to Professor Toshiyuki Murase, Professor Yoshiaki Hikasa, and Professor Takashi Takeuchi, Tottori University, and Professor Seiji Hobo, Kagoshima University, for reviewing the manuscript and their invaluable advices.

The author is particularly grateful for escorting the first step as a researcher and providing an optimum environment for the present study by Professor Takuo Sawada, Professor Toshio Takahashi and Associate Professor Yasushi Kataoka, Nippon Veterinary Life Science University. The author gratefully appreciates Associate Professor Noriyasu Sasaki in Nippon Veterinary Life Science University, Mr. Taku Tsukamoto in Shimadzu Corporation, Associate Professor Norihiko Ito in Tottori University for the elaborated guidance, invaluable advices and suggestions.

I appreciate the supporting my research by Mrs. Saki Hayashi, Mr. Koji Kawaguchi, Mr. Naoki Miyashita, and other students in Department of Veterinary Internal Medicine, Tottori University. I want to thank Husky, Hanzo, Kouta, Ririana, Daifuku, Won, Sabu and Ruigi belonging to Tottori University.

I would like to express my gratitude to my family and many friends for encouraging for my graduate life.

Finally, the author is greatly indebted to Coco, Nuts and Sam for their support throughout the present study.