Isolation of *Escherichia coli* Strains with AcrAB–ToIC 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 *blaTEM* 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–ToIC efflux pump, in addition to acquisition of specific resistance genes and genetic mutations.

KEYWORDS: 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.

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-ToIC, 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-ToIC 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), after incubation, the biochemical properties of these colonies were assessed using triple sugar iron agar (Nissui), 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 (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 (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., blaTEM and blaSHV 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     | ACGTACTAAGCAATGACTGG   | AGAAGTCGC CGTCGATAGAAC | –                         | PCR and sequencing  | [7]       |
| gyrB     | TGTAIGCGATGCTGAACCTG   | CTCAATAGCAAGTCGGAATA  | –                         | PCR and sequencing  | [7]       |
| parC     | TGTAIGCGATGCTGAACCTG   | CTCAATAGCAAGTCGGAATA  | –                         | PCR and sequencing  | [7]       |
| parE     | TACCGAG CTCCCTTGTGG    | GGCAATGTCGAGGACCATG   | –                         | PCR and sequencing  | [7]       |
| qnrA     | AGAAGATTTCACGCCAGG     | TGCCAGGCACAGTCTTTGAC  | –                         | PCR                 | [3]       |
| qnrB     | GGMATHGAAATTCGCGAGA   | TTTGCYGYCGCCAGTCGAA  | –                         | PCR                 | [3]       |
| qnrS     | GCAAGTTCATGGACAGGGT    | TCTAAACGTCGTTGCCG     | –                         | PCR                 | [3]       |
| aac (6')-Ib | TTGGATGCTCTATGAGTGGCA  | CTCGATGCTGTCGTTTT       | PCR and sequencing     | [19]                |
| qepA     | AACTGCTTACGGAGGAGAT    | GTCTACGCGATAACGCC      | –                         | PCR                 | [13]      |
| blaTEM   | ATGAGTATACACATTTCTGC   | TTACCAATGCTGTATTCA     | –                         | PCR and sequencing  | [14]      |
| blxEM    | ATGAGTATACACATTTCTGC   | TTACCAATGCTGTATTCA     | –                         | PCR and sequencing  | [14]      |
| catA1    | AGTGGCTCAATGTACCTATAAC | TTTGTAATCTCAAGCATTTCTGC | –                        | PCR                 | [15]      |
| catA2    | ACATTTGCTGCCTTTATCTGC  | TGGAAAGGATTACACAATCTGC | –                         | PCR                 | [15]      |
| catA3    | TTGGCGTAGGATAATTGTC    | TGGAGATGATGATGACCAC    | –                         | PCR                 | [15]      |
| floR     | CGCGTGTACCCTTCTGACC    | GATCGCGACATCGTGGTC     | –                         | PCR                 | [15]      |
| cmIa     | TTGGCAACATACGTCTGACAT  | ACACACGTGGATACCAACAG   | –                         | PCR                 | [25]      |
| acri     | CGGGCGACGACGACATACCC   | CGAACCACGGATACACTCT    | RT-PCR                  | [12]                |
| acrB     | CGGGCGTCAGACTGAAAGATAT | ACTCTCATGCAAGAAGGAGA   | TGGACGACGACGACGAAATACACC | RT-PCR              | This study|
| tolC     | CGGCGTGAGAGGAGGAGTC    | CCAAGTCAATACCCCATCTGTC | RT-PCR                  | This study          | This study|
| gapA     | ACGCGTAACTTTCGACAA     | GAAACGTTGCATACGACCT    | CAACGCTGGTCATCAACGAA    | 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 Gakuin University Veterinary Teaching Hospital (RGU; University) and animal clinics in the community (Community)

