Phylogenetic association of fluoroquinolone and cephalosporin resistance of D-O1-ST648 *Escherichia coli* carrying *bla*<sub>CMY-2</sub> from faecal samples of dogs in Japan

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This study aimed to investigate the genetic association between fluoroquinolone (FQ) and/or cephalosporin (CEP) resistance in *Escherichia coli* isolates from dogs, and the risk to human health. We characterized *E. coli* clinical isolates, derived from faecal samples of dogs attending veterinary hospitals, using phylogenetic grouping, determination of virulence factor (VF) prevalence, multilocus sequence typing (MLST) and O serotyping. The D group was the dominant phylogenetic group among strains resistant to FQ and/or CEP. In contrast, the dominant group among susceptible strains was group B2. Group D strains showed a significantly higher prevalence of VFs than strains belonging to groups A and B1, and were resistant to significantly more antimicrobials than group B2 strains. The phylogenetic distribution of FQ–CEP-resistant *E. coli* groups (FQ–CEPRECs) and FQ-resistant groups was significantly correlated (r=0.98), but FQ–CEPRECs and CEP-resistant *E. coli* groups were not correlated (r=0.58). Data from PFGE, O serotype and MLST analyses indicated that the majority of FQ-resistant strains derived from a particular lineage of phylogenetic group D: serotype O1 and sequence type (ST) 648. Some D-O1-ST648 strains carried *bla*<sub>CMY-2</sub>, showed multidrug resistance and possessed a higher prevalence of the VFs *kspMT*, *ompT* and PAI compared with other group D strains. Our data indicate that the emergence of FQ-CEP-resistant *E. coli* is based primarily on FQ-resistant *E. coli*. Moreover, as strains of the D-O1-ST648 lineage have been found in clinical isolates derived from humans at a relatively high frequency, our findings indicate that the spreading of D-O1-ST648 strains may cause serious difficulties in both veterinary and human clinical fields in the future.

INTRODUCTION

Companion animals are an integral part of the daily life of many humans, and their medical treatment is similar to that of humans. For example, antimicrobials used for humans are also used in the treatment of companion animals. Inappropriate and excessive use of these antibiotics has led to increased resistance against these agents (Bergman et al., 2009). Recently, common clonal groups of fluoroquinolone (FQ)-resistant extra-intestinal *Escherichia coli* [serotype 25 (O25), sequence type (ST)131] have frequently been isolated from the human clinical field but has also been isolated from companion animals, although at an apparently lower rate (Platell et al., 2011). These results suggest that transmission of FQ-resistant extra-intestinal *E. coli* is caused mainly by transmission from humans to dogs. However, this evidence also suggests the opposite possibility: that some unknown clonal groups that are dominant in companion animals also harbour antimicrobial-resistant bacteria, and that transmission of these strains to humans poses a public health risk to humans.

Abbreviations: AMP, ampicillin; CEP, cephalosporin; CEPRECs, strains resistant to CEPs but not FQs; CFZ, cefazolin; CHL, chloramphenicol; CPD, cefpodoxime; DSM, dihydrostreptomycin; ENR, enrofloxacin; ESBL, extended-spectrum β-lactamases; FQ, fluoroquinolone; FQRECs, strains resistant to FQs but not CEPs; GEN, gentamicin; KAN, kanamycin; MLST, multilocus sequence typing; NAL, nalidixic acid; OTC, oxytetracycline; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region; VF, virulence factor.
are frequently isolated from humans. For example, extended-spectrum β-lactamase (ESBL)-producing E. coli frequently show FQ resistance (Ruppé et al., 2009). Emergence of these FQ–CEP RECs has become a serious clinical problem.

