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Isolation of *Escherichia coli* Strains with AcrAB–TolC Efflux Pump-Associated Intermediate Interpretation or Resistance to Fluoroquinolone, Chloramphenicol and Aminopenicillin from Dogs Admitted to a University Veterinary Hospital

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ABSTRACT. Understanding the prevalence of antimicrobial-resistance and the relationship between emergence of resistant bacteria and clinical treatment can facilitate design of effective treatment strategies. We here examined antimicrobial susceptibilities of *Escherichia coli* isolated from dogs admitted to a university hospital (University hospital) and companion animal clinics (Community clinics) in the same city and investigated underlying multidrug-resistance mechanisms. The prevalence of *E. coli* with intermediate and resistant interpretations to ampicillin (AMP), enrofloxacin (ENR) and chloramphenicol (CHL) was higher in the University hospital than in the Community clinics cases. Use of antimicrobials, including fluoroquinolone, was also significantly higher in the University hospital than in the Community clinics cases. Upon isolation using ENR-supplemented agar plates, all ENR-resistant isolates had 3–4 nucleotide mutations that accompanied by amino acid substitutions in the quinolone-resistance-determining regions of *gyrA*, *parC* and *parE,* and 94.7% of all isolates derived from the University hospital showed AMP and/or CHL resistance and possessed bla_{TEM} and/or *catA1*. The average mRNA expression levels of *acrA*, *acrB* and *tolC* and the prevalence of organic solvent tolerance, in isolates derived from ENR-supplemented agar plates were significantly higher in the University hospital than in the Community clinics isolates. Thus, *E. coli* derived from the University hospital cases more often showed concomitant decreased susceptibilities to aminopenicillins, fluoroquinolones and CHL than did those derived from the Community clinics; this was related to an active AcrAB–TolC efflux pump, in addition to acquisition of specific resistance genes and genetic mutations.

KEY WORDS: AcrAB, antimicrobial resistance, canine, efflux pump, *Escherichia coli.*

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Excessive and inappropriate use of antimicrobial agents leads to the generation and spread of antimicrobial-resistant bacteria [1, 26]. Resistant bacteria encountered in companion animal medicine also represent a potential hazard to human health [2, 23, 25], because companion animals live in close proximity with humans and receive medical treatment, including antimicrobials used for humans [22].

Understanding the prevalence of antimicrobial resistance and the mechanisms involved allows estimation of the association between the emergence of resistant bacteria and clinical treatments. This is important for devising effective treatment strategies against bacterial infections in companion animals and for reducing the risk of transmission of antimicrobial-resistant bacteria from companion animals to humans.

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Medical treatment of companion animals consists of primary medical care in companion animal clinics in the community and secondary medical care in university-related veterinary hospitals. Generally, university-related veterinary hospitals favor heavier and/or more frequent exposure to antimicrobial agents in seriously ill animals. Most previous surveillance studies of antimicrobial resistance in companion animals have taken place either in the community or in university hospitals; previous studies typically did not distinguish between or compare these settings [7, 22, 25] and mostly did not clarify the actual antimicrobials used. Therefore, comprehensive surveillance, including obtaining information on actual antimicrobial use, should be carried out in both primary and secondary medical care settings within the same region to investigate the extent of antimicrobial resistance in companion animals.

Fluoroquinolones are broad-spectrum antimicrobials and are amongst the most important antimicrobial agents used to treat a variety of bacterial infections, not only in humans, but also in companion animals. Thus, emergence of fluoroquinolone-resistant bacteria due to antimicrobial treatment may present a serious challenge in clinical treatment of bacterial infections [2, 8]. Therefore, surveillance of fluoroquinolone-resistant bacteria could offer important information for the control of infectious diseases.

Fluoroquinolone resistance is mainly caused by chromosomal mutations in the quinolone-resistance-determining region (QRDR) of topoisomerase IV and DNA gyrase [3, 6, 10, 29]. Moreover, plasmid-mediated quinolone-resistance genes (PMQRs), such as *qnr*, *aac (6')-Ib-cr* and *qepA*, have been reported in Gram-negative bacteria, including *E. coli* [12]. Furthermore, the overexpression of efflux pumps, mainly AcrAB–TolC, in *E. coli* concomitantly decreases susceptibility to fluoroquinolones [19]. Detailed investigations of these fluoroquinolone resistance mechanisms are important for elucidating the differences in mechanisms underlying emergence of fluoroquinolone-resistant *E. coli* isolates between primary and secondary medical care and could provide beneficial information for controlling *E. coli* infection in each type of facility.

In this study, we examined the antimicrobial-susceptibility of *E. coli* isolates derived from dogs sampled in a university hospital and in community companion animal clinics located in the same city. We also investigated the multidrug-resistance mechanisms involved, including AcrAB–TolC function.

MATERIALS AND METHODS

Clinical histories and condition of host dogs: In total, 173 cotton rectal swabs were collected from 93 dogs treated at Rakuno Gakuen University (RGU) Veterinary Teaching Hospital (Ebetsu, Japan; University hospital) and from 80 dogs treated at 8 companion animal clinics (10 samples per clinic, from different dogs) in the community of Ebetsu (Community clinics) from June to December 2005 (regardless of the clinical condition seen for the animal). All dogs admitted to the University hospital had also visited the Community clinics previously.

