

Effects of oral orbifloxacin on fecal coliforms in healthy cats: a pilot study

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ABSTRACT. The study objective was to determine the effect of oral orbifloxacin (ORB) on antimicrobial susceptibility and composition of fecal coliforms in cats. Nine cats were randomized to two groups administered a daily oral dose of 2.5 and 5.0 mg ORB/kg for 7 days and a control group (three cats per group). Coliforms were isolated from stool samples and were tested for susceptibilities to ORB and 5 other drugs. ORB concentration in feces was measured using high-performance liquid chromatography (HPLC). The coliforms were undetectable after 2 days of ORB administration, and their number increased in most cats after termination of the administration. Furthermore, only isolates of *Escherichia coli* were detected in all cats before administration, and those of *Citrobacter freundii* were detected after termination of the administration. *E. coli* isolates exhibited high ORB susceptibility [Minimum inhibitory concentration (MIC), $\leq 0.125 \mu\text{g/ml}$] or relatively low susceptibility (MIC, $1-2 \mu\text{g/ml}$) with a single *gyrA* mutation. *C. freundii* isolates largely exhibited intermediate ORB susceptibility (MIC, $4 \mu\text{g/ml}$), in addition to resistance to ampicillin and cefazolin, and harbored *qnrB*, but not a *gyrA* mutation. HPLC revealed that the peaks of mean concentration were 61.3 and $141.0 \mu\text{g/g}$ in groups receiving 2.5 and 5.0 mg/kg, respectively. Our findings suggest that oral ORB may alter the total counts and composition of fecal coliform, but is unlikely to yield highly fluoroquinolone-resistant mutants of *E. coli* and *C. freundii* in cats, possibly because of the high drug concentration in feces.

KEY WORDS: antimicrobial resistance, fecal coliforms, feline, orbifloxacin

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Orbifloxacin (ORB) is a synthetic antimicrobial agent of the fluoroquinolone (FQ) class and has a wide range of antibacterial activity and high bioavailability [14]. In companion animal medicine, ORB is approved for treatment of several bacterial infections, such as urinary tract infection and skin infection, and has been widely used in many countries [1, 32].

The development of FQ-resistant bacteria increases the risk of FQ treatment failure in companion animals. In addition to an effect on animal health, the prevalence of FQ-resistant bacteria may have important consequences for human public health, if the resistant isolates or resistance determinants are transmitted to humans from their pets [15, 20]. Understanding the development of FQ resistance is important not only from a veterinary perspective but also from a global public health perspective. FQ resistance is mainly acquired by the modification of target enzymes, i.e. DNA gyrase and topoisomerase IV; however, it may also involve the acquisition of plasmid-mediated quinolone resistance (PMQR) determinants [13]. Such acquisition of FQ resistance is closely associated with selective pressure resulting from the use of FQ drugs [25].

Coliforms, including *Escherichia coli*, are representative commensal bacteria in the gut of animals and can act as an indicator of antimicrobial resistance [11, 30]. Notably,

most FQ drugs after administration migrate to the gut and urine [21, 24]; therefore, the gut flora, including coliforms, is likely exposed to FQs in animals administered with the drugs. To assess the effect of FQ use on the fecal or gut flora, experiments with FQ administration have been previously conducted on various animals, such as pigs [4], chickens [22] and dogs [29]. However, the similar experiments have not yet been performed on cats, a representative species of companion animals.

In this study, we assessed antimicrobial susceptibility, bacterial species and the number of fecal coliforms in cats treated with two specific doses of ORB, as well as untreated cats. We also determined several genetic mechanisms of FQ resistance in coliform bacteria isolated from treated and untreated cats.

