



Research article

In vitro antibacterial activity of levofloxacin against Gram-negative bacteria isolated from canine urinary tract infections

Kananuch Vasuntrarak¹, Koranis Patthanachai¹, Piyawan Charoenlertkul¹, Suphachai Nuanualsuwan^{2,3},
Patrrarat Chanchaithong⁴, Thanawit Siha-umphai¹ and Nipattra Suanpairintr^{1,3,*}

¹ Department of Pharmacology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

² Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

³ Center of Excellence for Food and Water Risk Analysis (FAWRA), Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

⁴ Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

Abstract

Urinary tract infection (UTI) is a common bacterial disease in dogs, and the increasing prevalence of antimicrobial resistance among uropathogens has complicated therapeutic management. This study aimed to evaluate the in vitro antibacterial activity of levofloxacin against Gram-negative bacteria isolated from dogs with UTIs. A total of 284 Gram-negative bacterial isolates were obtained from urine samples of dogs diagnosed with UTIs. Bacterial identification was performed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The in vitro antibacterial activity of levofloxacin was evaluated by determining the Minimum inhibitory concentration (MIC) and Mutant prevention concentration (MPC) using the agar dilution method. Low susceptibility rates were observed among Enterobacterales, including *Escherichia coli* (*E. coli*) (26.96%), *Klebsiella pneumoniae* (*K. pneumoniae*) (31.37%), and *Proteus mirabilis* (*P. mirabilis*) (36.84%), whereas *Pseudomonas aeruginosa* exhibited higher susceptibility (72.13%). MPC values were consistently higher than MICs, particularly for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, suggesting an increased risk of resistance selection at high bacterial densities. Pharmacokinetic/pharmacodynamic (PK/PD) analysis indicated that the standard oral dose of levofloxacin (25 mg/kg) is likely effective only against susceptible strains, with *P. mirabilis* being the only species to achieve both MIC- and MPC-based PK/PD targets. Although levofloxacin may be effective against certain canine UTI pathogens, its use should be guided by bacterial identification and susceptibility testing. Therefore, levofloxacin should be considered a second-line therapeutic option rather than an empirical first-line treatment for canine UTIs.

Keywords: Dogs, Levofloxacin, Minimum inhibitory concentration (MIC), Mutant prevention concentration (MPC), Urinary tract infection (UTI)

Corresponding author: Nipattra Suanpairintr, Department of Pharmacology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: nipattra.d@chula.ac.th. Tel: 02-218-9731.

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INTRODUCTION

Urinary tract infection (UTI) is a common bacterial disease in dogs, and factors such as sex, age, and breed may influence its occurrence. Due to anatomical predisposition, female dogs are generally at higher risk, whereas age and breed may contribute through associated comorbidities and abnormalities of the urinary tract (Mendóza-López et al., 2017; Adsanychan et al., 2019; Sørensen et al., 2019; Mustapha and Goel, 2020; Thassakorn et al., 2025). Canine UTIs can be caused by both gram-negative and Gram-positive organisms. Among the Gram-negative pathogens, *Escherichia coli* (*E. coli*) is the most frequently isolated (Hernando et al., 2021; Darwich et al., 2021; Yudhanto et al., 2022; Temmerman et al., 2024; Facchin et al., 2025), followed by *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Hernando et al., 2021; Temmerman et al., 2024).

The International Society for Companion Animal Infectious Diseases (ISCAID) recommends that empirical antimicrobial treatment be based on regional data on pathogen prevalence and resistance (Weese et al., 2019). First-line agents include amoxicillin, amoxicillin–clavulanic acid, and trimethoprim–sulfonamides. The use of broader-spectrum drugs, such as nitrofurantoin, fluoroquinolones, and third-generation cephalosporins are advised only when resistance to first-line treatments is confirmed or the clinical condition requires their use (Weese et al., 2019).

Levofloxacin, a third-generation fluoroquinolone, has bactericidal activity against a broad spectrum of Gram-negative, Gram-positive, aerobic and some anaerobic bacteria through the inhibition of DNA gyrase and topoisomerase IV (Sitovs et al., 2021). The ISCAID guidelines include levofloxacin for canine UTI treatment at an oral dose of 25 mg/kg every 24 hours (Weese et al., 2019) and has demonstrated clinical efficacy in skin infections and UTIs in dogs (Casas et al., 2019). Pharmacokinetic studies have shown that levofloxacin has good oral absorption and high bioavailability in dogs, ranging from 60.9% to 104% (Landoni and Albarellos, 2019; Madsen et al., 2019; Urzúa et al., 2020). Although urinary concentrations have not been reported, its systemic clearance suggests substantial urinary excretion, which supports its potential as a therapeutic option for canine UTIs (Madsen et al., 2019). Although its use is more common in human medicine, interest in levofloxacin in veterinary medicine is growing, especially for extra-label use in the managing of antimicrobial-resistant infections (Papich, 2021). The global emergence of resistance to first- and second-generation fluoroquinolones has led to levofloxacin use in veterinary medicine in several countries, including Thailand, where fluoroquinolone resistance among Gram-negative canine uropathogens has become an increasing concern. A study conducted in Chiang Mai reported enrofloxacin resistance in *E. coli* isolates from canine UTIs at a rate of 55.9% (Amphaiphan et al., 2021), whereas *E. coli* accounted for 80.4% of multidrug-resistant isolates from a veterinary teaching hospital in Nakhon Pathom (Soonthornsit et al., 2022). A more recent conducted study in Bangkok documented resistance rates of 79.5% to enrofloxacin and 78.2% to marbofloxacin among *E. coli* isolates from canine UTIs (Jariyapamornkoon et al., 2024). Collectively, these findings highlight the need for evaluating levofloxacin as a potential therapeutic option for resistant UTIs in canines. However, there remain only limited susceptibility data for levofloxacin against canine uropathogens. Given the increasing resistance to commonly used fluoroquinolones, it is crucial to evaluate the in vitro antibacterial activity of levofloxacin in order to guide appropriate clinical use. Because Gram-negative organisms, particularly *E. coli*, are the most frequently isolated uropathogens and are commonly associated with concerns about antimicrobial resistance in our hospital setting, this study focused on Gram-negative bacterial isolates associated with canine UTIs.