University hospital cases (15 male and 20 female dogs) included those with tumor, cataract, glaucoma, keratitis, hip dysplasia, Cushing syndrome and herniated intervertebral discs. Community clinic cases (27 male and 24 female dogs) included those undergoing castration, panovario-hysterectomy or treatment for urinary tract infections, cystitis, chronic diarrhea, dermatitis, otitis externa, gingivitis, pharyngitis and keratitis. Dogs were aged 0–16 years (University dogs: 8.2 ± 3.7 y [mean ± SD]; Community dogs: 5.5 ± 4.2 y). The 6-month history of antimicrobial use prior to sampling was also compared for the 54 dogs admitted to the University hospital and the 56 dogs admitted to the Community clinics.

Bacterial isolates: Canine rectal samples were collected before commencing clinical treatment. Samples were streaked on deoxycholate–hydrogen sulfide–lactose (DHL) agar (Nissui, Tokyo, Japan) and incubated for 24 hr at 37°C. Colonies of suspected *E. coli* growing on these DHL agar plates were picked and subcultured on nutrient agar (Nissui). After incubation, the biochemical properties of these colonies were assessed using triple sugar iron agar (Nissui), lysine indole motility medium (Nissui) and cytochrome oxidase tests. Final identification of *E. coli* was performed using API20E codes (bioMérieux, Tokyo, Japan). The 173 canine samples were also assessed on DHL agar supplemented with 4 μg/m*l* of enrofloxacin (ENR; Bayer, Osaka, Japan).

Susceptibility testing: Susceptibilities to a panel of antimicrobials were examined using the agar dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [5]. Mueller–Hinton (MH) agar was obtained from Oxoid (Basingstoke, U.K.). Ampicillin (AMP), amoxicillin (AMX), cefazolin (CFZ), cephalexin (LEX), gentamicin (GEN), kanamycin (KAN), dihydrostreptomycin (DSM), oxytetracycline (OTC) and chloramphenicol (CHL) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), and cefpodoxime (CPD) was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan). *Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29212, *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as controls. Resistant, intermediate and susceptible interpretations were as defined by CLSI guidelines [5]. Resistance to DSM (≥32 μg/m*l*) and OTC (≥16 μg/m*l*) was microbiologically defined as described in the Japanese Veterinary Antimicrobial-resistance Monitoring System [18]. Intermediate interpretations for DSM and OTC were defined as having two-fold lower minimum inhibitory concentration (MIC) than those of the resistance category. Phe-Arg-β-naphthylamide (PAβN; Sigma-Aldrich; final concentration: 20 μg/m*l*) was used as an efflux-pump inhibitor [24].

Organic solvent tolerance: Organic solvent tolerance (OST) was investigated as previously described [28] with slight modifications. An overnight culture of *E. coli* was diluted with 0.9% NaCl (approximately 1×10^7 cells/m*l*). A drop of cell suspension (5 μl) was spotted onto MH agar medium to form a circle with a diameter of 8 mm. The surface of the agar was overlaid with a mixture (3:1, 1:1, or 1:3 [vol/vol]) of *n*-hexane (96.0% pure; Kishida Chemical Co., Ltd., Osaka, Japan) and cyclohexane (>99% pure; Merck KGaA, Darmstadt, Germany) to a depth of 3 mm. Cyclohexane is an organic solvent known to be a more effective agent against *E. coli* than *n*-hexane [28]. Bacterial growth was assessed after the plates were incubated at 37°C for 16–18 hr in a sealed vessel. Confluent growth of the cells (confluent) was considered to be indicative of tolerance to the solvent tested. When only a few colonies (<10) grew on the plate or when no growth was observed, the cells were considered to be sensitive to the solvent tested (non-confluent). Each experiment was performed 3 times, and averages were calculated.