MATERIALS AND METHODS

Study design and enrolled cats: Nine domestic short-haired cats living at a research facility were enrolled; mean age was 6.11 ± 1.71 years, and body weight was 3.51 ± 0.42 kg. All cats were selected from a research colony maintained under standard laboratory conditions at the Tottori University. None of the cats had received antimicrobials for at least six months prior to the study, and all cats were deemed healthy based on a physical examination and a hematological examination. The cats were randomized into three groups (groups A, B and C) of three cats each. The cats in groups A and B received a daily oral dose of 5 and 2.5 mg ORB/kg (DS Pharma Animal Health, Osaka, Japan), respectively, for 7 consecutive days, as approved dosages in Japan. The cats in group C served as a control. Further, the cats were fed commercial dry cat food and received no medication other

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than ORB during the study. All cats were housed in separate cages located in one room, and direct contact among the cats was prevented during the study. Only when administered with ORB, the cats were separately led out of each cage. Authors contacting with the cats wore a new disposable glove in each case. This study was approved by Tottori University Animal Use Committee (approval number, 13-T-29).

Isolation and identification of fecal coliforms: Stool samples were collected from each of the 9 cats on days 1, 3, 5, 7, 9, 11, 13, 15 and 17. On day 1, the sample was obtained before drug administration. Serial 10-fold dilutions were then prepared from 1 g of each stool sample in 0.1% peptone water. Once the appropriate dilution was prepared, 0.1 ml was plated onto eosin methylene blue (EMB) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing either no drugs or ORB at the concentration of 2 µg/ml, which was defined as the concentration of intermediate susceptibility in to the Clinical and Laboratory Standards Institute Guidelines [8]. On EMB agar, coliform colonies develop a metallic luster, other gram-negative bacteria appear colorless, and gram-positive bacteria cannot grow. The inoculated plates were incubated at 35°C for 24 hr, and the number of coliform bacteria was enumerated as colony-forming units (CFU)/g of feces. A maximum of 10 coliform colonies per cat per sampling were picked up and subjected to identification of bacterial species. Isolates were confirmed to be *E. coli* by gram staining, the typical colony shape on deoxycholate hydrogen sulphide lactose agar (Nissui Pharmaceutical Co., Ltd.) and detection of the *uid* gene by PCR [3]. When isolates were not identified as *E. coli*, they were identified using the API 20E Kit (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan). After bacterial identification, the isolates were stored in 10% skim milk at -80°C for antimicrobial susceptibility testing and genetic analysis. The remaining stool samples were frozen at -80°C until high performance liquid chromatography (HPLC) analysis.

Antimicrobial susceptibility testing: Susceptibility testing against ORB was conducted using the agar dilution method, according to the Clinical and Laboratory Standards Institute Guidelines [9]. In addition, susceptibilities to ampicillin (AMP), cefazolin (CEZ), tetracycline (TET), chloramphenicol (CHL), kanamycin (KAN) and trimethoprim/sulfamethoxazole (SXT) were determined using the disk diffusion method [9]. The results were interpreted as per criteria of the CLSI guidelines [8]. *E. coli* ATCC 25922 was used as the quality control strain. In CEZ-resistant isolates, AmpC β-lactamase and extended-spectrum β-lactamase (ESBL) were phenotypically screened using cefoxitin disks (30 µg/disk) and cefotaxime (3 µg), respectively; the results were considered positive, if the inhibition zone diameters were ≤14 and ≤27 mm, respectively [10, 28]. Further, AmpC-positive isolates were defined as derepressed AmpC mutants or inducible AmpC producers, as previously described [18].

Analysis of the mechanism of FQ resistance: Isolates with an ORB MIC of ≥1 µg/ml were assessed for the presence of mutations of the quinolone resistance-determining region (QRDR) and PMQR determinants.

The QRDR of the *gyrA* gene was amplified by PCR with

previously described primers [12]. The resulting amplicons were bidirectionally sequenced using the same primers. The QRDR of the *parC* gene was also amplified and sequenced using previously described primers [12], when QRDR mutations were detected in *gyrA*.

PMQR genes were detected using multiplex PCR as previously reported [7]. Any ambiguous PCR results were clarified with repeat assays. PCR products were then randomly selected and bidirectionally sequenced with the same primers for confirmation.

