Prominence of an O75 Clonal Group (Clonal Complex 14) among Non-ST131 Fluoroquinolone-Resistant Escherichia coli Causing Extraparticulate Infections in Humans and Dogs in Australia

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Fluoroquinolone (FQ)-resistant extraparticulate pathogenic Escherichia coli (FQr ExPEC) strains from phylogenetic group B2 are undergoing epidemic spread. Isolates belonging to phylogenetic group B2 are generally more virulent than other E. coli isolates; therefore, resistance to FQs among group B2 isolates is concerning. Although clonal expansion of sequence type 131 (ST131) is a major factor, the contribution of additional clonal groups has not been quantified. Group B2 FQr ExPEC isolates from humans (n = 250) and dogs (n = 12) in Australia were screened for ST131, a recently recognized and rapidly emerging multidrug-resistant and virulent clonal group that is important in both human and companion animal medicine. Non-ST131 isolates underwent virulence genotyping, PCR-based O typing, partial multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and FQ resistance mechanism analysis. Of 49 non-ST131 isolates (45 human, 4 canine), 49% (24 human, 2 canine) represented the more virulent phylogenetic group B2, have been shown to possess both pathotypic and phylogenetic similarities (18, 24).

In our previous study (32), 262 (250 human, 12 canine) phylogenetic group B2 FQr E. coli isolates were identified by PCR-based phylotyping among 702 FQr ExPEC clinical isolates obtained from humans and dogs in eastern Australia (2). The human isolates were obtained from two Brisbane private pathology laboratories processing samples from both hospitals (approximately 80 to 85% of submissions) and community clinics (approximately 15% of submissions) between October 2007 and October 2008, and the canine isolates were from four veterinary pathology laboratories (serving Brisbane, Sydney, and Melbourne) processing samples from both community clinics and referral hospitals between October 2007 and October 2009. Group B2 isolates underwent PCR-based screening for ST131-specific single-nucleotide polymorphisms (SNPs) in mdh and gyrB (15); this identified 213 (205 human, 8 canine) isolates as ST131 and 49 (45 human, 4 canine) as non-ST131 (32). For comparative purposes, four historical fluoroquinolone-susceptible phylogenetic group B2 O75 blood isolates from humans with urosepsis (strains 2H19, PM3, V21, and V8) (19) were included.

O typing and further isolate selection. As O types may be a primary indicator of clonal association, the 49 FQr non-ST131 group B2 isolates underwent PCR-based O typing to detect 13 known clonal group- and/or

Materials and Methods

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O typing and further isolate selection. As O types may be a primary indicator of clonal association, the 49 FQr non-ST131 group B2 isolates underwent PCR-based O typing to detect 13 known clonal group- and/or
sips-associated rfb variants (O1, O2, O4, O6, O11, O12, O15, O16, O18, O25a, O25b, O75, and O157) (3, 4). Twenty-six isolates belonging to O75 (i.e., 24 human and 2 canine isolates) were further characterized, as detailed below. In addition, the remaining 2 non-O75 canine isolates and 6 (30%; randomly selected) of the 21 remaining non-O75 human isolates were also characterized to determine the level of diversity among the non-O75 isolates.

Susceptibility testing. The 34 selected isolates underwent disk diffusion testing for susceptibility to 19 antimicrobials, including amikacin, amoxicillin-clavulanic acid, ampicillin, aztreonam, cefepime, cefoxitin, ceftazidime, cephalexin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, imipenem, nitrofurantoin, piperacillin, piperacillin-tazobactam, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole (5, 6). Resistance was defined as nonsusceptibility (i.e., resistance or intermediate susceptibility) according to standardized interpretative criteria (5, 6). An isolate’s resistance score was the number of antimicrobials to which the isolate demonstrated resistance, whereas the resistance profile was the combination of antimicrobials to which the isolate exhibited resistance. Resistance profiles were examined to provide information about the extent and specific patterns of resistance, plus provide some (albeit weak) indication regarding clonality.

Virulence gene profiling and lactose fermentation determination. Isolates were screened for 52 ExPEC-associated virulence marker genes and their variants using established multiplex PCR assays (11, 13, 19, 21). Here, the term “virulence genes” is used to include proven or putative virulence-associated genes and associated variants. Virulence scores reflected the total number of virulence genes identified, with adjustment for multiple detection of certain operons (i.e., pap, sfa-loc, and kps). Virulence genotype similarity between isolates was calculated as the number of virulence genes concordantly detected in both isolates divided by the total number of virulence genes detected in either isolate. Virulence profiles provide some indication of the organism’s virulence potential (as reflected in the total number and particular combination of constituent virulence genes), and they may have associations with specific clonal groups and thus provide another marker for clonality independently of their virulence implications. Lactose fermentation was determined by plating on MacConkey agar.