FQ resistance is mainly caused by amino acid substitutions in the quinolone resistance-determining region (QRDR) of gyrA and gyrB, genes that encode DNA gyrase subunits, and in parC and parE, which encode topoisomerase IV subunits (Hopkins et al., 2005). On the other hand, third-generation CEP resistance is caused mainly by plasmid-mediated ESBLs and ampC genes, such as blaCTX-M and blaCMY, in E. coli and Klebsiella pneumoniae (Pérez-Pérez & Hanson, 2002). Therefore, the main mechanisms underlying acquisition of FQ and CEP resistance should be independent. However, previous studies have reported a significant correlation between the occurrence of FQ resistance and CEP consumption, and between CEP resistance and FQ consumption in E. coli (Bergman et al., 2009). Recent studies have indicated that a reason for this phenomenon may be the co-localization of ESBL genes and plasmid-mediated quinolone resistance (PMQR) determinants on the same genetic platforms (Kim et al., 2009a; Lavigne et al., 2006). However this cannot be the only reason, as FQ–CEP RECs, which do not possess PMQR determinants, have also frequently been found (Ambrozic Avgustin et al., 2007; Kirchner et al., 2011).

Phylogenetic groups (A, B1, B2 and D) and virulence factor (VF) genes have been investigated as epidemiological markers in intestinal and extra-intestinal E. coli isolates in humans and animals (Clermont et al., 2000; Johnson et al., 2009; Takahashi et al., 2009; Yokota et al., 2012). In general, extra-intestinal virulent strains frequently belong to group B2, and to a lesser extent to group D. Less virulent indigenous intestinal strains are frequently from group A or B1 (Clermont et al., 2000). Several previous studies have shown that the phylogenetic group prevalent among FQ-resistant E. coli differs from that among FQ-susceptible E. coli. Takahashi et al. (2009) showed that, although phylogenetic group B2 was dominant, its prevalence was significantly less in FQ-resistant E. coli (49%) than in FQ-susceptible E. coli (78%) from human urinary isolates. Our own recent study indicated that FQ-resistant strains isolated from human clinical samples were mainly of the B2 group (80.4%) and in Japan, most (94.4%) of these B2 strains belonged to the O25:H4-ST131 lineage (Yokota et al., 2012). The other FQ-resistant strains were predominantly from group D. In isolates derived from dogs, the dominant groups among FQ-resistant E. coli were B1 and D (both 34%), and among FQ-susceptible E. coli was B2 (53%) (Johnson et al., 2009). Therefore phylogenetic grouping, which is a simple and inexpensive method, could be a useful marker indicating the association between genetic background and pathogenicity or antimicrobial resistance, and so could support epidemiological analysis in clinical E. coli isolates.

In this study, we characterized E. coli clinical isolates derived from dogs attending veterinary hospitals to elucidate the association of FQ and CEP resistance by using genetic analysis, including the determination of phylogenetic groups and VF prevalence. We also investigated the hazard to human health via canine FQ- and CEP-resistant extra-intestinal pathogenic E. coli by using multilocus sequence typing (MLST) and O serotyping.

METHODS

Bacterial strains. One hundred and forty E. coli strains were isolated from 173 canine rectal samples using cotton swabs. These samples were recovered from dogs as follows: 93 dogs treated at Rakuno Gakuen University Animal Hospital (Ebetsu, Japan; strains named RE) and 80 dogs treated at eight community animal clinics (Ebetsu, Japan; strains named CE) in 2005. The case histories of the dogs were varied and included urethritis, cystitis, otitis externa, chronic diarrhoea, dermatitis, tumour, herniated intervertebral discs, keratitis, Cushing’s syndrome and septicemia. Canine rectal samples were collected immediately before administration of receiving any clinical treatment when the dogs visited the clinics or the university hospital. The percentages of dogs treated with FQ, CEP and any antimicrobials (for 6 months prior to sampling) were 22.2, 33.3 and 60.5%, respectively. The samples were incubated for 24 h at 37 °C in deoxycholate hydrogen sulfide lactose agar (Nissui) and subcultured in nutrient agar (Nissui). After incubation, the biochemical properties of these cultures were studied using triple-sugar iron medium (Nissui), lysine indole motility medium (Nissui) and an oxidase test. Final identification of E. coli was performed using API20E (bioMérieux). We classified the 140 isolated strains into groups according to FQ and/or CEP resistance as follows: 22 FQ–CEP RECs, 14 strains resistant to FQs but not CEPs (FQRECs), 10 strains resistant to CEPs but not FQs (CERP RECs) and 94 strains susceptible to both antimicrobials. Among the 94 susceptible strains, we randomly selected 35 and performed the following analyses. FQ- and CEP-resistant strains were defined as strains having MICs higher than 4 μg ml⁻¹ for enrofloxacin (ENR) and higher than 8 μg ml⁻¹ for cepodoxime (CPD) following antimicrobial susceptibility testing.