Enterobacterial repetitive intergenic consensus sequence-based PCR: Enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) was carried out to investigate epidemiologic relationship among the PMQR-positive isolates. The procedure was slightly modified from the previous studies [27, 31]. Briefly, the PCR reaction was set up in a 20 µl reaction volume containing 2 µl of a 10 × ExTaq buffer, 1 U of ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan), 0.25 mM each of the dNTPs, 10–30 ng of bacterial DNA and 20 pmol of each primer (i.e. ERIC1R and ERIC2). DNA amplifications were performed with an initial denaturation (7 min at 94°C) followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°C) and extension (8 min at 65°C) with a final extension (15 min at 65°C).

Measurement of ORB concentration in feces: Stool samples were obtained from cats in groups A and B on days 1, 3, 5, 7 and 9 and from cats in group C on days 1 and 9. The concentration of ORB in feces was determined at the Research Institute for Animal Science in Biochemistry and Toxicology (Sagamihara, Japan). In brief, ORB in stool samples was extracted with acetonitrile containing 1% formic acid and purified via liquid–liquid partition, salting-out and a mini-column (Oasis MAX[®], Nihon Waters K.K., Tokyo, Japan). ORB was analyzed using an HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a binary pump, an autosampler, a column heater and a fluorescence detector. For separation, Supelcosil Abzplus (Sigma–Aldrich Co., LLC, Tokyo, Japan) was concurrently used with LiChroCART 4-4 LiChrospher 100 RP-18 Guard Column (Merck, Tokyo, Japan). Limitation of quantitation was determined as 0.1 µg/g. The quality control was carried out by analyzing stool samples together with samples of known concentrations (i.e. 0.2 and 2 µg/g) every time.

Statistical analysis: Standard one-way analysis of variance with the Tukey–Kramer multiple comparison test was used to compare the mean age and body weight of the enrolled cats, and mean CFU/g of stool samples among groups A, B and C. The Mann–Whitney *U* test was used to compare mean concentrations of ORB in feces between groups A and B. Fisher's exact test was used to compare rates of antimicrobial resistance between groups and periods (i.e. before and after treatment). A *P* value of <0.05 was considered as statistically significant in all analyses.

RESULTS

Enrolled cats: There were no differences in age or body weight among the three groups (*P*>0.05). No adverse effects

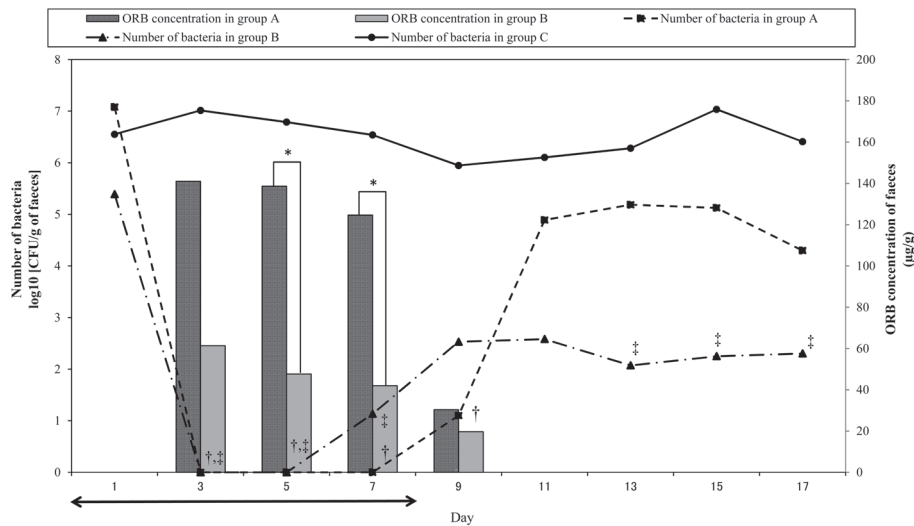


Fig. 1. The means of the total number of coliforms and ORB concentration in the feces of cats in groups A, B and C during the test schedule. In groups A and B, ORB was administered orally daily at a dose of 5.0 and 2.5 mg/kg, respectively, on days 1–7 (↔). See Supplemental Figs. 1 and 2 for the total number of coliforms and ORB concentration, respectively, in each cat. †, ‡ Significantly lower number of coliforms in groups A and B, respectively, compared with group C ($P < 0.05$). * There were significant differences between groups A and B ($P < 0.05$).

of the drug were noted in any cat.