Minimum inhibitory concentration (MIC) is a standard method for the assessment of bacterial susceptibility to antimicrobial agents and is typically

determined using a bacterial inoculum of 10^5 colony-forming units per milliliter (CFU/mL) (CLSI, 2021). However, this inoculum may not represent clinical infections with high bacterial loads, such as those in UTIs (Matsukawa et al., 2007; Scott et al., 2015). To better capture the dynamics of resistance emergence under such conditions, Mutant prevention concentration (MPC), which is defined as the lowest antimicrobial concentration that prevents the growth of first-step resistant mutants, is determined using a high-density bacterial inoculum ($\geq 10^9$ CFU/mL) to reflect the natural frequency of resistance mutations (10^6 – 10^8 CFU/mL) (Dong et al., 1999; Blondeau, 2009). Although MIC testing identifies concentrations inhibiting susceptible bacterial populations, it may not detect resistant subpopulations, particularly in infections with high bacterial burdens. In contrast, by targeting both susceptible organisms and resistant mutants, MPC testing provides a more comprehensive assessment, making it a valuable tool for the optimization of antimicrobial dosing strategies and minimization of resistance development (Blondeau et al., 2004; Blondeau, 2009). In this study, we aimed to evaluate the in vitro antibacterial activity of levofloxacin against Gram-negative bacterial isolates from canine UTIs by determining the MIC and MPC values. These data will provide support for evidence-based antimicrobial selection and dosing regimens, helping to optimize treatment outcomes while minimizing the risk of resistance development.

MATERIALS AND METHODS

Bacterial Isolates

This study included 284 Gram-negative bacterial isolates obtained from the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. All isolates were recovered from the urine samples of dogs diagnosed with UTIs at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, between 2017 and 2021. The inclusion criteria for isolate selection were Gram-negative bacterial isolates obtained from urine samples collected via cystocentesis or catheterization. According to the Veterinary Diagnostic Laboratory criteria, pure cultures yielding $\geq 10^3$ CFU/mL from cystocentesis samples and $\geq 10^4$ to 10^5 CFU/mL from catheterized samples were considered indicative of UTI (Wood, 2017; Weese et al., 2019). The Veterinary Diagnostic Laboratory previously identified all isolates using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany). The isolates included 115 *E. coli*, 51 *K. pneumoniae*, 57 *P. mirabilis*, and 61 *P. aeruginosa*. Samples were stored at -80°C in tryptic soy broth (TSB; Difco, USA) supplemented with 20% glycerol for future analysis. This study was conducted in accordance with the faculty regulations and approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (protocol code IBC 2231004).

Levofloxacin Stock Solution

Levofloxacin (98.0–102.0% on an anhydrous basis) was purchased from Sigma-Aldrich, USA (batch No. 0000521831). The stock solution was prepared following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021) by dissolving levofloxacin in sterile distilled water, with 1 mol/L NaOH added dropwise until complete dissolution. Stock solutions of the drugs were prepared fresh daily, protected from light, and kept at -20°C until use (Liu et al., 2013).

Determination of MIC

The MICs of levofloxacin against all bacterial isolates were determined using the agar dilution method, following the CLSI guidelines (CLSI, 2021). Stock isolates stored at -80°C were cultured on tryptic soy agar (TSA; Difco, USA) and incubated at 37°C for 20 hours. The bacterial suspensions were prepared from revived

isolates in 0.85% saline and adjusted to a 0.5 McFarland standard, then diluted 1:10 in 0.85% saline before inoculation onto Mueller–Hinton agar (MHA; Difco, USA) plates containing serial 2-fold concentrations (0.008–256 µg/mL) of levofloxacin. The inoculation was performed using a 48-pin replicator (Sigma-Aldrich, USA). The final inoculum on the agar was approximately 0.4×10^5 CFU/spot. Inoculation began with an antibiotic-free control plate, followed by levofloxacin plates in ascending concentrations, and ended with a second control plate to check for contamination and carryover. All plates were incubated under aerobic conditions at 37°C for 16 to 20 hours (CLSI, 2021). We determined the viable counts for each inoculum using 10-fold serial dilution and the spread plate method on TSA. All isolates were tested in triplicate, and *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were included as quality control strains (CLSI, 2021).