PFGE. For a more discriminating assessment of within- and across-species genomic commonality, the 34 selected Australian isolates and the 4 historical isolates underwent comparative pulsed-field gel electrophoresis (PFGE) analysis according to a standardized protocol (34). Dice coefficient-based similarity dendograms were constructed within BioNumerics software (Bio-Rad) according to the unweighted-pair group method with arithmetic mean. Isolates were considered to represent the same pulsotype if they exhibited ≥94% profile similarity, which approximates to a ≤3-band difference (39).

MLST. To classify the study isolates as to clonal group and to enable interlaboratory comparisons, the 34 selected Australian isolates underwent partial multilocus sequence typing (MLST) (for fumC, gyrb, and reca) according to the Achtman system (http://mlst.ucc.ie/mlst/dbs/Ecoli/). Subsequently, to give a full 7-locus MLST, the remaining 4 loci were analyzed for 9 of these isolates (4 canine, 5 human). The 4 historical O75 isolates also underwent full 7-locus MLST.

Detection of fluoroquinolone resistance genes. For the same 23 isolates, fluoroquinolone resistance-associated chromosomal gene mutations were determined by PCR amplification and pyrosequencing of the quinolone resistance-determining regions (QRDRs) within gyrA and parC (31). Screening for the plasmid-mediated quinolone resistance (PMQR) genes qnrA, qnrB, qnrS, and qepA was performed by multiplex PCR (31). A second PCR was done to identify isolates containing theaac(6’)-Ib-cr variant (31). The degree of homogeneity (or lack thereof) in the fluoroquinolone resistance genes may also aid as a marker for clonality and may be used to indicate divergence among FQR isolates.

Efflux pump activity testing. Two methods were used to indicate the level of efflux pump activity on the 23 representative isolates: MIC testing in the presence of an efflux pump inhibitor and an organic solvent tolerance test. For the former, susceptibility to 4 fluoroquinolones (enrofloxacin, ciprofloxacin, moxifloxacin, and pradofloxacin) was determined in duplicate in the presence of 64 μg/ml of l-phenylalanyl-arginyl-β-naphthylamide (PAβN; P 4157; Sigma) using broth microdilution (5, 6). Organic solvent tolerance was assessed as described previously (31).

Statistical methods. Comparisons of proportions were tested using Fisher’s exact test. Resistance and virulence scores were compared using Mann-Whitney U tests. Analyses were performed using Stata (version 10.0) software (Statacorp, College Station, TX). Diversity of resistance types and virulence types was compared using Simpson’s index of diversity (1-D) (38). The criterion for assuming statistical significance was a P value of <0.05.

RESULTS

O typing and susceptibility testing. Of the 49 non-ST131 FQR group B2 clinical isolates (45 human, 4 canine), approximately half (n = 26; 24 human, 2 canine) represented O-type O75. All 26 O75 isolates were confirmed to be FQR by disc diffusion and broth microdilution MIC determination. Coreistance to non-FQR antimicrobial agents was common among the 26 O75 isolates, with >75% of isolates exhibiting resistance to ampicillin (96%), piperacillin (96%), streptomycin (96%), trimethoprim-sulfamethoxazole (88%), and tetracycline (85%). Resistance to gentamicin (58%), cephalexin (8%), and amoxicillin-clavulanic acid (4%) was less prevalent. In contrast, all 26 O75 isolates were susceptible to amikacin, aztreonam, cefepime, cefoxitin, ceftazidime, chloramphenicol, imipenem, nitrofurantoin, and piperacillin-tazobactam. Resistance scores ranged from 3 to 10 (median, 8). Among the 26 isolates, 8 different resistance profiles were demonstrated (Table 1).

Among the non-O75 isolates examined (n = 8; 2 canine, 6 human), 1 canine isolate showed intermediate susceptibility to both enrofloxacin and ciprofloxacin by disk diffusion, whereas MIC testing indicated susceptibility at the breakpoint for both fluoroquinolones; therefore, this isolate was excluded from further study. Data were therefore collated for six randomly selected non-O75 human isolates and one non-O75 canine isolate. All seven isolates were resistant to ampicillin, the only non-FQR agent to which a >75% resistance prevalence was detected among these isolates. Other less prevalent resistances included cephalexin (71%), piperacillin (57%), tetracycline (57%), trimethoprim-sulfamethoxazole (57%), amoxicillin-clavulanic acid (43%), cefoxitin (29%), gentamicin (29%), streptomycin (29%), and chloramphenicol (14%). All seven non-O75 isolates were susceptible to amikacin, aztreonam, cefepime, ceftazidime, imipenem, nitrofurantoin, and piperacillin-tazobactam. Resistance scores ranged from 5 to 8 (median, 7; versus O75 isolates, P = 0.25). Compared with the O75 isolates (1-D = 0.71), resistance profiles were more diverse among the seven non-O75 isolates (1-D = 1), each of which had a unique profile (Table 1).