Total number of coliforms in cats: The total number of coliforms during the test schedule is shown in Fig. 1 and Supplemental Fig. 1. According to the one-way analysis of variance and the Tukey–Kramer multiple comparison test, pre-treatment coliform counts in groups A, B and C were not significantly different (7.08 ± 0.53 , 5.39 ± 0.72 and 6.55 ± 1.11 , respectively).

In groups A and B, within 3 days of ORB administration, the number of fecal coliforms decreased rapidly and continued to be significantly lower than that of group C ($P < 0.05$), until day 9 or 7, respectively. After cessation of ORB treatment, coliform counts in the cats of groups A and B mostly reached detectable levels by days 9 (cat 3) and 11 (cats 1 and 2) and by days 7 (cat 5) and 9 (cat 6), respectively. There were no significant differences in coliform counts between groups A and C from day 11 ($P > 0.05$). On the other hand, significant differences were found in coliform counts between groups B and C ($P < 0.05$) on days 13, 15 and 17, because of extremely low counts of coliform in cats 4 and 6. Significant differences between groups A and B were not seen during the study period ($P > 0.05$). No remarkable fluctuations were observed in coliform counts in the cats of group C during the study period.

Rates of antimicrobial resistance of coliforms in cats: By antimicrobial susceptibility testing, ORB resistance was not detected in groups A and B before the treatment. After the treatment, ORB resistance was found in group B (6.0%); however, there was no significant difference in the resistance rates before and after the treatment ($P > 0.05$). On the other hand, ORB resistance was not detected in group A after the treatment.

Compared to before treatment, rates of AMP resistance were significantly high in groups A and B after the treatment (0% vs. 26.2% and 0% vs. 76.2%, respectively, $P < 0.01$); rates of CEZ resistance were also significantly high in these groups after the treatment (0% vs. 26.2% and 0% vs. 76.2%, respectively, $P < 0.01$). There were significant differences in rates of resistance to the two drugs between groups A and B after the treatment ($P < 0.01$).

In group C, resistance to any antimicrobials tested was not detected during test period.

Changes in the composition of the bacterial population of fecal coliforms: Coliform species are shown in Table 1. On day 1, all isolates from cats in groups A, B and C were identified as *E. coli*.

After ORB treatment, the isolates of *Citrobacter freundii* were detected in all cats in groups A and B. In group A, *C. freundii* isolates were detected on days 11, 13, 15 and/or 17, whereas in group B, the bacteria were detected on days 7, 9, 11, 13 and/or 17. Furthermore, *E. coli* isolates were detected alone or along with *C. freundii* isolates in all cats except cat 4 (from group B), in whom coliforms were not detected until day 15 after treatment initiation, and only *C. freundii* isolates were detected on day 17. In group C, all isolates were identified as *E. coli*.

Using EMB agar containing ORB, coliforms were detected with $10^{3.08}$ CFU/g in cat 3 (from group A) on day 15 and with $10^{3.28}$ CFU/g in cat 4 (from group B) on day 17 after treatment initiation; these isolates were identified as *E. coli* and *C. freundii* (cat 3) and *C. freundii* alone (cat 4). No growth of coliforms was detected in the other cats by using ORB-containing EMB agar.

Coliform bacteria other than *E. coli* or *C. freundii* were

Table 1. Bacterial species of coliforms from stool samples obtained from cats during the test schedule

Group ^{a)}	Cat	Date of sampling (No. of isolates) ^{b)}								
		1	3	5	7	9	11	13	15	17
A	1	Ec	—	—	—	—	Cf	Ec	Ec	Ec
	2	Ec	—	—	—	—	Ec	Ec/Cf	Cf	Ec
	3	Ec	—	—	—	Ec	Ec	Ec	Ec ^{c)} /Cf ^{c)}	Ec/Cf
B	4	Ec	—	—	—	—	—	—	—	Cf ^{c)}
	5	Ec	—	—	Cf	Cf	Ec/Cf	Ec	Ec	Cf
	6	Ec	—	—	—	Cf	Cf	Cf	Ec	—
C	7	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec
	8	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec
	9	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec	Ec

a) Groups A and B were treated with 5 and 2.5 mg ORB/kg, respectively. Group C was control (untreated). b) ORB was administered orally during days 1–7. Ec: *Escherichia coli*; Cf: *Citrobacter freundii*. c) The isolates were also detected using EMB agar containing ORB (2 µg/ml) and exhibited low (MIC: 1 µg/ml) or intermediate susceptibility to ORB (MIC: 2–4 µg/ml).