The lowest concentration of levofloxacin that completely inhibited visible colony formation was recorded as the MIC. Because UTI-specific breakpoints for levofloxacin have not been established in dogs, we interpreted the MIC values using the canine-specific breakpoints for skin and soft tissue infections for Enterobacterales and *P. aeruginosa* in accordance with CLSI VET01S (CLSI, 2024). The breakpoints for Enterobacterales were susceptible ≤ 0.5 µg/mL, intermediate 1 µg/mL, and resistant ≥ 2 µg/mL, whereas for *P. aeruginosa* they were susceptible ≤ 1 µg/mL, intermediate 2 µg/mL, and resistant ≥ 4 µg/mL. Because canine UTI-specific breakpoints are unavailable, we applied the criteria conservatively, and isolates categorized as intermediate were classified as resistant.

Determination of MPC

The MIC measures bacterial susceptibility at standard inoculum levels, whereas the MPC evaluates the suppression of resistant subpopulations at higher bacterial densities typical of UTIs. Levofloxacin-susceptible isolates were selected for MPC determination using a modified method based on Blondeau (2009). To generate a high-density bacterial inoculum, each isolate was initially cultured on three MHA plates and incubated under aerobic conditions at 37°C for 20 hours. All pure bacterial colonies from 3 plates were then transferred and cultured into 100 mL of freshly prepared Mueller-Hinton broth (MHB; Difco, USA) and incubated under the same conditions for 20 hours. The resulting suspension was centrifuged at $5,000 \times g$ for 30 minutes at 4°C and the bacterial pellet was resuspended in a 3-mL fresh, chilled MHB.

The suspension was adjusted with 0.85% sterile saline to approximately 10^{10} CFU/mL using a spectrophotometer (Thermo Scientific, Canada) with an absorbance of ≥ 1.0 at 600 nm. Aliquots of 100 µL from the high-density suspension were spread onto MHA plates containing levofloxacin at varying concentrations (1×, 2×, 4×, 8×, 16×, 32×, 64×, 128×, 256×, and 512×MIC). The plates were incubated under aerobic conditions at 37°C, with growth assessed every 24 hours. Growth was considered absent when no colonies were detected after 48 hours. MPC were tested in triplicate (Blondeau, 2009). To confirm bacterial concentrations, the viable counts were determined for each inoculum using serial dilution and the spread plate method on TSA. The MPC was recorded as the lowest levofloxacin concentration suppressing bacterial growth.

Data Analysis

We conducted descriptive statistical analyses using Microsoft Excel (Microsoft 365). The MIC and MPC ranges, 50th and 90th percentiles, and susceptibility rates were calculated for each bacterial species. For susceptible isolates, we also determined MIC(S), MIC(S)₅₀, and MIC(S)₉₀, along with MPC/MIC ratios. We generated the MIC and MPC distribution graphs using Microsoft Excel (Microsoft 365).

Pharmacokinetic/Pharmacodynamic Estimation

For concentration-dependent antimicrobials like fluoroquinolones, therapeutic efficacy is primarily determined by the ratio of the area of the drug concentration curve from time 0 to 24 hours to MIC (AUC_{0-24}/MIC) and secondarily by the ratio of the maximum plasma concentration to MIC (C_{max}/MIC) (Papich, 2014). We evaluated the pharmacokinetic/pharmacodynamic (PK/PD) profiles of levofloxacin for the treatment of canine UTIs using published PK data obtained following a single oral dose of 23.7 mg/kg in dogs, approximating the standard recommended dose of 25 mg/kg, with an AUC_{0-24} of 167.7 $\mu\text{g}\cdot\text{h}/\text{mL}$ and a C_{max} of 15.5 $\mu\text{g}/\text{mL}$ (Madsen et al., 2019). In vitro plasma protein binding of levofloxacin in dogs has previously been reported as $23.7 \pm 3.8\%$ (Madsen et al., 2019). However, the selected PK parameter values were used without accounting for plasma protein binding. The MIC_{50} , MIC_{90} , $MIC(S)_{50}$, and $MIC(S)_{90}$ values were used to calculate the C_{max}/MIC and AUC_{0-24}/MIC ratios, thereby assessing the adequacy of the current dosing regimen. Additionally, MPC_{50} and MPC_{90} values were incorporated into the analysis to compute C_{max}/MPC and AUC_{0-24}/MPC ratios, providing insight into the potential of the dosing regimen to suppress the emergence of resistant bacterial subpopulations. This analysis was performed using plasma-derived PK parameters, because urinary PK data for levofloxacin in dogs are currently unavailable.

RESULTS

MIC of Levofloxacin

Figure 1 shows the MIC distribution of levofloxacin against 284 Gram-negative bacterial isolates from dogs with UTIs, with MIC ranges, MIC_{50} , MIC_{90} , and susceptibility rates summarized in Table 1. According to CLSI (2024), the susceptible breakpoint for levofloxacin is $MIC \leq 0.5 \mu\text{g}/\text{mL}$ for *E. coli*, *K. pneumoniae*, and *P. mirabilis*, and $MIC \leq 1 \mu\text{g}/\text{mL}$ for *P. aeruginosa*. Among the isolates, *E. coli* showed the lowest susceptibility rate ($n=31/115$, 26.96%), followed by *K. pneumoniae* ($n=16/51$, 31.37%) and *P. mirabilis* ($n=21/57$, 36.84%), whereas *P. aeruginosa* demonstrated a higher susceptibility rate ($n=44/61$, 72.13%).

Table 1 Minimum inhibitory concentrations (MICs) of levofloxacin against 284 bacterial isolates from canine urinary tract infections.