Determination of QRDR mutations, PMQRs, β-lactamases and CHL-resistance genes: Mutations in QRDRs of *gyrA*, *parC*, *parE* and *gyrB* were examined by direct DNA sequencing of PCR products, as described by Everett *et al*. [9]. PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac (6') Ib-cr* and *qepA*) were detected by PCR using specific primers (Table 1) and direct DNA sequencing [4, 15, 21]. To identify the AMP-resistance mechanism, β-lactamase genes, viz., bla_{TEM} and bla_{SHV}, were detected by PCR and direct DNA sequencing [16]. CHL-resistance genes, viz., *catA1*, *catA2*, *catA3*, *floR* and *cmlA*, were detected by PCR as described in previous studies [17, 27]. Nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Table 1. Sequences of oligonucleotides and fluorescence-labeled oligonucleotides used for PCR, direct sequencing and real-time RT-PCR in this study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fluorescent probe (5′–3′)	Purpose	Reference
gyrA	ACGTACTAGGCAATGACTGG	AGAAGTCGC CGTCGATAGAAC	–	PCR and sequencing	[7]
gyrB	TGTATGCGATGTCTGAACTG	CTCAATAGCAGCTCGGAATA	–	PCR and sequencing	[7]
parC	TGTATGCGATGTC TGAACTG	CTCAATAGCAGCTCGGAATA	–	PCR and sequencing	[7]
parE	TACCGAG CTGTTCCTTGTGG	GGCAATGTGCAGACCAT CAG	–	PCR and sequencing	[7]
qnrA	AGAGGATTTCTCACGCCAGG	TGCCAGGCACAGATCTTGAC	–	PCR	[3]
qnrB	GGMATHGAAATTCGCCACTG[a]	TTTGCYGYYCGCCAGTCGAA[a]	–	PCR	[3]
qnrS	GCAAGTTCATTGAACAGGGT	TCTAAACCGTCGAGTTCGGCG	–	PCR	[3]
aac (6')-Ib	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	–	PCR and sequencing	[19]
qepA	AACTGCTTGAGCCCGTAGAT	GTCTACGCCATGGACCTCAC	–	PCR	[13]
bla_{TEM}	ATGAGTATTCAACATTTTCG	TTACCAATGCTTAATCAGTG	–	PCR and sequencing	[14]
bla_{SHV}	ATGCGTTATATTCGCCTGTG	TTAGCGTTGCCAGTGCTCGA	–	PCR	[14]
catA1	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	–	PCR	[15]
catA2	ACACTTTGCCCTTTATCGTC	TGAAAGCCATCACATACTGC	–	PCR	[15]
catA3	TTCGCCGTGAGCATTTTG	TCGGATGAGTATGGGCAAC	–	PCR	[15]
floR	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	–	PCR	[15]
cmlA	TTGCAACAGTACGTGACAT	ACACAACGTGTACAACCAG	–	PCR	[25]
acrA	CTATCACCCTACGCTCTATCTTC	GCGCGCACGAACATACC	CGAACCCGGATCACACTCT	RT-PCR	[12]
acrB	GCGGTCGTGTGAAGAAAGTTTA	ACTCCCAACGAGAAGAGGAGAA	TGACCATCAGCAGCACGAACATACCAGT	RT-PCR	This study
tolC	GGTACGTTGAACGAGCAGGATC	CCATCAGCAATAGCATTCTGTTCC	CTGGCACTGAACAATGCGCTGAGCAA	RT-PCR	This study
gapA	AAAGGCGCTAACTTCGACAA	GAACGGTGGTCATCAGACCT	CAACGATAACTTCGGCATCA	RT-PCR	This study

a) M, A, or C; H, A, or C or T; Y, C, or T.

Table 2. Antimicrobial susceptibility of *E. coli* strains derived from dogs attending Rakuno Gakuen University Veterinary Teaching Hospital (RGU; University) and animal clinics in the community (Community)

Antimicrobial (break point, μg/m*l*)	Groups	Range (μg/m*l*)	MIC_{50} (μg/m*l*)	MIC_{90} (μg/m*l*)	Number of strains (%)			
					S	I	R	I + R
AMP	University	2–>128	4	>128	44 (59.5)*	4 (5.4)	26 (35.1)	30 (40.5)*
(≥32)	Community	0.5–>128	4	>128	50 (75.8)	0	16 (24.2)	16 (24.2)
AMX	University	1–>128	16	>128	32 (43.2)**	15 (20.3)**	27 (36.5)	42 (56.8)**
(≥32)	Community	4–>128	4	>128	50 (75.8)	0	16 (24.2)	16 (24.2)
CFZ	University	1–>128	2	>128	61 (82.4)	1 (1.4)	12 (16.2)	13 (17.6)
(≥32)	Community	1–>128	2	>128	57 (86.3)	0	9 (13.6)	9 (13.6)
LEX	University	8–>128	8	>128	54 (73.0)	5 (6.8)	15 (20.3)	20 (27.0)
(≥32)	Community	4–>128	8	>128	47 (71.2)	9 (13.6)	10 (15.1)	19 (28.8)
CPD	University	<0.125–>128	0.5	128	62 (83.8)	0	12 (16.2)	12 (16.2)
(≥8)	Community	0.25–>128	0.5	128	57 (86.4)	0	9 (13.6)	9 (13.6)
KAN	University	1–>128	2	32	66 (89.2)	1 (1.4)	7 (9.5)	8 (10.8)
(≥64)	Community	2–>128	2	>128	59 (89.4)	0	7 (10.6)	7 (10.6)
GEN	University	0.5–>128	1	8	67 (90.5)	0	7 (9.5)	7 (9.5)
(≥16)	Community	0.5–64	1	2	62 (93.9)	0	4 (6.1)	4 (6.1)
DSM	University	2–>128	4	>128	55 (74.3)	1 (1.4)	18 (24.3)	19 (25.7)
(≥32)	Community	2–>128	4	>128	48 (72.7)	1 (1.5)	17 (25.8)	18 (27.3)
OTC	University	2–>128	2	>128	56 (75.7)	3 (4.1)	15 (20.3)	18 (24.3)
(≥16)	Community	1–>128	2	>128	50 (75.8)	0	16 (24.2)	16 (24.2)
CHL	University	4–>128	8	64	54 (73.0)*	9 (12.2)	11 (14.9)*	20 (27.0)*
(≥32)	Community	4–>128	8	8	61 (92.4)	3 (4.5)	2 (3.0)	5 (7.6)
ENR	University	0.01–128	0.03	64	59 (79.7)*	0	15 (20.3)*	15 (20.3)*
(≥4)	Community	0.01–64	0.03	16	61 (92.4)	0	5 (7.5)	5 (7.6)

AMP: Ampicillin, AMX: Amoxicillin, CFZ: Cefazolin, CHL: Chloramphenicol, CPD: Cefpodoxime, DSM: Dihydrostreptomycin, ENR: Enrofloxacin, GEN: Gentamicin, KAN: Kanamycin, LEX: Cephalexin, OTC: Oxytetracycline. S: Susceptible, I: Intermediate, R: Resistant. *P<0.05, **P<0.01; difference versus Community.