Table 2. Antimicrobial susceptibility and FQ resistance mechanisms of coliforms from cats before and after ORB administration

Group ^{a)}	Cat	Period	Isolates		MIC range of orbifloxacin (µg/ml)	QRDR mutation		PMQR	Susceptibility to other antimicrobials
			Species ^{b)}	n		GyrA	ParC		
A	1	Pre-treatment	Ec	10	0.063				Susceptible
		Post-treatment	Ec	30	0.063–0.125				Susceptible
			Cf	10	4	None		<i>qnrB</i>	AMP CEZ
	2	Pre-treatment	Ec	10	0.063–0.125				Susceptible
		Post-treatment	Ec	22	0.031–0.125				Susceptible
			Cf	18	4	None		<i>qnrB</i>	AMP CEZ
	3	Pre-treatment	Ec	10	0.063				Susceptible
			Ec	5	0.031–0.063				Susceptible
		Post-treatment	Ec	39	1	S83L ^{d)}	None	None	Susceptible
			Cf	6	4	None		<i>qnrB</i>	AMP CEZ
			Ec ^{c)}	4	1–2	S83L ^{d)}	None	None	Susceptible
			Cf ^{c)}	6	4	None		<i>qnrB</i>	AMP CEZ
4	Pre-treatment	Ec	10	0.063–0.125				Susceptible	
	Post-treatment	Cf	10	4	None		<i>qnrB</i>	AMP CEZ	
		Cf ^{c)}	10	4	None		<i>qnrB</i>	AMP CEZ	
5	Pre-treatment	Ec	10	0.063				Susceptible	
	Post-treatment	Ec	9	0.063–0.25				Susceptible	
		Cf	25	4	None		<i>qnrB</i>	AMP CEZ	
6	Pre-treatment	Ec	10	0.063				Susceptible	
	Post-treatment	Ec	10	0.063–0.125				Susceptible	
		Cf	25	4	None		<i>qnrB</i>	AMP CEZ	
			5	8	None		<i>qnrB</i>	AMP CEZ	
C	7	All	Ec	89	0.063–0.125			Susceptible	
	8	All	Ec	90	0.063–0.125			Susceptible	
	9	All	Ec	87	0.063–0.125			Susceptible	

a) Groups A and B were treated with 5 and 2.5 mg ORB/kg, respectively. Group C was control (untreated). b) Ec: *Escherichia coli*, Cf: *Citrobacter freundii*. c) The isolates were detected using EMB agar containing ORB (2 µg/ml). d) S83L: codon position 83, serine-to-leucine mutation.

not identified during the test schedule.

Resistance mechanisms in *E. coli* and *C. freundii*: As for ORB resistance, *E. coli* isolates were highly susceptible (MIC, 0.031–0.25 $\mu\text{g/ml}$) in cats 1 and 2 (from group A), and 4, 5 and 6 (from group B) not only before treatment but also after treatment (Table 2). On the other hand, isolates with low or intermediate susceptibility (MIC, 1–2 $\mu\text{g/ml}$) were predominantly detected in cat 3 (from group 1) after treatment, and these isolates had a single QRDR mutation in *gyrA* at codon position 83 (serine to leucine: S83L). In group C, all *E. coli* isolates were highly susceptible to ORB (MIC, 0.063–0.125 $\mu\text{g/ml}$) during the test schedule. All *C. freundii* isolates from cats 1–5 demonstrated intermediate susceptibility (MIC, 4 $\mu\text{g/ml}$), whereas the resistant isolates (MIC, 8 $\mu\text{g/ml}$) were barely detectable only in cat 6 on days 9, 11 and 13. According to PCR and sequencing results, none of the *C. freundii* isolates had QRDR mutations in *gyrA*, but carried *qnrB*, one of the PMQR genes.