Parameters	<i>E. coli</i> (n = 115)	<i>K. pneumoniae</i> (n = 51)	<i>P. mirabilis</i> (n = 57)	<i>P. aeruginosa</i> (n = 61)
Range of MIC ($\mu\text{g}/\text{mL}$)	0.125-64	0.125-256	0.125-128	0.125-64
MIC_{50} ($\mu\text{g}/\text{mL}$)	8	16	4	0.5
MIC_{90} ($\mu\text{g}/\text{mL}$)	32	64	32	64
Susceptibility (%)	26.96	31.37	36.84	72.13

MIC: minimum inhibitory concentration, MIC_{50} : minimum inhibitory concentration required to inhibit the growth of 50% of organisms, MIC_{90} : minimum inhibitory concentration required to inhibit the growth of 90% of organisms

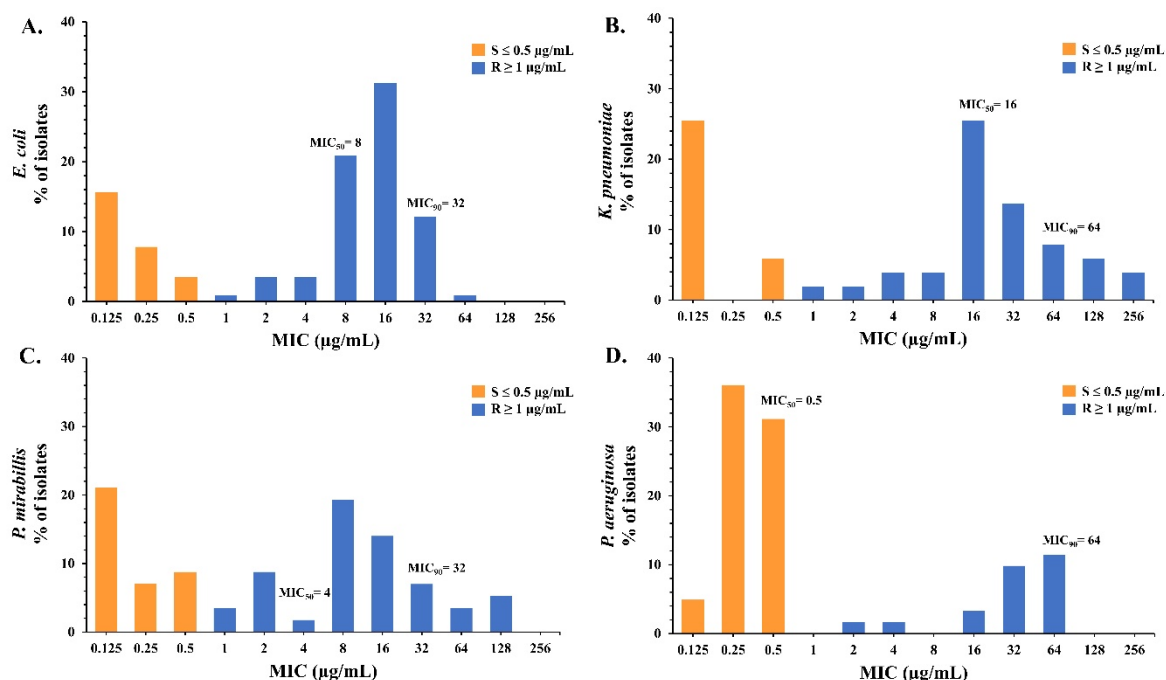


Figure 1 Distribution of Minimum inhibitory concentrations (MICs) of levofloxacin against 284 Gram-negative bacterial isolates from canine urinary tract infections. Panel A: *E. coli* (n = 115); Panel B: *K. pneumoniae* (n = 51); Panel C: *P. mirabilis* (n = 57); Panel D: *P. aeruginosa* (n = 61). S, susceptible breakpoint; R, resistant breakpoint.

MPC of Levofloxacin

A total of 112 levofloxacin-susceptible isolates were subjected to MPC determination, and Figure 2 shows the distribution of the MPC values. Table 2 summarizes the MIC(S) ranges; MIC(S)₅₀, MIC(S)₉₀, and MPC ranges; MPC₅₀, MPC₉₀, and MPC/MIC(S) ratios; and susceptibility rates for each species. Because formal MPC breakpoints have not been established, we interpreted susceptibility and resistance using MIC breakpoints (CLSI, 2024). When the bacterial inoculum was increased in the MPC assay, the susceptibility decreased in *E. coli* and *K. pneumoniae* to 29.03% (n = 9/31) and 25.00% (n = 4/16), respectively. We observed a marked reduction in *P. aeruginosa*, with susceptibility decreasing to 9.09% (n = 4/44), whereas *P. mirabilis* maintained a high susceptibility rate of 90.48% (n = 19/21). The MPC₉₀/MIC(S)₉₀ ratios were 32 for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, whereas *P. mirabilis* showed a lower ratio of 1.

Table 2 Minimum inhibitory concentrations (MICs), Mutant prevention concentrations (MPCs), and MPC/MIC ratios of 112 levofloxacin-susceptible bacterial isolates from canine urinary tract infections.