Susceptibility testing. Susceptibilities to a panel of 10 antimicrobials were studied by the agar dilution method, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008), using an inoculum of 10⁸ c.f.u. Mueller–Hinton agar was obtained from Oxoid, and ENR and CEP from Bayer. Ampicillin (AMP), ceftazolin (CFZ), CDP, gentamicin (GEN), kanamycin (KAN), dihydrostreptomycin (DSM), oxytetracycline (OTC) and chloramphenicol (CHL) were obtained from Sigma-Aldrich, and nalidixic acid (NAL) from Wako Junyaku. Resistance to DSM and OTC was defined by the Japanese Veterinary Antimicrobial Resistance Monitoring System (National Veterinary Assay Laboratory, 2009).

Detection of QRDR mutations, PMQRs and β-lactamase genes. Mutations in the QRDR of gyrA, parC, parE and gyrB were detected by direct DNA sequencing of PCR-amplified products, as described in previous studies (Sorlozano et al., 2007, Yang et al., 2004). β-Lactamase genes were detected by PCR and direct DNA sequencing. blaTEM, blaSHV and the chromosomal ampC promoter region were detected as described by Ruppé et al. (2009). Plasmid-mediated ampC was detected according to Pérez-Pérez & Hanson (2002), and bleCTX-M was detected as defined by Xu et al. (2005). The subtypes of detected β-lactamase genes were determined by nucleotide sequences using a BigDye Terminator v3.1 Cycle Sequencing kit with a 3130 Genetic Analyzer (Applied Biosystems). PMQR genes
[qnrA, qnrB, qnrS, aac(6’)-Ib-cr and qepA] were detected by PCR and direct DNA sequencing (Kim et al., 2009b).

**Phylogenetic groups, VF profile and pathogenicity island carrying uropathogenic-specific protein (PAIusp) subtypes.**

Phylogenetic groups were determined by a dichotomous decision tree, as reported previously, based on detection of chuA and yjaA and DNA fragment TspE4.C2, as determined by PCR (Clermont et al., 2000). PAIusp subtype (Ia, Ib, Ila or Iib) was determined by PCR, as reported previously (Kanamaru et al., 2006). Profiling of 18 VF genes (aer, afa, cnf1, cvaC, ETTT, fimH, fyuA, hly, iheA, iroN, iha, kpsMT, ompT, PAI, pap, sfa/foc, traT and usp) was also carried out by multiplex PCR, as reported previously (Takahashi et al., 2006).

**PFGE.** PFGE was performed according to the Pulsed Net USA protocol using XbaI (Takara) (The National Molecular Subtyping Network for Foodborne Disease Surveillance, 2004). To further investigate the association between FQ and CEP resistance and its pathogenicity, we focused on strains from group D. A CHEF-DR III (Bio-Rad Laboratories) system was used with the following running conditions: 19 h at 11.3 °C, at a voltage of 6 V, ramped with an initial forward time of 2.2 s and a final forward time of 54.2 s. After electrophoresis, the gels were stained with ethidium bromide and photographed. All fragment sizes within the gel were normalized using a DNA Size Standards Ladder (Bio-Rad Laboratories). PFGE profiles were digitized for analysis using BioNumerics version 5.10 software (Applied Maths). The similarity coefficients were calculated using the Dice algorithm. The unweighted pair group method with an arithmetic mean (UPGMA) algorithm was used for clustering with a 0.59 % band position tolerance window and 0.51 % optimization. The most relevant clusters and subclusters in the dendrograms were determined by calculating the similarity cut-off value, as described previously (McLellan et al., 2003).

**O serotyping.** Serotype was determined by a slide agglutination test using *E. coli* O antisera (Denka Seiken). Serotype was also determined by PCR according to the method of Clermont et al. (2007).

**MLST.** MLST was performed using seven conserved housekeeping genes (adk, fumC, gpyB, icd, mdh, purA and recA), as described previously (Tartof et al., 2005).