Table 3. Prevalence of concomitant antimicrobial resistance on ENR-resistant *E. coli* isolates derived from non-supplemented agar

Isolates	Prevalence of concomitant resistance (%)									
	AMP	AMX	CEZ	LEX	CPD	KAN	GEN	DSM	OTC	CHL
ENR-resistant (20)	90.0**	90.0**	75.0**	80.0**	70.0**	15.0	30.0**	65.0**	35.0	40.0**
ENR-susceptible (120)	20.0	20.8	5.0	7.5	5.8	9.2	4.2	18.3	20.0	2.5

AMP: Ampicillin, AMX: Amoxicillin, CFZ: Cefazolin, CHL: Chloramphenicol, CPD: Cefpodoxime, DSM: Dihydrostreptomycin, ENR: Enrofloxacin, GEN: Gentamicin, KAN,:Kanamycin, LEX: Cephalexin, OTC: Oxytetracycline. **P<0.01.

Real-time reverse transcription-PCR: Overnight cultures of *E. coli* isolates were diluted 1:100 in LB broth and grown to the mid-logarithmic phase. RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at −80°C until used. The concentration of RNA was determined spectrophotometrically (BioSpectrometer, Eppendorf, Hamburg, Germany). Gene expression (*acrA*, *acrB* and *tolC*) was estimated by quantitative reverse transcription (RT) TaqMan-PCR. The respective primer pairs and probes (Table 1) used for *acrB*, *tolC* and *gapA* in this study were designed according to the sequence of *E. coli* strain K12 substrain MG1655, which is deposited in GenBank (accession number U00096). The probes were labeled by the manufacturer (Sigma-Aldrich) with the reporter dye 6-carboxyfluorescein (6′-FAM) at the 5′-end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3′-end. Purified RNA (2.5 *n*g) was used in one-step RT and real-time PCR amplification. RT-PCR amplification mixtures (20 μl) contained purified RNA, 2× QuantiTect Probe RT-PCR Master Mix, 0.2 μl of QuantiTect RT Mix (QuantiTect Probe RT-PCR kit, Qiagen), 0.2 μM of probe and 0.5 μM forward and reverse primers. The cycle conditions comprised 20-min reverse transcription at 50°C; a 15-min initial activation step at 95°C; and 45 cycles each of 55°C for 1 min and at 60°C for 30 sec in a LightCycler 480 (Roche, Mannheim, Germany). Expression of *gapA* was used to normalize expression ratios. *E. coli* strain AG100 (K-12 *argE3 thi-1 rpsL xyl mtl* D (*gal-uvrB*) *supE44*) was kindly donated by Dr Helen I. Zgurskaya (University of Oklahoma, Norman, OK, U.S.A.) and used as a reference strain. All experiments were performed 3 times, and averages were calculated.

Statistical analysis: Statistical significance of differences between the isolates obtained from dogs admitted to the 2

Table 4. Organic solvent tolerance (OST) of *E. coli* strains derived from dogs in attending Rakuno Gakuen University Veterinary Teaching Hospital (RGU; University) and animal clinics in the community (Community)

OST (*n*-hexane: cyclohexane)	Groups	Non-confluent	Confluent
3:1	University	32 (43.2)**	42 (56.8)**
	Community	55 (83.3)	11 (16.7)
1:1	University	60 (81.1)**	14 (18.9)**
	Community	63 (95.5)	3 (4.5)
1:3	University	67 (90.5)	7 (9.5)*
	Community	65 (98.5)	1 (1.5)

Values indicate the number of *E. coli* isolates and (percentage of the total). *P<0.05, **P<0.01; statistical difference versus Community.

Table 5. Status of antimicrobial use in dogs attending Rakuno Gakuen University Veterinary Teaching Hospital (RGU; University) and animal clinics in the community (Community)

Antimicrobial use	University	Community
Average number of antimicrobials used for each dog	1.4**	0.8
Frequency of dogs treated by fluoroquinolone	24.1%**	14.3%
Frequency of dogs treated by all antimicrobials	74.1%**	50.0%

We could obtain antimicrobial use history for 6 months prior to sampling from 54 dogs in the University and 56 dogs in the Community. **P<0.01; statistical difference versus Community.

types of treatment facilities was determined by Student's *t*-test and Fisher's exact test. *P*-value <0.05 was considered statistically significant.

RESULTS

Antimicrobial-resistance profile of canine E. coli isolates: There was a significant difference in the ages (P<0.05), but not in the gender distribution of the dogs admitted to the University hospital or the Community clinics.

Seventy-four *E. coli* isolates were obtained from 93 rectal samples from dogs admitted to the University hospital (79.6%) and 66 isolates from 80 rectal samples obtained from dogs admitted to the Community clinics (82.5%), after culture on DHL agar plates that had not been supplemented with ENR. There was no significant difference in the frequency of *E. coli* isolation between dogs admitted to the 2 types of treatment facilities (P>0.05).