Parameters	<i>E. coli</i> (n = 31)	<i>K. pneumoniae</i> (n = 16)	<i>P. mirabilis</i> (n = 21)	<i>P. aeruginosa</i> (n = 44)
Range of MIC(S) (μg/mL)	0.125-0.5	0.125-0.5	0.125-0.5	0.125-0.5
MIC(S) ₅₀ (μg/mL)	0.125	0.125	0.125	0.25
MIC(S) ₉₀ (μg/mL)	0.5	0.5	0.5	0.5
Range of MPC (μg/mL)	0.25-128	0.25-64	0.125-32	0.5-128
MPC ₅₀ (μg/mL)	1	2	0.5	4
MPC ₉₀ (μg/mL)	16	16	0.5	16
Susceptibility (%)	29.03	25.00	90.48	9.09
MPC ₅₀ / MIC(S) ₅₀	8	16	4	16
MPC ₉₀ / MIC(S) ₉₀	32	32	1	32

MIC(S): minimum inhibitory concentration of susceptible isolates, MIC(S)₅₀: minimum inhibitory concentration required to inhibit the growth of 50% of susceptible isolates, MIC(S)₉₀: minimum inhibitory concentration required to inhibit the growth of 90% of susceptible isolates, MPC: mutant prevention concentration, MPC₅₀: mutant prevention concentration required to prevent the growth of 50% of resistant sub-population, MPC₉₀: mutant prevention concentration required to prevent the growth of 90% of resistant sub-population

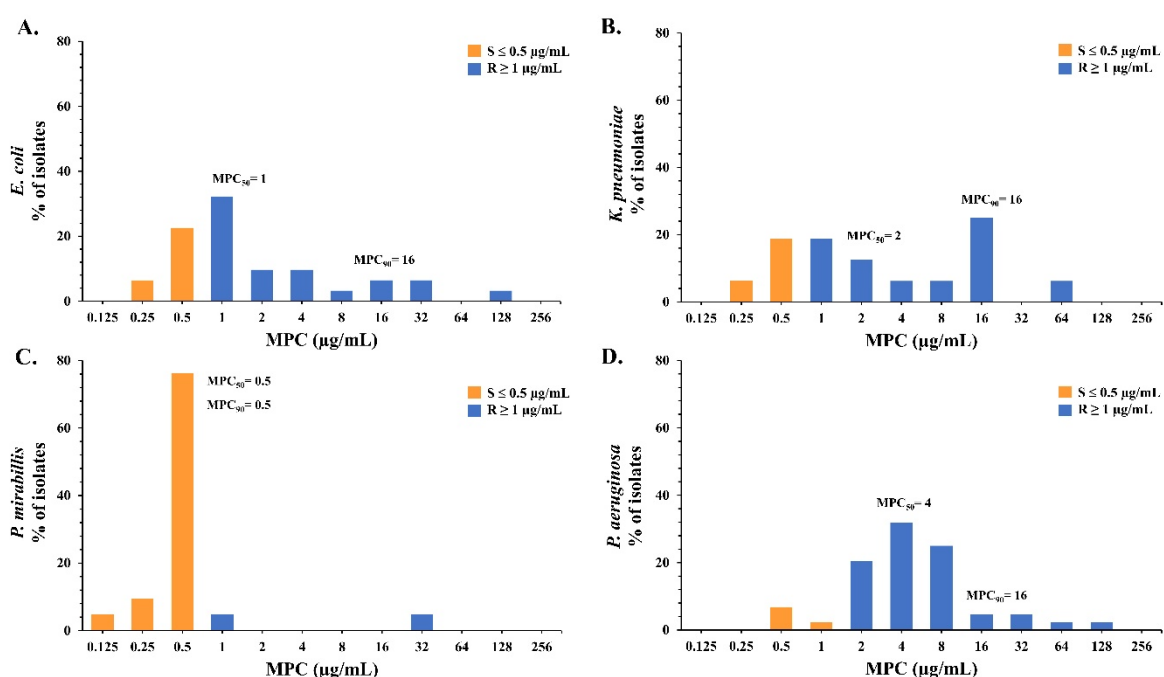


Figure 2 Distribution of Mutant prevention concentrations (MPCs) of levofloxacin against 112 levofloxacin-susceptible isolates. Panel A: *E. coli* (n = 31); Panel B: *K. pneumoniae* (n = 16); Panel C: *P. mirabilis* (n = 21); Panel D: *P. aeruginosa* (n = 44). S, susceptible breakpoint; R, resistant breakpoint.

PK/PD Estimation

Table 3 summarizes the estimated PK/PD results of levofloxacin. In this study, we used the optimal PK/PD targets for fluoroquinolones, defined as AUC₀₋₂₄/MIC ratios greater than 100–125 and C_{max}/MIC ratios ≥ 8–12 (Papich, 2014), to predict the clinical efficacy. PK/PD integration based on MIC₅₀ and MIC₉₀ values for *E. coli*, *K. pneumoniae*, and *P. mirabilis* showed that both the AUC₀₋₂₄/MIC and C_{max}/MIC ratios were below these efficacy thresholds. In contrast, based on MIC values, *P. aeruginosa* achieved target thresholds. For PK/PD analysis based on

MPC, *P. mirabilis* achieved AUC₀₋₂₄/MPC and C_{max}/MPC ratios above the target thresholds at both MPC₅₀ and MPC₉₀. In contrast, the MPC-based PK/PD indices for *K. pneumoniae* and *P. aeruginosa* remained below the efficacy thresholds.