**Statistical analysis.** Statistical significance between two groups was determined by Fisher’s exact test. Statistical significance among three or more groups was determined by the Mann–Whitney U test. *P* values of <0.05 were considered statistically significant.

**RESULTS**

**Association of antimicrobial susceptibility and phylogenetic groups**

All FQ-resistant strains (22 FQ–CEPRECs and 14 FQRECs) had three or four mutations in QRDRs and only one FQREC strain possessed a PMQR, aac(6’)-Ib-cr (strain CE13). Among the 32 CEP-resistant strains (22 FQ–CEPRECs and 10 CEPRECs), bla<sub>CMY-2</sub> (18 strains), bla<sub>CTX-M-14</sub> (four strains) and bla<sub>CTX-M-27</sub> (one strain) were found (data not shown). Other strains shared a mutation (involving nt 32 or 42) or a nucleotide insertion (14–15insG or 11–12insT) in the chromosomal ampC promoter regions (data not shown) which led to overexpression of AmpC (Mulvey et al., 2005; Ruppé et al., 2009). Although bla<sub>TEM-1</sub> was found in 18 (14 FQ–CEPRECs and four CEPRECs) of the 32 CEP-resistant strains, no other bla<sub>TEM</sub> that altered the activity of TEM-1 towards extended-spectrum cephalosporins was found (data not shown).

The mean number of antimicrobial agents to which strains were resistant was lowest in strains of phylogenetic group B2 (Table 1) and was highest in strains of group D. In particular, the prevalence of CEP resistance (CFZ and CPD) and NAL and ENR resistance was significantly higher in group D than in groups B1 and B2 (*P* value of CEP resistance was 0.035 vs group B1 and 0.003 vs group B2; *P* value of NAL and ENR resistance was 0.013 vs group B1 and 0.004 vs group B2). The prevalence of AMP and CHL resistance in group D was also higher than in strains of group B2. These findings indicate that group D strains tended to be multidrug-resistant compared with strains from other phylogenetic groups.

Among the 35 susceptible strains, those from group B2 were most common among the susceptible strains (14 isolates) compared with other phylogenetic groups (next most common were group B1, 12; group D, 5; and group A, 4). On the other hand, strains from group D were significantly more common among the 14 FQRECs (seven were from group D, three were from group B1 and two each were from groups A and B2) and the 22 FQ–CEPRECs (13 were from group D and three each were from groups A, B1 and B2). There was a significant correlation between FQRECs and FQ–CEPRECs in the distribution of phylogenetic groups (*r* = 0.98, *P* = 0.019), whereas no correlation was found between CEPRECs (10 strains; four were from group D, three each were from groups A and B1) and FQ–CEPRECs (*r* = 0.58).

Of the β-lactamase genes, bla<sub>CMY-2</sub> was detected in 18 strains across all phylogenetic groups; however, nine of these strains belonged to group D. Four strains possessed bla<sub>CTX-M-14</sub>: one strain each from groups A and B1 and two strains from group D. One strain possessing bla<sub>CTX-M-27</sub> was found in group B2.

**Association of VFs and phylogenetic groups**

Group B2 strains harboured VF genes significantly more frequently, in particular sfa/foc, iroN, cnf1, hly, ompT, PAI and usp, than strains from other groups (*P*<0.01; Table 2). However, ET TT was markedly less frequent in group B2 than in other groups. Group D demonstrated the second highest prevalence of VFs. Groups A and B1 both demonstrated a low prevalence of VFs, except for iroN and cvaC, which were more prevalent in group B1. The usp gene was found in groups B2 and D. The PAI<sub>usp</sub> subtypes identified were Ia (in five strains), Ib (three strains) and Ila (eight strains); all PAI<sub>usp</sub>-positive strains were from group B2. Moreover, all four PAI<sub>usp</sub>-positive strains from group D were of type Ila (data not shown).