| Antimicrobial (break point, µg/ml) | Groups          | Range (µg/ml) | MIC<sub>50</sub> (µg/ml) | MIC<sub>90</sub> (µg/ml) | Number of strains (%) |
|-----------------------------------|-----------------|---------------|---------------------------|--------------------------|-----------------------|
| AMP                               | University      | 2–>128        | 4                         | >128                     | 44 (59.5)*            |
|                                   | Community       | 0.5–>128      | 4                         | >128                     | 50 (75.8)             |
| (≥32)                             | Community       | 1–>128        | 16                        | >128                     | 32 (43.2)**           |
| (≥32)                             | Community       | 4–>128        | 4                         | >128                     | 50 (75.8)             |
| AMP                               | Community       | 1–>128        | 2                         | >128                     | 61 (82.4)             |
| AMX                               | Community       | 1–>128        | 2                         | >128                     | 57 (86.3)             |
| (≥32)                             | Community       | 8–>128        | 8                         | >128                     | 54 (73.0)             |
| (≥32)                             | Community       | 4–>128        | 8                         | >128                     | 47 (71.2)             |
| CPD                               | University      | <0.125–>128   | 0.5                       | 128                      | 62 (83.8)             |
| (≥8)                              | Community       | 0.25–>128     | 0.5                       | 128                      | 57 (86.4)             |
| KAN                               | University      | 1–>128        | 2                         | 32                       | 66 (89.2)             |
| (≥64)                             | Community       | 2–>128        | 2                         | >128                     | 59 (89.4)             |
| GEN                               | University      | 0.5–>128      | 1                         | 8                        | 67 (90.5)             |
| (≥16)                             | Community       | 0.5–64        | 1                         | 2                        | 62 (93.9)             |
| DSM                               | University      | 2–>128        | 4                         | >128                     | 55 (74.3)             |
| (≥32)                             | Community       | 2–>128        | 4                         | >128                     | 48 (72.7)             |
| OTC                               | University      | 2–>128        | 2                         | >128                     | 56 (75.7)             |
| (≥16)                             | Community       | 1–>128        | 2                         | >128                     | 50 (75.8)             |
| CHL                               | University      | 4–>128        | 8                         | 64                       | 54 (73.0)*            |
| (≥32)                             | Community       | 4–>128        | 8                         | 8                        | 61 (92.4)             |
| ENR                               | University      | 0.01–128      | 0.03                      | 64                       | 59 (79.7)*            |
| (≥24)                             | Community       | 0.01–64       | 0.03                      | 16                       | 61 (92.4)             |

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<0.01; **P<0.001; difference versus Community.

| Number of strains (%) |
|-----------------------|
| I + R                 |

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 ng) 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 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 | 20.0 | 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.
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.

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 cases (*P<0.05*).

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 QR-DRs accompanied by changes in 3 or 4 amino acids in QR-DRs. The *aac (6') Ih-cr*, one of the genes encoding PMQRs, was detected in only 1 strain. In total, more than 70% of 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).
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/ml possessed \( \text{bla}_{\text{TEM-1}} \), and 100% of isolates with a CHL MIC of >128 µg/ml possessed \( \text{catA1} \) (Table 6). Expression levels of \( \text{acrA} \), \( \text{acrB} \) and \( \text{tolC} \) 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 \( \text{acrB} \) expression, while isolates with an intermediate interpretation to AMP also exhibited OST and had higher \( \text{acrB} \) expression than did isolates that were AMP-susceptible (data not shown).

Among dogs from which we isolated \( \text{E. coli} \) 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 \( \text{E. coli} \) 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 \( \text{E. coli} \) 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 \( \beta \)-lactam antimicrobials (Table 6).