Table 3 PK/PD integration calculated using plasma PK parameters of levofloxacin following a single oral dose (approximately 25 mg/kg) in dogs.

PK/PD	Parameters	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>P. aeruginosa</i>
PK/MIC	AUC ₀₋₂₄ /MIC ₅₀	20.96	10.48	41.93	335.4
	C _{max} /MIC ₅₀	1.94	0.97	3.88	31
	AUC ₀₋₂₄ /MIC ₉₀	5.24	2.62	5.24	2.62
	C _{max} /MIC ₉₀	0.48	0.24	0.48	0.24
PK/MIC(S)	AUC ₀₋₂₄ /MIC(S) ₅₀	1,341.60	1,341.60	1,341.60	670.80
	C _{max} /MIC(S) ₅₀	124	124	124	62
	AUC ₀₋₂₄ /MIC(S) ₉₀	335.40	335.40	335.40	335.40
	C _{max} /MIC(S) ₉₀	31	31	31	31
PK/MPC	AUC ₀₋₂₄ /MPC ₅₀	167.70	83.85	335.40	41.93
	C _{max} /MPC ₅₀	15.50	7.75	31	3.88
	AUC ₀₋₂₄ /MPC ₉₀	10.48	10.48	335.4	10.48
	C _{max} /MPC ₉₀	0.97	0.97	31	0.97

AUC₀₋₂₄: area of the drug concentration curve from time zero to 24 hours, C_{max}: peak plasma concentration, MIC₅₀: minimum inhibitory concentration required to inhibit the growth of 50% of organisms, MIC₉₀: minimum inhibitory concentration required to inhibit the growth of 90% of organisms, MIC(S)₅₀: minimum inhibitory concentration required to inhibit the growth of 50% of susceptible isolates, MIC(S)₉₀: minimum inhibitory concentration required to inhibit the growth of 90% of susceptible isolates, MPC₅₀: mutant prevention concentration required to prevent the growth of 50% of resistant sub-population, MPC₉₀: mutant prevention concentration required to prevent the growth of 90% of resistant sub-population, Plasma PK parameters were adopted from Madsen et al. (2019).

DISCUSSION

MIC of Levofloxacin

In this study, we evaluated the in vitro antibacterial activity of levofloxacin against 284 Gram-negative bacterial isolates from canine UTIs. The high level of levofloxacin resistance among *E. coli* isolates observed in this study is consistent with global concerns regarding fluoroquinolone resistance and reports of multidrug-resistant, including levofloxacin-resistant, *E. coli* in UTI cases (Jalil and Al Atbee, 2022; Mareş et al., 2024). Although researchers have reported resistance to levofloxacin in *E. coli* from various animal sources (Chen et al., 2014; Anes et al., 2020), data from canine UTIs remain limited. In contrast to our findings, a study from the United States reported a high susceptibility of canine and feline *E. coli* uropathogens to levofloxacin, including enrofloxacin-resistant isolates (Liu et al., 2013). For *K. pneumoniae*, susceptibility data for levofloxacin in animals are scarce, with most available information derived from human studies (Lin et al., 2016; Anes et al., 2020). Thus, our findings provide important additional data on the susceptibility of this pathogen to levofloxacin in dogs. With regard to *P. mirabilis*, the susceptibility rate observed in our study was considerably lower than the 92.50% reported in a study of canine and feline UTIs in Portugal (Marques et al., 2019). In contrast to Enterobacterales isolates, *P. aeruginosa* showed a relatively high susceptibility rate (n = 44/61, 72.13%), which is consistent with a previous report of 84.80% susceptibility in canine clinical isolates (Park et al., 2020), providing support for the potential use of levofloxacin for treatment. Although the MIC₅₀ remained within the susceptible breakpoint, the MIC₉₀ exceeded the threshold (≤ 1 µg/mL) (CLSI, 2024). This may be explained by a bimodal MIC distribution, in which most isolates remained susceptible while a smaller resistant subpopulation shifted the MIC₉₀ to a higher level. This finding highlights the heterogeneity of susceptibility and emphasizes the importance of antimicrobial susceptibility testing before clinical use.

The variability in levofloxacin susceptibility profiles may be attributed to differences in sample sources, animal species, and antimicrobial use practices across countries. In addition, the methodologies used for susceptibility testing and sample sizes can significantly influence the representativeness of results.

A comparison across studies is complicated by differences in breakpoint standards. Veterinary-specific levofloxacin breakpoints have historically been unavailable, and human breakpoints, which are generally higher than current veterinary criteria, have been commonly applied. Canine-specific breakpoints for Enterobacterales and *P. aeruginosa* were first introduced in 2020 (CLSI, 2020) and later updated in CLSI VET01S, 7th edition (CLSI, 2024), which we used in this study. However, veterinary UTI-specific breakpoints for levofloxacin remain unavailable. Thus, we applied the canine breakpoints established for skin and soft-tissue infections as the closest available reference criteria. This may have affected the interpretation of susceptibility, as urinary drug concentrations may differ from plasma concentrations and potentially provide greater antibacterial activity at the site of infection. Consequently, our findings should be interpreted cautiously. In addition, we classified intermediate isolates as resistant to provide a more conservative assessment of susceptibility and minimize the potential risk of resistance selection, particularly in the context of further MPC evaluation. However, this approach may result in higher reported resistance rates and limit a direct comparison with studies using standard CLSI interpretive categories.