All *E. coli* isolates were susceptible to the 5 tested antimicrobials, whereas all *C. freundii* isolates were resistant to AMP and CEZ. All *C. freundii* isolates were phenotypically confirmed to be inducible AmpC producers, but not ESBL producers.

Genetic relationship between *C. freundii* isolates from cats: Four *C. freundii* isolates each from cats 1–6 (from groups A and B) were selected and subjected to ERIC-PCR. As the result, all of these isolates had the identical banding pattern (data not shown).

ORB concentration in feces of ORB-treated cats: The changes in ORB concentration in feces are shown in Fig. 1 and Supplemental Fig. 2. On day 1 before treatment initiation, ORB was not detected in stool samples from any cat. ORB was detected in cats of groups A and B between days 3 and 9 after treatment initiation. Highest concentration of ORB was determined in cats 2 and 6 of groups A and B, respectively, between days 3 and 7. The peaks of mean concentration in groups A and B were observed on day 3 (141.0 and 61.3 $\mu\text{g/g}$, respectively). On days 5 and 7, significantly higher concentrations of ORB were detected in group A than in group B ($P < 0.05$). On the other hand, ORB was not detected in all of cats in group C on days 1 and 9.

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the effects of oral FQ on the fecal flora of cats. We have demonstrated that the counts of coliform bacteria significantly decreased during and after the period for administration of ORB. A similar reduction in the number of coliforms was reported in dogs treated with enrofloxacin [29]. Thus, these data indicate that FQ administration reduces the number of coliforms in the fecal flora of cats and dogs. As for the composition of the bacterial population of the fecal coliform bacteria, only *E. coli* was detected in all cats on day 1, whereas isolates of *C. freundii* and *E. coli* were detected as a dominant species of all cats in groups A and B after cessation of ORB treatment. Few reports have addressed the effect of antimicrobial treatment on the

composition of the bacterial population of fecal or gut flora in animals. Johnson *et al.* [17] detected the emergence of *Streptococcus* spp. and *Corynebacterium* spp. in cats after the administration of metronidazole. Lawrence *et al.* [19] confirmed the increase in the number of enterococcal cells in dogs after the administration of cefovecin. Although there were several differences in test condition between studies, our and their results suggest that administration of antimicrobials can cause increase, in addition to decrease, of specific bacterial species of fecal or gut flora, which may be explained by microbial substitution.

Pharmacokinetics of ORB has not yet been evaluated in animals treated with different doses of this antibiotic. In this study, we found clear differences in ORB concentrations in feces between the two doses (5.0 and 2.5 mg/kg). This dose-dependent kinetics of ORB should be considered when administering this drug. Moreover, our results reveal remarkably high concentrations of ORB in feces of cats receiving either the high-dose or low-dose treatment. Thus, ORB is likely to be mostly excreted into the feces after oral administration: a notion supported by a previous study [21], revealing that a higher concentration of ORB is present in bile acid than in serum of cats after ORB treatment. These pharmacokinetic properties of the drug may be responsible for the significant reduction of fecal coliforms in cats after treatment.

Our study showed some variations in the numbers of coliforms and ORB concentrations in feces between cats administered with the drug, suggesting that effect of ORB treatment on fecal coliforms and pharmacokinetics of the drug may vary by individual. Such interindividual variations should be taken into account when administering the drug for cats.

Susceptibility testing revealed that no ORB-resistant *E. coli* appeared because all isolates failed to develop more than one QRDR mutation: the necessary condition for the acquisition of FQ resistance [12]. Our results strongly contradict the study by Aly *et al.* [2], wherein all fecal *E. coli* isolates exhibit high-level resistance to enrofloxacin after treatment of dogs with the drug. In general, the concept of a mutant selection window has considerable implications for the acquisition of FQ resistance, and antimicrobials at concentrations beyond the mutant prevention concentration (MPC) can prevent the development of FQ-resistant mutants [5]. In our study, the fecal ORB concentration in cats treated with the doses of 2.5 and 5.0 mg/kg far exceeds the MPC value of *E. coli* (0.5–32 $\mu\text{g/ml}$); this result has been confirmed by another study [26]. These findings suggest that oral ORB poses a low risk of selection of highly FQ-resistant mutants among fecal *E. coli* isolates in cats because of high gut levels.