**DISCUSSION**

In this study, \( \text{E. coli} \) isolates with resistant or an intermediate interpretation to aminopenicillins, CHL, or fluoroquinolone were more frequently obtained from dogs admitted to the University 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 \( \text{E. coli} \) 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 \( \text{E. coli} \) 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 \( \text{acrA} \), \( \text{acrB} \) and \( \text{tolC} \) 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 \( \text{E. coli} \) 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 group   | Canine case history          | Antimicrobial use for 6 months before sampling | QRDR mutations* | MIC (µg/ml) | β-lactamase gene | PMQR | CP-re- resistance gene | Expression level* |
|----------------|------------------------------|-----------------------------------------------|-----------------|------------|------------------|------|------------------------|-----------------|
| University group |                              |                                               |                 |            |                  |      |                        |                  |
| RE18 Mastocytoma | CFZ, LEX                     | L N I G - -                                  | >128            | >128 128 (+16)³ | 32 (+16)³ | N.D. | N.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. | catI                  | 2.31 3.77 4.65  |
| RE28 Rhabdomyosarcoma | CFZ, LEX                    | L N I G - -                                  | >128            | >128 128 (+32) | 64 (+16) | blcAβI-t-l   | N.D.          | 2.17 7.13 3.46  |
| RE33 Mastocytoma | CFZ, LEX, OFX                | L N I G - -                                  | >128            | 2 64 (+8) 16 (+8) | 16 (+8) | N.D. | N.D.                  | 0.75 2.68 1.87  |
| RE61 Herniated intervertebral discs | LEX                        | L N I G - -                                  | >128            | <128 <128 <128 <128 | 32 (+4) | N.D. | N.D.                  | 2.16 2.9 3.95    |
| RE63 Unknown | AMP                          | L N I V - -                                  | >128            | 16 (+8) 16 (+8) | 16 (+8) | N.D. | N.D.                  | 0.89 0.96 1.77  |
| RE20 Lung tumor | AMC, CFZ, ENR                | L N I V - -                                  | >128            | 64 (+8) 16 (+8) | 16 (+8) | N.D. | N.D.                  | 0.102 1.01 1.72  |
| RE2 Tumor of the breast | GEN, FRM                   | L N I V - -                                  | >128            | >128 >128 >128 >128 | 32 (+4) | N.D. | N.D.                  | 1.56 1.2 2.49    |
| RE72 Glaucoma | CFZ, OFX, ORB               | L N I T - -                                  | >128            | 1 64 (+8) 16 (+8) | 16 (+8) | N.D. | catI                  | 1.54 2.67 4.09  |
| RE80 Oral tumor | None                         | L N I T - -                                  | >128            | 0.5 N.D. N.D. N.D. | N.D.  | N.D. | N.D.                  | 1.15 1.58 2.56  |
| RE4 Osteosarcoma | AMP, AMX, FRM               | L N I - E660A                                | >128            | >128 >128 >128 >128 | 64 (+16) | N.D. | catI                  | 0.76 0.104 1.29  |
| RE64 Oral tumor | CFZ                          | L N I - E660A                                | >128            | >128 >128 >128 >128 | 64 (+16) | N.D. | catI                  | 0.8 0.104 1.29  |
| RE65 Unknown | None                         | L N I - E660A                                | >128            | >128 >128 >128 >128 | 64 (+16) | N.D. | catI                  | 1.94 1.67 2.55  |
| RE62 Multiple myeloma | ENR, MIN                   | L N I - S458A                                | >128            | 4 128 (+16) 16 (+8) | 8 (+4)  | N.D. | N.D.                  | 1.15 1.58 2.56  |
| RE26 Unknown | CFZ, LEX                     | L N I - 4 (+4)                               | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 0.84 1.28 1.60  |
| RE50 Keratitis | OFX, ORB                    | L N I - 4 (+4)                               | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 1.32 1.65 2.74  |
| RE54 Biopsy of vertebral body | CFZ                       | L N I - 4 (+4)                               | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | catI                  | 1.54 2.67 4.09  |
| RE17 Cushing syndrome | CFZ                    | L N I V - 16 (+8)                             | 1              | 64 (+8) 16 (+8) | 16 (+8) | N.D. | N.D.                  | 1.36 2.76 3.87  |
| RE39 Herniated intervertebral discs | AMP                        | L N I V - 16 (+8)                             | 1              | 64 (+8) 16 (+8) | 16 (+8) | N.D. | catI                  | 1.18 3.28 3.74  |
| Community group |                              |                                               |                 |            |                  |      |                        |                  |
| CE7 Otis externa | LEX, GEN                     | L N I A - -                                  | >128            | >128 >128 >128 >128 | 64 (+16) | N.D. | N.D.                  | 0.88 1.4 2.01  |
| CE5 Unknown | None                         | L N I G - -                                  | >128            | 0.5 64 (+8) 16 (+8) | 8 (+4)  | N.D. | N.D.                  | 0.25 0.37 0.43  |
| CE6 Unknown | LEX, CFZ, GEN                | L N I G - -                                  | >128            | >128 >128 >128 >128 | 64 (+4) | N.D. | N.D.                  | 0.63 2.12 2.02  |
| CE10 Diarrhea | None                         | L N I V - 4 (+4)                              | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 0.84 1.28 1.60  |
| CE14 Unknown | None                         | L N I V - 4 (+4)                              | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 1.34 1.71 1.52  |
| CE10 Unknown | None                         | L N I V - 4 (+4)                              | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 1.34 1.71 1.52  |
| CE12 Gingivitis | CLI                         | L N I S458T                                  | >128            | 2 128 (+8) 8 (+4) | 8 (+4)  | N.D. | nusS                 | 0.24 0.46 0.56  |
| CE13 Diarrhea | SXT                          | L N I - S458T                                 | >128            | 2 128 (+8) 8 (+4) | 8 (+4)  | N.D. | nusS                 | 0.91 1.6 1.82  |
| CE3 Pharyngitis | AMP, LEX, OFX               | L N G R - -                                  | >128            | 32 32 (+8) 8 (+4) | 8 (+4)  | N.D. | nusS                 | 0.9 0.9 1.25    |
| CE4 Unknown | None                         | L N G R - -                                  | >128            | 32 32 (+8) 8 (+4) | 8 (+4)  | N.D. | nusS                 | 0.95 1.51 1.7    |
| CE8 Keratitis | LEX, LVX                     | L N I - 4 (+4)                               | 0.25            | 0.25 16 (+8) 8 (+4) | 8 (+4)  | N.D. | N.D.                  | 0.97 1.34 1.43  |