MPC of Levofloxacin

To determine the MPC, we selected 112 levofloxacin-susceptible isolates for analysis. Because we included only susceptible isolates, the MIC(S) values representing the MIC distribution within this subgroup were calculated to more accurately reflect their baseline susceptibility. We then used these MIC(S) values to compute the MPC/MIC ratio. As anticipated, the MPC values were consistently higher than the corresponding MIC values, reflecting the greater antimicrobial concentrations required to suppress the growth of first-step resistant mutants in high-density bacterial populations (Zhao and Drlica, 2001; Drlica, 2003; Blondeau, 2009). In this study, the susceptibility of *E. coli* and *K. pneumoniae* decreased markedly at higher bacterial densities, with MPC₅₀ and MPC₉₀ values exceeding susceptibility breakpoints, indicating reduced activity under high-inoculum conditions. This is consistent with previous reports that showed substantially higher MPC than MIC values for *E. coli* uropathogens (Liu et al., 2013). In contrast, *P. mirabilis* remained susceptible, with MPC₅₀ and MPC₉₀ within the breakpoint values, indicating maintained activity against both susceptible and first-step mutant populations. For *P. aeruginosa*, the susceptibility decreased markedly under high bacterial density, with most MIC-susceptible isolates reclassified as resistant based on MPC. This finding is consistent with the results of previous studies (Hansen et al., 2006) and highlights the potential for resistance selection under high-inoculum conditions, emphasizing the value of MPC assessment for this pathogen.

The MPC/MIC ratio reflects the width of the gap between the MPC and MIC, known as the mutant selection window (MSW), which representing the concentration range in which resistant subpopulations may be selectively enriched. A wider MSW indicates a greater likelihood of resistance selection, whereas a narrower window suggests a lower risk (Drlica and Zhao, 2007). The MPC/MIC ratios varied among bacterial species, indicating differences in the potential for resistance suppression. Higher MPC₉₀/MIC(S)₉₀ ratios (32× in *E. coli*, *K. pneumoniae*, and *P. aeruginosa*) reflect a larger gap between inhibitory and mutant-preventing concentrations, which increases the likelihood that drug exposure may fall within the MSW. In contrast, the lower ratio observed in *P. mirabilis* (1×) indicates minimal separation between MIC and MPC, suggesting a reduced risk of selecting resistant subpopulations. Therefore, achieving urinary concentrations

above the MPC is critical; however, urinary PK data for levofloxacin in dogs are limited. If concentrations do not exceed the MPC, exposure may remain within the MSW, potentially promoting resistance.

Susceptible strains of *P. mirabilis* demonstrated the lowest potential for levofloxacin resistance selection under the evaluated conditions. The narrow MSW observed in this study suggests that the wild-type population may lack critical first-step resistance mutations. However, these findings should be interpreted cautiously, as they might have been influenced by the limited number of isolates and the lack of relevant clinical data. Further studies involving larger numbers of clinical isolates and characterization of resistance determinants are warranted to better understand resistance selection in *P. mirabilis* associated with canine UTIs.

Although the MIC is traditionally used as a PD parameter to guide antimicrobial dosing regimens, growing evidence supports the utility of the MPC as a more effective tool for optimizing fluoroquinolones dosing strategies aimed at preventing resistance (Vilalta et al., 2014; Zhang et al., 2018; Blondeau and Fitch, 2021; Blondeau and Fitch, 2024). The MPC represents the lowest drug concentration required to inhibit the growth of first-step resistant mutants and has therefore been suggested as an important parameter to incorporate into PK/PD modeling (Zhang et al., 2022). However, its application in routine clinical practice remains limited due to the requirement for high bacterial inoculum and extended incubation time, as well as labor-intensive manual procedures that lack automation compared with standard MIC testing (Lin et al., 2022). MPC values can vary considerably depending on the bacterial strain, drug properties, inoculum size, and mutation type, with resistant strains typically exhibiting higher MPC values than wild-type strains (Gianvecchio et al., 2019). Given this variability, MPC determinations should be interpreted on a case-by-case basis, and their limitations should be carefully considered when integrating them into strategies of resistance mitigation (Jariyapamornkoon et al., 2024).

It is important to consider that the bacterial isolates included in this study were obtained from dogs presented to a referral hospital, where prior antimicrobial treatment may have contributed to the emergence of multidrug-resistant organisms (Rampacci et al., 2018; Scarpellini et al., 2025). However, detailed clinical metadata, such as prior antimicrobial exposure, clinical presentation, and host characteristics, were not available because the isolates were archived samples submitted to the Veterinary Diagnostic Laboratory, and case-related clinical information was inaccessible, representing a limitation of this study. Future studies that incorporate clinical metadata may provide a more comprehensive understanding of the factors influencing levofloxacin susceptibility and resistance selection in canine UTIs.