Virulence genotypes and lactose fermentation. Among the 26 O75 isolates, 17 different virulence markers were detected overall,
with individual isolates having a median virulence score of 12 (range, 11 to 13). Virulence genotypes were highly similar, with pairwise similarity values ranging from 96.5% to 100%. The O75 isolates’ consensus virulence genotype included the F10 papA allele (P fimbia structural subunit variant), iha (adhesin-siderophore receptor), fimH (type 1 fimbrae), sat (secreted autotransporter toxin), fyuA (yersiniabactin receptor), iutA (aerobactin receptor), kpsMII (group 2 capsule), usp (uropathogenic specific protein), ompT (outer membrane protease), and malX (pathogenicity island marker), with variation limited to presence/absence of the K1 and K5 group 2 capsule variants (Table 2). None of the detected virulence markers differed in prevalence between human and companion animal O75 isolates.

In contrast, among the non-O75 isolates, 41 (79%) of the 52 virulence scores were identical among the non-O75 isolates (79% similarity to the two main clusters. Notably, both K1 (outer membrane protease), and malX (pathogenicity island marker), with variation limited to presence/absence of the K1 and K5 group 2 capsule variants (Table 2). None of the detected virulence markers differed in prevalence between human and companion animal O75 isolates.

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Our findings support four main conclusions regarding the recent worldwide emergence of FQ-resistant phylogenetic group B2 E. coli. First, the O75/ST1193 isolates account for half of the non-ST131 FQ-resistant group B2 population in this collection. Second, these isolates represent the apparent emergence of FQ-resistant variants of an O75-associated clonal group (CC14, founder strain PM2) from FQ-susceptible O75/ST131 isolates. Third, the virulence genotypes of the historical FQ-susceptible O75/CC14 isolates were highly similar to those of the present FQ-resistant Australian isolates (Table 2). They included a typical V21-containing PfG, and all the virulence genes found in the Australian O75/ST1193 isolates (cluster II, pulsotype 1297, Fig. 1).

Our findings indicate that FQ-resistant phylogenetic group B2 E. coli isolated from extraintestinal infections are highly clonal, as indicated by both PFGE and MLST and their possession of a relatively homogeneous repertoire of virulence genes, resistance profiles, and FQ-resistant mechanisms. Although this conclusion is based on a small number of isolates, O75/ST1193 isolates from both humans and dogs also exhibit clonality and whether FQ-resistant O75/ST1193 isolates represent this newly recognized, FQ-resistant O75-associated clonal group, the O75 isolates identified in the current study are likely to be descendants of a more long-standing clonal lineage than the apparent emergence of FQ-resistant variants of an O75-associated clonal group. The recent worldwide emergence of FQ-resistant phylogenetic group B2 E. coli has been associated with the apparent emergence of FQ-resistant variants of an O75-associated clonal group, the O75 isolates identified in the current study are likely to be descendants of a more long-standing clonal lineage than the apparent emergence of FQ-resistant variants of an O75-associated clonal group. Our findings indicate that FQ-resistant phylogenetic group B2 E. coli isolated from extraintestinal infections are highly clonal, as indicated by both PFGE and MLST and their possession of a relatively homogeneous repertoire of virulence genes, resistance profiles, and FQ-resistant mechanisms. Although this conclusion is based on a small number of isolates, O75/ST1193 isolates from both humans and dogs also exhibit clonality and whether FQ-resistant O75/ST1193 isolates represent this newly recognized, FQ-resistant O75-associated clonal group, the O75 isolates identified in the current study are likely to be descendants of a more long-standing clonal lineage than the apparent emergence of FQ-resistant variants of an O75-associated clonal group.
groups, including O75 (22), and the frequent isolation of another successful FQr clonal group, i.e., ST131, from both humans and dogs (16, 31). It appears that fluoroquinolone resistance among phylogenetic group B2 isolates, at least in Australia, is emerging mainly by expansion of two resistant clonal groups—primarily ST131, as previously described (31), and ST1193, as described here—rather than by arising repeatedly by independent mutations in diverse genetic backgrounds.