Of all the canine *E. coli* isolates, 44.3% (62 of 140 isolates) were resistant to at least 1 antimicrobial agent tested with aminopenicillin resistance being the most frequent (approximately 30%); approximately 50% of aminopenicillin-resistant isolates were also resistant to cephalosporins (CFZ and CPD). Although there was no significant difference in the rate of resistance to AMP or AMX between isolates derived from University hospital cases and isolates derived from Community clinics cases, when considering isolates with resistance as well as those with an intermediate interpretation to AMP and AMX, this rate was significantly more prevalent in the University hospital than in the Community clinics samples (P<0.05, Table 2). The prevalence of CHL-resistant and ENR-resistant isolates was also significantly higher in the University hospital than in the Community clinics samples (Table 2).

In terms of susceptibilities to aminopenicillin and CHL among the 15 ENR-resistant isolates derived from the University hospital samples, 7 isolates showed resistance and/or intermediate interpretation to aminopenicillin, as well as resistance to CHL. Six of these isolates showed resistance and/or intermediate interpretation to aminopenicillin, but susceptibility to CHL, while the remaining 2 isolates showed susceptibility to both aminopenicillin and CHL (data not shown). Among the 5 ENR-resistant isolates derived from the Community clinics samples, 1 isolate showed both resistance to aminopenicillin and CHL, and 4 isolates showed resistance and/or an intermediate interpretation to aminopenicillin, but susceptibility to CHL (data not shown). The prevalence of resistance to aminoglycosides (KAN, GEN and DSM) and OTC was not significantly different between isolates derived from the University hospital cases and from the Community clinics cases. ENR-resistant isolates frequently demonstrated concomitant resistance to aminopenicillins, cephalosporins, GEN, DSM and CHL (Table 3). Prevalence of OST was significantly higher in isolates from the University hospital cases than from the Community clinics cases (Table 4).

The average number of antimicrobials used for each dog was significantly higher in the University hospital than in the Community clinics cases (Table 5). The frequencies of dogs treated with any antimicrobials and with fluoroquinolones were also significantly higher in the University hospital than in the Community clinics cases (Table 5). In addition, prevalence of fluoroquinolone-resistant isolates was significantly higher in dogs that had been treated with fluoroquinolones compared with that in dogs that had not been treated with this agent (P<0.05; data not shown).

Isolation of fluoroquinolone-resistant E. coli using ENR-supplemented DHL agar plates: To investigate fluoroquinolone-resistance mechanisms and the occurrence of multidrug resistance involving fluoroquinolone, we selected ENR-resistant *E. coli* on ENR-supplemented DHL agar plates (Fig. 1). Rate of resistance to aminopenicillins, cephalosporins, GEN, DSM, OTC and CHL was significantly higher in isolates obtained from ENR-supplemented DHL agar plates than those obtained from DHL agar plates that had not been supplemented with ENR. Isolates obtained from ENR-supplemented DHL agar plates were most frequently AMP resistant.

We further characterized the 31 *E. coli* isolates derived from ENR-supplemented DHL agar plates (Table 6). All ENR-resistant isolates had nucleotide substitutions in QRDRs accompanied by changes in 3 or 4 amino acids in QRDRs. The *aac (6') Ib-cr,* one of the genes encoding PMQRs, was detected in only 1 strain. In total, more than 70% of

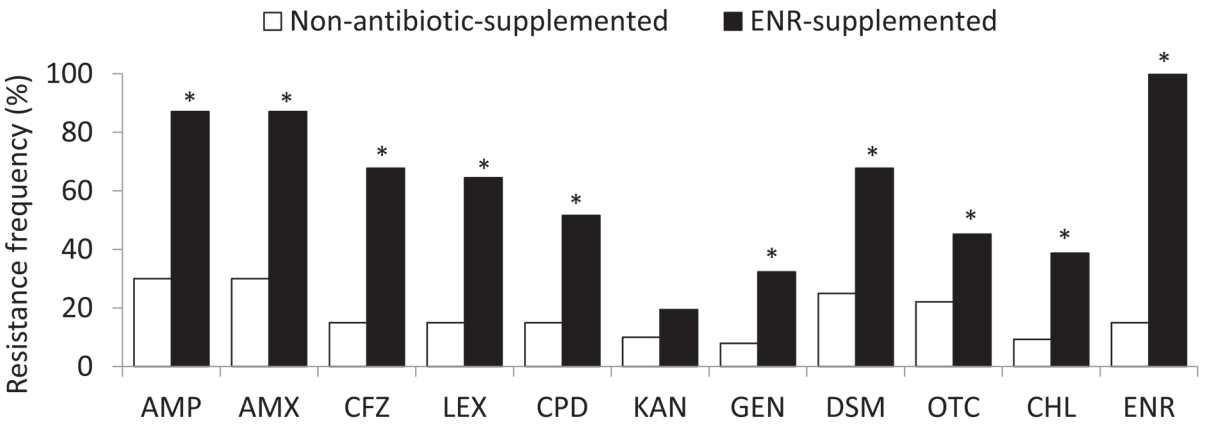

Fig. 1. Influence of enrofloxacin selection on isolation frequencies of *E. coli* isolated from canine rectal samples. AMP, ampicillin; AMX, amoxicillin; CFZ, cefazolin; CHL, chloramphenicol; CPD, cefpodoxime; DSM, dihydrostreptomycin; ENR, enrofloxacin; GEN, gentamicin; KAN, kanamycin; LEX, cephalexin; OTC, oxytetracycline. *Statistical difference for isolation with deoxycholate hydrogen sulfide lactose (DHL) medium without antimicrobials; $P<0.05$.

the ENR-resistant isolates had resistance or intermediate interpretation to AMP and/or CHL, and 74% of isolates with an AMP MIC of>128 μg/m*l* possessed $bla_{\text{TEM-1}}$, and 100% of isolates with a CHL MIC of>128 μg/m*l* possessed *catA1*(Table 6). Expression levels of *acrA*, *acrB* and *tolC* and the effect of PAβN were higher in CHL–resistance and CHL–intermediate interpretable isolates than in CHL-susceptible isolates (Table 6). Isolates exhibiting OST had high *acrB* expression, while isolates with an intermediate interpretation to AMP also exhibited OST and had higher *acrB* expression than did isolates that were AMP-susceptible (data not shown).

