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Virulence genotypes and phylogenetic background of fluoroquinolone-resistant and susceptible *Escherichia coli* urine isolates from dogs with urinary tract infection☆

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ABSTRACT

The origins and virulence potential of fluoroquinolone-resistant (FQ-R) *Escherichia coli* from dogs with urinary tract infection (UTI) are undefined. Therefore, fluoroquinolone-resistant ($n = 38$) or susceptible ($n = 62$) *E. coli* urine isolates from dogs with UTI were characterized for phylogenetic group (A, B1, B2, D) and 61 virulence-associated genes by multiplex PCR, then were compared according to these characteristics. Compared with fluoroquinolone-susceptible (FQ-S) isolates, the fluoroquinolone-resistant isolates exhibited significantly lower prevalences for most virulence genes studied (albeit higher prevalences for several, including *iutA*: aerobactin receptor), significantly fewer virulence genes per isolate, and shifts away from virulence-associated group B2. Nonetheless, 26% of fluoroquinolone-resistant isolates qualified as extraintestinal pathogenic *E. coli* (ExPEC), suggesting possible human virulence potential. The findings call into question whether the fluoroquinolone-resistant *E. coli* encountered in dogs arise through conversion of fluoroquinolone-susceptible canine resident strains to resistance, or instead are imported from an external source. They also identify dogs as a possible reservoir of drug-resistant ExPEC for transmission to other pets and humans.

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1. Introduction

Escherichia coli is a leading cause of urinary tract infections in dogs and humans (UTI) (Ling, 1995; Russo and Johnson, 2003). Most *E. coli* UTI episodes are caused by extraintestinal pathogenic *E. coli* (ExPEC) from phylogenetic group B2 (Johnson et al., 2003a; Russo and Johnson, 2000). The rising prevalence in *E. coli* of resistance to antimicrobial agents, including fluoroquinolones, has created a need to define the origins, transmission pathways, and virulence potential of the resistant strains (Cooke et al., 2002; Garau et al., 1999; Goettsch et al., 2000).

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Among human-source *E. coli*, antimicrobial resistance is often associated with reduced virulence and shifts toward non-B2 phylogenetic groups, suggesting a possible external source for the resistant strains (Johnson et al., 2004, 2003b; Moreno et al., 2006b; Vila et al., 2002). In contrast, resistant and susceptible *E. coli* from food animals or retail meats are quite similar, consistent with a common origin, e.g. the host's gut microflora (Johnson et al., 2005a, 2006a, 2005d, 2003c, 2007).

Which (if either) paradigm holds for the antimicrobial-resistant *E. coli* encountered in dogs is unknown. Accordingly, in the present study we characterized a collection of fluoroquinolone-resistant (FQ-R) and fluoroquinolone-susceptible (FQ-S) *E. coli* urine isolates from the late 1990s from dogs with UTI according to phylogenetic distribution and virulence profiles (Cooke et al., 2002; Singer and Cooke, 2002). We then compared the FQ-R and FQ-S populations with one another and with relevant external control groups.

2. Materials and methods

2.1. Isolates

Seventy-four isolates of *E coli* were obtained from the urine of dogs with UTI that were evaluated between September of 1996 and September of 1999 at a California veterinary medical teaching hospital (VMTH) (Cooke et al., 2002; Singer and Cooke, 2002). Of these 74 *E coli* isolates, 25 were resistant to enrofloxacin and 49 were susceptible. In addition, 13 enrofloxacin-resistant and 13 enrofloxacin-susceptible *E coli* isolates that were isolated from the urine of dogs at other veterinary hospitals in California during the same time period were included for comparison (Cooke et al., 2002; Singer and Cooke, 2002). It is unknown whether the dogs from which these isolates originated had any contact with the VMTH. Urine from many of the animals was collected by cystocentesis, but records of sample collection were not available for all dogs in this study. Resistance to enrofloxacin in each of these isolates was determined by the microbroth dilution method (Sensititre plate for UTI, Trek Diagnostics Inc., Westlake, Ohio), with resistance to enrofloxacin defined as an MIC $>$ 16 ug/mL. The assay was performed as described by the manufacturer and appropriate quality control organisms were included. Enrofloxacin-resistant isolates were defined as being FQ-R and enrofloxacin-susceptible isolates as being FQ-S. Study isolates were selected without regard for their susceptibility to antimicrobial agents other than enrofloxacin, and these data were not recorded or analyzed.

2.2. Phylogenetic group and extended virulence genotypes

Major *E. coli* phylogenetic group (A, B1, B2, and D) and 49 ExPEC-associated virulence genes, including 3 alleles of *papG* (P fimbriae adhesin molecule) and 12 alleles of *papA* (P fimbriae structural subunit), were detected by using established multiplex PCR-based assays (Clermont et al., 2000; Johnson and Stell, 2000; Johnson et al., 2000). Testing was done in duplicate using two separately prepared boiled lysates of each isolate as template DNA, in conjunction with relevant positive and negative controls. Isolates were operationally regarded as ExPEC if positive for $\geq$2 of *papA* and/or *papC*, *sfa*/*foc* (S and F1C fimbriae), *afa*/*dra* (Dr-binding adhesins), *iutA* (aerobactin system), and *kpsM* II (group 2 capsule) (Johnson et al., 2003c). The virulence score was the number of virulence genes detected, adjusted for multiple detection of the *pap*, *sfa*/*foc*, and *kpsM* II operons.