PK/PD Estimation

Previous studies have demonstrated that the oral administration of levofloxacin at the recommended dose of approximately 25 mg/kg in dogs yields an AUC₀₋₂₄ of 167.7 µg·h/mL and a C_{max} of 15.5 µg/mL (Madsen et al., 2019). Although the optimal PK/PD targets for fluoroquinolones remain under discussion, AUC₀₋₂₄/MIC ratios >100–125 and C_{max}/MIC ratios ≥8–12 are widely accepted as predictive of clinical efficacy (Preston et al., 1998; Thomas et al., 1998; McKellar et al., 2004; Papich, 2014). In the present study, PK/PD integration based on MIC₅₀ and MIC₉₀ values for *E. coli*, *K. pneumoniae*, and *P. mirabilis* revealed that AUC₀₋₂₄/MIC and C_{max}/MIC ratios fell below these efficacy thresholds. These results suggest that the standard oral dose of levofloxacin may be insufficient for treating infections caused by these pathogens, particularly when higher MICs are present. In contrast, *P. aeruginosa* showed AUC₀₋₂₄/MIC and C_{max}/MIC ratios that exceeded the target thresholds when calculated using MIC₅₀, but not MIC₉₀, which indicate that although the current dosing regimen may be effective in approximately half of the cases, it may not provide consistent coverage across all isolates.

When the focus is placed exclusively on levofloxacin-susceptible isolates, all bacterial species demonstrated $AUC_{0-24}/MIC(S)$ ratios that exceeded 125 and $C_{max}/MIC(S)$ ratios ≥ 12 . These results suggest that levofloxacin at the recommended dose is likely to be effective for treating infections caused by susceptible Gram-negative uropathogens. These findings further highlight the importance of conducting antimicrobial susceptibility testing before the initiation of levofloxacin therapy. PK/PD analysis supports the use of oral levofloxacin at 25 mg/kg when pathogens fall within susceptibility breakpoints ($MIC \leq 0.5 \mu\text{g/mL}$ for Enterobacterales and $\leq 1 \mu\text{g/mL}$ for *P. aeruginosa* (CLSI, 2024).

PK/PD integration based on MPC values varied by species. Only *P. mirabilis* achieved the target AUC_{0-24}/MPC and C_{max}/MPC ratios at both MPC_{50} and MPC_{90} , suggesting that levofloxacin at the recommended dose may reduce the likelihood of mutant selection. In contrast, the current regimen may only partially limit mutant selection in *E. coli* and appears to be insufficient for *K. pneumoniae* and *P. aeruginosa*. These PK/PD results may help inform dosing considerations for levofloxacin in canine UTI cases.

This study has several limitations that should be considered when interpreting the findings. The PK parameters were derived from a single dataset following the oral administration of levofloxacin at approximately 25 mg/kg, a dose consistent with current clinical recommendations; however, this might not capture interindividual variability in drug exposure and attainment of the PK/PD target. Moreover, in this study we based the PK/PD analysis on plasma concentrations. Although fluoroquinolones are generally considered to achieve high urinary concentrations (Frazier et al., 2000; Daniels et al., 2014; Dowling, 2024), direct evidence for levofloxacin in dogs is limited; however, its systemic clearance suggests substantial urinary excretion (Madsen et al., 2019). Consequently, plasma-based PK/PD indices may underestimate antibacterial activity at the site of infection, particularly in UTIs. For example, isolates predicted to have suboptimal PK/PD target attainment based on plasma data may still exhibit a clinical response if urinary drug concentrations exceed the MIC or even the MPC. These findings should therefore be interpreted cautiously, and studies that incorporate urinary PK are needed to better predict clinical outcomes. In addition, in vitro findings may not fully predict in vivo efficacy because treatment outcomes are influenced by multiple factors, including host condition, immune status, bacterial load, and environmental variables.

CONCLUSIONS

In this study we evaluated the in vitro activity of levofloxacin against Gram-negative uropathogens from canine UTIs. The MIC results revealed high resistance rates among *E. coli*, *K. pneumoniae*, and *P. mirabilis*, whereas *P. aeruginosa* exhibited comparatively greater susceptibility. MPC and PK/PD analyses indicated that the recommended oral dose of levofloxacin (25 mg/kg) is likely effective for only susceptible strains, emphasizing the need for susceptibility testing before treatment. *P. mirabilis* displayed the most favorable MPC/MIC and PK/PD profiles, whereas other species posed a higher risk of resistance selection at high bacterial densities ($\geq 10^9$ CFU/mL) used for MPC determination. These findings highlight the importance of individualized antimicrobial selection and support the integration of both MIC and MPC data into therapeutic decision-making to optimize clinical outcomes and limit the development of resistance. Levofloxacin remains a viable option for canine UTIs caused by susceptible pathogens; however, to preserve its clinical utility, its use should be judicious and guided by laboratory data. Future investigations that incorporate urinary drug concentration measurements are warranted to better reflect in vivo efficacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Kananuch Vasuntrarak: Conceptualization (Equal); Formal analysis (Lead); Investigation (Equal); Methodology (Equal); Writing – original draft (Lead).

Koranis Patthanachai: Formal analysis (Supporting); Investigation (Supporting).

Piyawan Charoenlertkul: Formal analysis (Supporting); Investigation (Supporting).

Suphachai Nuanualsuwan: Conceptualization (Supporting); Supervision (Supporting); Writing – review and editing (Supporting).

Patrrarat Chanchaithong: Conceptualization (Supporting); Supervision (Supporting); Writing – review and editing (Supporting).

Thanawit Siha-umphai: Conceptualization (Supporting); Formal analysis (Supporting); Investigation (Equal); Methodology (Equal).

Nipattra Suanpairintr: Conceptualization (Equal); Methodology (Equal), Project administration (Lead); Supervision (Lead); Writing – review and editing (Lead).

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