Among dogs from which we isolated *E. coli* on ENR-supplemented DHL agar plates, the frequency of dogs treated with any antimicrobials was significantly higher in the University hospital (89.5%) than in the Community clinics (58.3%) cases (Table 6). In contrast, the frequency of dogs treated with fluoroquinolones was not significantly different between the University hospital (31.6%) and Community clinics (25.0%) cases. Twenty-seven of 31 *E. coli* isolates obtained on ENR-supplemented DHL agar plates showed resistance or an intermediate interpretation to AMP and/or CHL. Among the 27 dogs from which we isolated *E. coli* with resistant or an intermediate interpretation to AMP and/or CHL on ENR-supplemented DHL agar plates, 18 dogs had been treated with fluoroquinolone and/or β-lactam antimicrobials (Table 6).

DISCUSSION

In this study, *E. coli* isolates with resistant or an intermediate interpretation to aminopenicillins, CHL or fluoroquinolone were more frequently obtained from dogs admitted to the Universal hospital than from those admitted to the Community clinics. Remarkably, isolates with resistance to fluoroquinolones more frequently showed resistance to aminopenicillins, cephalosporins, GEN, DSM and CHL, as compared with fluoroquinolone-susceptible isolates. This result suggested that the difficulty of providing effective antimicrobial treatment increases in secondary medical care. It indicated a need to investigate the mechanism underlying the emergence of this multidrug-resistance phenotype.

To characterize in detail the fluoroquinolone-resistant isolates obtained from the University hospital and Community clinics studied here, we investigated antimicrobial-resistance mechanisms of *E. coli* isolates derived from dogs using ENR-supplemented DHL agar plates. All ENR-resistant isolates obtained from ENR-supplemented DHL agar plates possessed 3 or 4 mutations in QRDRs. A previous study showed that *in vitro* exposure to fluoroquinolone caused mutations in QRDRs and AcrAB–TolC overexpression [13]. This may indicate that *in vivo* fluoroquinolone exposure can also cause an increase in fluoroquinolone-resistant *E. coli* possessing multiple mutations in QRDRs and AcrAB–TolC overexpression. Indeed, prevalence of fluoroquinolone-resistant isolates was significantly higher in dogs that had been treated with fluoroquinolones compared with that in dogs that had not been treated with this agent, as determined using on DHL agar plates that had not been supplemented with ENR. Moreover, fluoroquinolone-resistant isolates derived from the University hospital had higher levels of *acrA*, *acrB* and *tolC* expression than did such isolates obtained from the Community clinics, as determined using ENR-supplemented DHL agar plates. These findings suggested that the high prevalence of fluoroquinolone-resistant *E. coli* isolates derived from the University hospital may have been caused by frequent fluoroquinolone use in the University hospital and/or continuous fluoroquinolone use in the Community clinics and the University hospital. This may have resulted in development of a mechanism that decreased fluoroquinolone susceptibility, viz., overexpression of AcrAB–TolC.

In this study, CHL, in addition to ENR was another agent to which isolates derived from the University hospital showed a significantly higher prevalence of resistance than

Table 6. Characterization of antimicrobial and organic solvent susceptibility, QRDR mutations, existence of resistant genes and expression levels of AcrAB in *E. coli* isolates derived by ENR-supplemented agar