**PFGE analysis, O serotype and MLST in phylogenetic group D**

To investigate further the association between FQ and CEP resistance, we focused on strains from group D, which were
Companion animals harbour antimicrobial-resistant bacteria in common with those harboured by humans, and possess a higher risk to human health because these clinical field settings have been used in the past. In this study, we showed a significant relationship between FO and CEP resistance, and demonstrated that a common E. coli clone was frequently isolated from dogs in Japan. Group D was the dominant phylogenetic group, whereas group B was the dominant phylogenetic group, and group D was isolated from dogs in Japan. The prevalence of antimicrobial resistance was calculated as the total number of antimicrobial-resistant strains divided by the total number of isolates in each phylogenetic group. Significance was determined using the Kruskal–Wallis test and Dunn’s multiple comparison test (P < 0.05). The prevalence of antimicrobial resistance was significantly higher in group D than in other groups (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group A than in group B (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group B than in group D (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group A than in group C (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group B than in group C (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group A than in group D (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group B than in group D (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group A than in group D (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group B than in group D (P < 0.05). The prevalence of antimicrobial resistance was significantly lower in group A than in group D (P < 0.05). 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Table 2. Prevalence of VFs in *Escherichia coli* phylogenetic groups

<table>
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<th>Phylogenetic group (no. strains)</th>
<th>Adhesin</th>
<th>Iron uptake</th>
<th>Toxin</th>
<th>Cell protection</th>
<th>Other</th>
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<td>fimH</td>
<td>iha</td>
<td>pap</td>
<td>sfa/foc</td>
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<tr>
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<tr>
<td></td>
<td></td>
<td>(50.0)</td>
<td></td>
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<td></td>
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<tr>
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<td>3</td>
<td>1</td>
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<tr>
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<td></td>
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<td>(4.8)</td>
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<td>14</td>
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<td></td>
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<td>(73.7)</td>
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<tr>
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<td>(96.6)</td>
<td>(6.9)</td>
<td>(3.4)</td>
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<td>D-O1-ST648 (10)</td>
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<tr>
<td></td>
<td></td>
<td>(90.0)</td>
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<tr>
<td>Other D (strains belonged phylogenetic group D except ST648) (19)</td>
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<td>0</td>
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<td></td>
<td></td>
<td>(90.0)</td>
<td>(31.6)</td>
<td>(10.5)</td>
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<p>|                                  | No. of strains (%) with indicated VF genes | Mean no. VFs* |</p>
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<td>(63.2)‡‖</td>
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<td>(94.7)‡‖</td>
<td>(42.1)‡‖</td>
<td>(94.7)‡‖</td>
<td>(84.2)‡‖</td>
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<td>(8.9)‡</td>
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<td>(73.7)†‖</td>
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<td>D-O1-ST648 (10)</td>
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<td>Other D (strains belonged phylogenetic group D except ST648) (19)</td>
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*aer*, aerobactin; *afa*, afimbrial adhesion; *cnf1*, cytotoxic necrotizing factor 1; *cvaC*, colicin V; *ETTT*, type III secretion system; *fimH*, type 1 fimbriae adhesion; *fmuA*, yersiniabactin receptor for ferric yersiniabactin uptake; *hly*, α-haemolysin; *ibeA*, invasion of brain endothelium; *adhe*, catecholate siderophore receptor; *iha*, iron-regulated gene A homologue adhesion; *kpsMT*, group 2 capsule synthesis; *ompT*, outer-membrane protease T; *PAI*, pathogenic island marker of CFT073; *pap*, P fimbriae; *sfa/foc*, S/F1C fimbriae; *traT*, serum resistance associates; *usp*, uropathogenic-specific protein.

*Mean number of VFs was calculated as the total number of VFs divided by the total number of isolates in each phylogenetic group.

†Significant difference versus group A (*P*<0.05).

‡Significant difference versus group B1 (*P*<0.05).

§Significant difference versus group B2 (*P*<0.05).

‖Significant difference versus group D (*P*<0.05).