Historically, FQr extraintestinal infection isolates have tended to represent the less virulent phylogenetic groups D and A (10, 36). In contrast, phylogenetic group B2 E. coli isolates, which tend to possess an enhanced array of virulence genes, typically have been more susceptible to antimicrobials, including FQs (14). In conflict with this classic paradigm, two clonal groups within group B2, i.e., ST131 and the O75-associated ST1193 clonal group described here, possess an extensive virulence gene repertoire (15, 25), together with a multidrug resistance phenotype. Thus, this O75 clonal group and ST131 demonstrate that resistance and enhanced virulence can no longer be regarded as mutually exclusive within E. coli. Concurrence of resistance and virulence within epidemic ExPEC clonal groups was demonstrated previously by the phylogenetic group D-derived clonal groups CC69 (clonal group A; O11/O17/O73/O77/K52:H18) and O15:K52:H1, albeit with resistance to trimethoprim-sulfamethoxazole rather than fluoroquinolones (1, 17, 20). A corollary of this finding is that group B2-derived clonal groups clearly can no longer be presumed to be susceptible to clinically important antimicrobials, as was true until recently.

The O75 ST1193 clonal group isolates possessed nearly identical virulence genotypes that included the F10 papA allele, iha, fimH, sat, vat, fyuA, iutA, kpsMII, usp, ompT, and malX, differing only for presence of the K1 or K5 group 2 capsule variants. Although according to virulence genotype and PFGE profile our Australian O75 isolates closely resembled an FQ-susceptible O75 urosepsis isolate from Seattle, WA, from the 1980s (isolate 2H19), they lacked traT and most pap operon genes (19), which, in contrast, were present in strain 2H19. Moreover, both 2H19 and the Australian O75 isolates lacked clbB and clbN, which were present in the other historical O75 urosepsis isolates. On a broad scale, most O75 isolates appear to belong to a prevalent clonal complex (CC14) that has been widely geographically distributed for decades, if not longer (23). Certainly, phylogenetic group B2 and serogroup O75 have been associated with urinary tract infection and sepsis for over half a century, and the virulence traits seen in

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**FIG 1** Dendrogram of XbaI pulsed-field gel electrophoresis profiles of 38 phylogenetic group B2 Escherichia coli isolates, 34 human O75 isolates, 4 canine O75 isolates, 6 human non-O75 isolates, 2 canine non-O75 isolates, and 4 historical O75 isolates.
our Australian O75 isolates are known to be broadly disseminated among O75 strains (7, 28, 29). Interestingly, all isolates belonging to the O75/ST1193 clonal group were lactose nonfermenters, which may have implications regarding detection in a clinical microbiology setting.

PFGE analysis resolved two main subgroups (i.e., clusters) among the O75 isolates, one characterized by the K1 capsule (cluster I) and the other (which included historical isolate 2H19) by the K5 capsule (cluster II). Within each cluster, the constituent human and canine isolates were intermingled, without evident species-specific segregation. Although the number of companion animal isolates is low, our identification of highly genetically similar human and veterinary E. coli O75 isolates, which coincides with our previous demonstration of clonality among human and canine ST131 isolates, suggests the potential for transmission between humans and their pets and/or vice versa.

The homogeneity of the Australian FQr O75 ST1193 isolates suggests recent dissemination of successful new FQr clonal variants among both humans and dogs. In contrast, a lesser degree of homogeneity exists among the historical O75 isolates, further evidence that the Australian O75/ST1193 isolates recently emerged and spread from a common ancestor, most likely within the lineage represented by the historical O75 isolate 2H19. This is supported by the identical pattern of a double nonsynonymous mutation (S83L [TCG to TTG] and D87N [GAC to AAC]) plus a single silent mutation (V85V [GGT to GTC]) in gyrA and a single nonsynonymous mutation in parC (S801 [AGC to AGT]) in all Australian FQr O75 isolates compared to the varied gyrA and parC mutations observed in the non-O75 group.

In conclusion, we have documented a high prevalence of clonally related FQr serogroup O75 EsPEC isolates from ST1193 among humans and dogs in Australia. Further studies of current and historical FQr isolate collections are required to determine if this is a local or broader (e.g., global) phenomenon and to pinpoint in time the development of FQ resistance among O75 isolates. Furthermore, there is a global need for strategic surveillance and control schemes for FQ extraintestinal E. coli with high virulence potential, particularly given the emergence and spread of multiresistant group B-derived clonal groups such as ST131 and, now, ST1193 (CC14).

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