Compared with *E. coli* isolates, *C. freundii* isolates show higher ORB MIC values. Such low susceptibilities to FQs may give a competitive advantage to *C. freundii* when administered with ORB. Most strains of *C. freundii* maintained intermediate sensitivity to ORB. Among the tested PMQR genes, *qnrB* was detected in all *C. freundii* isolates. This finding can be explained by a study by Jacoby *et al.* [16],

revealing that chromosomal *qnrB* is prevalent in *C. freundii* isolates. On the other hand, no QRDR mutations of *gyrA*, the basis of the FQ resistance mechanism [23], were identified in *C. freundii* isolates. Cesaro *et al.* [6] reported that QRDR mutations can be more effectively suppressed in *qnr*-positive *E. coli* strains than in *qnr*-negative *E. coli* strains; this property of *qnr* may elucidate our present results, namely that *qnrB*-positive *C. freundii* isolates fail to acquire strong ORB resistance. It should be considered that gut flora of cats can act as a reservoir of *qnr*-positive bacteria, which are possibly selected as a result of FQ use. In coliform-positive samples after ORB treatment, *qnr*-positive *C. freundii* isolates were detected more frequently in group B (8 of 11 samples) than in group A (5 of 13 samples). This finding implies that low-dose treatment of ORB might facilitate the selection of *qnr*-positive bacteria, compared with high-dose treatment. However, to clarify this point, further large-scale studies would be needed.

As for susceptibilities to the antimicrobials other than FQs, all *E. coli* isolates exhibited susceptibility to all the tested antimicrobials both before and after ORB treatment. As a result, multidrug-resistant *E. coli* isolates, which were reported in dogs after enrofloxacin treatment [2], were not detected in the present study. On the other hand, all *C. freundii* isolates exhibited resistance to AMP and CEZ by the production of AmpC, but not ESBLs. This finding resulted in the significant increase of resistance rates to AMP and CEZ in isolates of *E. coli* and *C. freundii* after ORB treatment. Similarly, the high prevalence of ampC in this bacterial species was previously found in human isolates in a study by Kanamori *et al.* [18]. Our study suggests that FQ use poses a risk of coselection of AmpC-producing *C. freundii* isolates in feces of cats.

ERIC-PCR revealed that *C. freundii* isolates, which were *qnrB*-positive and AmpC-producing, from cats 1–6 (from groups A and B) were clonal or genetically identical. This result indicates that *C. freundii* isolates spread clonally in the cats; however, the cause of the clonal spread could not be identified. One hypothesis is cross-contamination between the cats during the study, despite deliberate efforts to prevent this. A similar phenomenon was reported previously [19]. Another is that ORB treatment might select *C. freundii* isolates that had spread horizontally among enrolled cats before the study. In either case, more aggressive and effective measures to prevent any transfer of bacteria would be needed for future studies.

There were several study limitations. Firstly, this study was carried out as a pilot study by using a small number of cats, and thus, the present results might be somewhat biased. Secondly, the effect of ORB treatment remains to be clarified in cats with clinical signs or household cats, because we used healthy experimental cats in research settings. Furthermore, this study covered only coliform bacteria. Fecal or gut flora in cats is composed of a variety of aerobic and anaerobic bacteria [17], in addition to coliform bacteria. Therefore, this study could not entirely clarify an effect of ORB treatment on fecal flora of cats.

Nevertheless, we have described the effects of ORB ad-

ministration on fecal coliforms in healthy cats. We revealed alterations in bacterial composition, e.g. selection of *qnrB*- and AmpC-positive *C. freundii* isolates, in addition to a decrease in the total number of coliforms. Moreover, we could not detect strongly FQ-resistant mutants among isolates of *E. coli* and *C. freundii*. Further studies using household cats with and without clinical signs are required to assess clinical and public health implications of the effects of FQ use on the fecal flora of cats.

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