Strain	Canine case history	Antimicrobial use for 6 months before sampling	QRDR mutations[a]						MIC (μg/ml)				β-lactamase gene	PMQR	CP-resistance gene	Expression level[e]		
			GyrA		ParC			ParE	AMP	CPD	ENR	CHL				*acrA*	*acrB*	*tolC*
			S83	D87	S80	E84	A108											
University group																		
RE18	Mastocytoma	CFZ, LEX	L	N	I	G	-	-	>128	>128	128 (×16)[c]	32 (×16)[c]	N.D.	N.D.	N.D.[d]	2.62	3.76	3.27
RE21	Abdominal tumor	AMP, CFZ,LEX, ENR	L	N	I	G	-	-	>128	>128	128 (×32)	>128	N.D.	N.D.	*catA1*	2.31	3.77	4.65
RE28	Rhabdomyosarcoma	CFZ, LEX	L	N	I	G	-	-	>128	>128	128 (×32)	64 (×16)	bla_{TEM-1}	N.D.	N.D	2.17	7.13	3.46
RE33	Mastocytoma	CFZ, LEX, OFX	L	N	I	G	-	-	>128	>128	128 (×32)	>128	bla_{TEM-1}	N.D.	*catA1*	2.81	14.29	5.88
RE61	Herniated intervertebral discs	LEX	L	N	I	G	-	-	>128	0.5	64 (×8)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.43	2.34	3.21
RE63	Unknown	AMP	L	N	I	G	-	-	16 (×8)[c]	2	64 (×8)	16 (×8)	N.D.	N.D.	N.D.	0.75	2.68	1.87
RE20	Lung tumor	AMC, CFZ, ENR	L	N	I	V	-	-	>128	>128	32 (×4)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	2.16	2.9	3.95
RE2	Tumor of the breast	GEN, FRM	L	N	I	-[b]	T	-	>128	>128	128 (×16)	32 (×16)	N.D.	N.D.	N.D.	2.8	2.87	4.07
RE72	Glaucoma	CFZ, OFX, ORB	L	N	I	-	T	-	>128	1	64 (×8)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	0.89	0.96	1.77
RE80	Oral tumor	None	L	N	I	-	T	-	>128	1	64 (×8)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.02	1.01	1.72
RE4	Osteosarcoma	AMP, AMX, FRM	L	N	I	-	-	E460A	>128	>128	64 (×16)	>128	bla_{TEM-1}	N.D.	*catA1*	1.36	1.5	2
RE64	Oral tumor	CFZ	L	N	I	-	-	E460A	>128	0.5	64 (×16)	>128	bla_{TEM-1}	N.D.	*catA1*	0.76	1.08	1.7
RE65	Unknown	None	L	N	I	-	-	E460A	>128	0.5	64 (×16)	>128	bla_{TEM-1}	N.D.	*catA1*	0.8	1.04	1.29
RE22	Multiple myeloma	ENR, MIN	L	N	I	-	-	S458A	>128	4	128 (×16)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.94	1.67	2.55
RE26	Unknown	CFZ, LEX	L	N	I	-	-	-	4 (×4)	0.25	16 (×8)	8 (×4)	N.D.	N.D.	N.D.	1.1	1.58	2.36
RE50	Keratitis	OFX, ORB	L	N	I	-	-	-	>128	>128	128 (×16)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.32	1.65	2.74
RE54	Biopsy of vertebral body	CFZ	L	N	I	-	-	-	>128	2	32 (×16)	>128	bla_{TEM-1}	N.D.	*catA1*	1.54	2.67	4.09
RE17	Cushing syndrome	CFZ	L	W	I	G	-	-	16 (×8)	1	64 (×8)	16 (×8)	N.D.	N.D.	N.D.	1.36	2.76	3.87
RE39	Herniated intervertebral discs	AMP	L	W	I	G	-	-	>128	>128	256 (×16)	>128	bla_{TEM-1}	N.D.	*catA1*	1.18	3.28	3.74
						Total (%)		R	84.2	63.1	100	52.6*				1.83*	3.48**	3.06**
								I + R	94.7	52.6	100 (×13.9)[c]	94.7** (×8.6*)[c]						
Community group																		
CE7	Otitis externa	LEX, GEN	L	N	I	A	-	-	>128	>128	128 (×16)	>128	N.D.	N.D.	*catA1*	0.88	1.4	2.01
CE5	Unknown	None	L	N	I	G	-	-	>128	2	128 (×16)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.44	1.63	1.22
CE6	Unknown	LEX, CFZ, GEN	L	N	I	G	-	-	>128	>128	64 (×4)	8 (×4)	N.D.	N.D.	N.D.	0.25	0.37	0.43
CE10	Diarrhea	None	L	N	I	V	-	-	4 (×4)	0.5	64 (×8)	4 (×2)	N.D.	N.D.	N.D.	0.63	2.12	2.02
CE14	Unknown	None	L	N	I	V	-	-	>128	0.5	128 (×16)	4 (×2)	bla_{TEM-1}	N.D.	N.D.	0.84	1.26	1.6
CE1	Unknown	None	L	N	I	-	T	-	>128	>128	64 (×8)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	0.9	1.27	1.32
CE9	Otitis externa	LEX, GEN, LVX	L	N	I	-	-	E460A	>128	>128	32 (×8)	16 (×8)	bla_{TEM-1}	N.D.	N.D.	1.7	1.89	1.72
CE12	Gingivitis	CLI	L	N	I	-	-	S458T	>128	0.5	32 (×4)	4 (×4)	bla_{TEM-1}	N.D.	N.D.	1.34	1.71	1.52
CE13	Diarrhea	SXT	L	N	I	-	-	S458A	>128	2	128 (×8)	8 (×4)	N.D.	*aac (6')-Ib-cr*	N.D.	0.24	0.46	0.56
CE3	Pharyngitis	AMP, LEX, OFX	L	G	R	-	-	-	>128	32	32 (×8)	8 (×4)	bla_{TEM-1}	N.D.	N.D.	0.91	1.6	1.82
CE4	Unknown	None	L	G	R	-	-	-	>128	32	32 (×4)	8 (×8)	bla_{TEM-1}	N.D.	N.D.	0.5	0.9	1.25
CE8	Keratitis	LEX, LVX	L	G	I	-	-	-	>128	>128	32 (×8)	8 (×4)	N.D.	N.D.	N.D.	0.95	1.51	1.7
						Total (%)		R	91.7	58.3	100	8.3				0.97	1.34	1.43
								I + R	91.7	66.7	100 (×8.0)[c]	33.3 (×4.6)[c]						