§Significant difference versus other D (strains belonged phylogenetic group D except ST648) (*P*<0.05).
E. coli strains belonging to phylogenetic group B2 showed a high prevalence of VFs and were frequently isolated from human clinical specimens (Clermont et al., 2000; Naseer et al., 2012; Takahashi et al., 2009). Thus, it has been recognized that group B2 strains are important in human E. coli infections, and these FQRECs, CEPRECs and CEPRECs carry a risk in clinical antimicrobial treatment regimens. Among these, the FQ-resistant phylogenetic group of E. coli, B2-O25:H4-ST131, which frequently possesses blaCTX-M-15 and aac(6’)-Ib-cr, has spread worldwide in humans (Peirano & Pirout, 2010; Takahashi et al., 2006). We observed B2-O25:H4-ST131 E. coli in 75.9% (85/112 strains) of the FQ-resistant E. coli clinical isolates derived from humans in Hokkaido prefecture in Japan (Yokota et al., 2012). However, these strains did not carry the blaCTX-M-15 and aac(6’)-Ib-cr genes. In this study, we detected only two and three strains belonging to group B2 from FQRECs and FQ-CEPRECs, respectively, whereas group B2 was the most dominant group among the susceptible strains. In addition,
B2-O25:H4-ST131 FQ-resistant strains were not observed in this study. Although a previous study isolated B2-O25:H4-ST131 in E. coli isolates derived from dogs, the frequency was lower than those in isolates derived from humans (Peerano & Pitout, 2010; Platell et al., 2011; Takahashi et al., 2006; Yokota et al., 2012). Therefore, these results suggest that the risk of transmission of FQRECs, CEPRECs, and FQ–CEPRECs belonging to group B2 (including B2-O25:H4-ST131) from dogs and humans is low, and that these are not a major source of human E. coli infections.

On the other hand, we found that the major strain (27.8%, 10 of 36 FQ–CEPRECs/FQRECs) in the canine population in the clinical environment of Hokkaido prefecture in Japan was D-O1-ST648 (Fig. 1). This clonal group has also been reported in dogs; 42.9% (9/21 strains) of bla<sub>CMY-2</sub>-bearing CEPRECs obtained from stray dogs in Korea were D-ST648 (Tamang et al., 2012). Hadada et al. (2012) detected a D-O1-ST648 strain, possessing bla<sub>CTX-M-14</sub> and bla<sub>CMY-2</sub>-in CFZ-resistant extra-intestinal pathogenic E. coli strains from a cat from a different region in Japan. Therefore, these results suggest that ST648 has spread in companion animals possessing β-lactamase genes, at least in Asia.

Clinical ST648 isolates have been also reported worldwide in humans since 2011. Our recent study on human isolates derived from Hokkaido prefecture showed that D-O1-ST648 was the second-most prevalent genotype among FQ-resistant strains (5/112 strains; 4.5% of the FQ-resistant isolates, followed by ST131; unpublished results, Yokota et al., 2012). Little et al. (2012) reported that ST648 strains isolated from human blood cultures carried the S83L and D87N substitutions in GyrA, and possessed both bla<sub>CMY-2</sub> and bla<sub>IMP-4</sub> genes. Remarkably, ST648 strains have also been reported as possessing the New Delhi metallo-enzyme carbapenemase in the UK (Hornsey et al., 2011). The D-O1-ST648 strains in this study also carried these amino acid substitutions in GyrA, and some carried the bla<sub>CMY-2</sub> gene (Fig. 1). As described above, Naseer et al. (2012) reported that ST648 was detected in four European countries, Norway, the UK, Spain and Sweden, and this clonal group was the second major clonal group (followed by ST131) in ESBL-producing E. coli strains derived from human clinical specimens. These findings indicate the possibility that transmission of this clonal lineage occurs between humans and dogs. In addition, VF genes (kpsMT, ompT and PAI) were significantly more prevalent in D-O1-ST648 FQRECs/FQ–CEPRECs than in other group D strains (Table 2). This finding indicates that the spread of D-O1-ST648 FQRECs/FQ–CEPRECs may cause serious difficulties in the clinical field, as will the spread of B2-O25:H4-ST131 strains.

However, many previous reports lack clinically important information regarding FQ or CEP susceptibility, VF profiles, phylogenetic group and O serotype in ST648 strains. Therefore, comprehensive surveillance for and implementation of appropriate interventions against D-(O1)-ST648 strains are required.

In conclusion, the majority of canine FQRECs and FQ–CEPRECs in Hokkaido prefecture, Japan, had genetic background features in common, indicating that they belong to the D-O1-ST648 group. These D-O1-ST648 FQRECs/FQ–CEPRECs, which have spread among humans and their companion animals, showed high VF prevalence and multidrug resistance, and may pose a serious challenge to management of infections in the future.

**ACKNOWLEDGEMENTS**

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