Total (%) R 84.2 63.1 100 52.6* 1.83* 3.48** 3.06**

<i>1 + R 94.7 52.6 100 94.7** 4.07**</i>

<i>(×8.0)c) (×4.6)c)</i>

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 \( \mu g/\text{ml} \) that had been derived from ENR-supplemented DHL agar plates possessed \textit{catA1}. However, other resistant isolates with a CHL MIC of 32 and 64 \( \mu g/\text{ml} \) and an intermediate interpretation isolates with a CHL MIC of 16 \( \mu g/\text{ml} \) 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 cephalosporins, possessed \textit{bla}_{TEM-1}. However, isolates with intermediate interpretation to AMP did not possess any of the \( \beta \)-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., \textit{bla}_{TEM-1} or \textit{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 \( \mu g/\text{ml} \)) or resistance (32 or 64 \( \mu g/\text{ml} \)) 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 \textit{E. coli} isolates that have active AcrAB–TolC [28]. We observed that OST isolates with higher \textit{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 \( \mu g/\text{ml} \), also exhibited OST and higher \textit{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 \textit{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 \textit{E. coli} strains with QRDR mutations, \( \beta \)-lactamase gene and \textit{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 \textit{E. coli} isolates. Indeed, our results showed that ENR-resistant \textit{E. coli} isolates had higher rates of resistance to several antimicrobials compared with ENR-susceptible \textit{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 \textit{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 \textit{E. coli} isolates in dogs in the United States [2, 8]. Moreover, fluoroquinolone–aminopenicillin–CHL-resistant \textit{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 \textit{E. coli} isolates 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 \( \beta \)-lactamases, \textit{catA1} and QRDR mutations, but also to overexpression of the AcrAB–TolC efflux pump in canine \textit{E. coli}.

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