AMC: Amoxicillin-clavulanic acid, CLI: Clindamycin, FRM: Fradiomycin, LVX: Levofloxacin, MIN: Minocycline, OFX: Ofloxacin, ORB: Orbifloxacin, SXT: Trimethoprim-sulfamethoxazole. Antimicrobial use history reflects antimicrobial use during the 6 months prior to sampling. a) No strain had mutations in GyrB. b) Wild-type c) Fold-reduction of MIC by PAβN. d) N.D.: Not detected. e) mRNA expression levels derived from real-time RT-PCR (relative amount of AG100). *P<0.05, **P<0.01; statistical difference versus Community group.

did those derived from the Community clinics. All ENR-resistant isolates with a CHL MIC of >128 μg/m*l* that had been derived from ENR-supplemented DHL agar plates possessed *catA1*. However, other resistant isolates with a CHL MIC of 32 and 64 μg/m*l* and an intermediate interpretation isolates with a CHL MIC of 16 μg/m*l* did not possess any specific CHL-resistance gene. Among all antimicrobial agents that we tested, isolates with ENR resistance were most frequently co-resistant to aminopenicillins, and all the isolates showing resistance to AMP, but not to cepharosporins, possessed bla_{TEM-1}. However, isolates with intermediate interpretation to AMP did not possess any of the β-lactamase genes for which we tested. These results indicated that the main resistance mechanisms for fluoroquinolones, AMP and CHL involved by acquisition of mutations in QRDRs and a resistance-associated gene, e.g., bla_{TEM-1} or *catA1*, although there may also be other mechanisms that decreased their susceptibilities and conferred co-resistance to these agents.

To evaluate the mechanism underlying decreased susceptibilities and co-resistance to fluoroquinolone, aminopenicillins and CHL, we investigated AcrAB–TolC function, because AcrAB–TolC is a major resistance–nodulation–division (RND) family-type efflux pump that excretes several antimicrobials [14, 19, 20]. AcrAB overexpression increases the MICs of aminopenicillins and CHL to an intermediate interpretation (16 μg/m*l*) or resistance (32 or 64 μg/m*l*) level, and its effect is not limited to fluoroquinolone resistance [13, 21]. AcrAB–TolC is also known to cause the efflux of several organic solvents, which cause cell death by breaking down microbial membranes [11]; therefore, investigation of OST is useful for identifying *E. coli* isolates that have active AcrAB–TolC [28]. We observed that OST isolates with higher *acrB* expression and isolates with an intermediate interpretation to aminopenicillins and CHL, as well as isolates resistant to aminopenicillins and/or with CHL MICs of 32 and 64 μg/m*l*, also exhibited OST and higher *acrB* expression than did susceptible isolates, as seen by analysis using ENR-supplemented DHL agar plates. A higher prevalence of isolates with OST, decreased aminopenicillin susceptibility and decreased CHL susceptibility, was observed in isolates obtained from University hospital compared to those from Community clinics cases, as seen on agar plates without ENR supplementation. These results supported the notion that active AcrAB–TolC function contributes to a decrease in susceptibility to aminopenicillins and CHL MICs in some *E. coli* isolates obtained from dogs.

Our study revealed that the frequency of total antimicrobial treatment as well as fluoroquinolone use was significantly higher in the University hospital than in the Community clinics. This evidence suggested that the frequent use of antimicrobials in dogs admitted to the University hospital and/or their continuous use in dogs moving from the Community clinics to the University hospital facilitate selection of antimicrobial resistant *E. coli* strains with QRDR mutations, beta-lactamase gene and *catA1*. In addition, our study also revealed that dogs admitted to the University hospital tend to be treated with multiple antimicrobials. This approach may facilitate development of multidrug-resistant *E. coli* isolates. Indeed, our results showed that ENR-resistant *E. coli* isolates had higher rates of resistance to several antimicrobials compared with ENR-susceptible *E. coli* isolates, and ENR-resistant isolates derived from the University hospital cases on ENR-supplemented DHL agar showed a stronger development of the AcrAB–TolC than did ENR-resistant *E. coli* isolates derived from the Community clinics cases. We considered that these findings substantially reflect the situation in Japanese companion animal medicine, because the samples in this study were successively. In addition, a previous study also showed that AMP or ENR treatment led to the emergence of aminopenicillin–ENR–CHL-resistant *E. coli* isolates in dogs in the United States [2, 8]. Moreover, fluoroquinolone–aminopenicillin–CHL-resistant *E. coli* isolates with overexpression of AcrAB–TolC were frequently isolated from humans in university hospitals [28]. These findings indicate that the emergence of this multidrug-resistant phenotype may mirror the same phenomenon in human and companion animal clinical fields in several countries in some cases. In these cases, a clearer strategy for choice and use of antimicrobials suitable to treatments is required in order to prevent the emergence and spread of these fluoroquinolone-resistant *E. coli* with decreased susceptibilities to several other antimicrobials. In particular, we suggest that it may be important to share the history of antimicrobials usage across the first and secondary medical care settings of companion animals to avoid treatment with several antimicrobials in the same period and to avoid extensive, continuous treatment with the same class antimicrobial.

In conclusion, this study revealed that the higher prevalence of concomitant resistant and intermediate interpretations to fluoroquinolones, aminopenicillins and CHL in isolates from the University hospital than in isolates from the Community clinics was due not only to the acquisition of specific resistance mechanisms, such as β-lactamases, *catA1* and QRDR mutations, but also to overexpression of the AcrAB–TolC efflux pump in canine *E. coli*.

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