2.3. Statistical methods

Comparisons of proportions were tested using Fisher's exact test (two-tailed). Virulence scores were compared by using the Mann–Whitney *U*-test. Odds ratios and 95% confidence intervals were calculated by using stepwise multivariable logistic regression. The criterion for statistical significance was $P < .05$.

Correlations among variables were assessed by using correspondence analysis, which uses a covariance matrix based on χ^2 distances (Greenacre, 1992). The computation determines a plane defined by the two principal axes of the analysis, each of which represents a unique weighted composite of all the variables in the dataset. The first axis, F1, accounts for the largest part of the variance possible with a single composite variable. The second axis, F2, which is orthogonal to F1, accounts for the largest part of the remaining variance not accounted for by F1.

Similarity relationships among the individual isolates with respect to VF profiles and phylogenetic group were assessed by using principal coordinates analysis (PCoA), a multivariate technique related to correspondence analysis that allows one to plot the major patterns within a multivariate dataset, e.g. multiple loci and multiple samples (Peakall and Smouse, 2006). Using GenAlEx6 (Peakall and Smouse, 2006), PCoA was applied to the VF dataset as a way to collapse the multiple VFs for simplified among-group comparisons. As in correspondence analysis, each axis in PCoA represents a unique weighted composite of all the individual variables in the dataset. Individual isolates are assigned values on each axis based on their results for the study variables and each variable's weighting factor on the particular axis. Each successive axis captures the largest possible share of the residual variance not accounted for by previous axes. Values for each isolate from the first three PCA axes, which are the axes that capture most of variance within the dataset, were used in a one-way MANOVA to test for differences between FQ-R and FQ-S isolates.

3. Results

3.1. Phylogenetic distribution and virulence profiles: total population

Among the 100 *E. coli* urine isolates from dogs with UTI, the 38 FQ-R isolates differed significantly from the 62 FQ-S isolates according to phylogenetic group distribution, with FQ-R isolates being comparatively enriched for groups A ($P = .04$) and B1 ($P = .009$), and FQ-S isolates comparatively enriched for group B2 ($P < .001$) (Table 1).

Table 1
Bacterial traits significantly associated with enrofloxacin resistance among 100 *Escherichia coli* urine isolates from dogs with urinary tract infection.

Category	Trait[a,b,c]	Prevalence of trait, number (%)			*P* value, susceptible vs. resistant
		Total (percent of 100)	Susceptible (percent of 62)	Resistant (percent of 38)	
Adhesins	*papa*	21 (21)	18 (29)	3 (8)	.012
	papG allele III	16 (16)	14 (23)	2 (5)	.025
	sfa/focDE	26 (26)	24 (39)	2 (5)	<.001
Toxins	*hlyD*	21 (21)	18 (29)	3 (8)	.012
	cnf1	18 (18)	16 (26)	2 (5)	.014
	pic	9 (9)	9 (15)	0 (0)	.02
	clbB	21 (21)	19 (31)	2 (5)	.002
	clbN	23 (23)	21 (34)	2 (5)	.001
	vat	33 (33)	31 (50)	2 (5)	<.001
Siderophores	*iron*	33 (33)	27 (44)	6 (16)	.005
	iutA	39 (39)	13 (21)	26 (68)	<.001
Capsule	*kpsM* II	39 (39)	31 (50)	8 (21)	.006
Miscellaneous	*traT*	53 (53)	28 (45)	25 (66)	.047
	usp	41 (41)	35 (56)	6 (16)	<.001
	ibeA	24 (24)	20 (32)	4 (11)	.016
	ompT	45 (45)	41 (66)	4 (11)	<.001
	malX	49 (49)	38 (61)	11 (29)	.002
Phylogenetic group	Group A	14 (14)	5 (8)	9 (24)	.039
	Group B1	20 (20)	7 (11)	13 (34)	.009
	Group B2	36 (36)	33 (53)	3 (8)	<.001
	Group D	30 (30)	17 (27)	13 (34)	.47

[a] Accessory traits shown are those that yielded $P < .05$. Definitions: *papA*, P fimbriae structural subunit; *papG* allele III, P adhesin variant; *sfa/focDE*, S and F1C fimbriae; *hlyD*, alpha hemolysin; *cnf1*, cytotoxic necrotizing factor 1; *clbB* and *clbN*, colibactin; *vat*, vacuolating toxin; *pic*, autotransporter protease; *iroN*, salmochelin receptor; *iutA*, aerobactin receptor; *kpsM* II, group 2 capsule; *usp*, uropathogenic specific protein; *ibeA*, invasion of brain endothelium; *traT*, serum resistance-associated; *ompT*, outer membrane protease; *malX*, pathogenicity island marker.

[b] Accessory traits detected in ≥1 isolate each but not yielding $P < .05$ (percent of isolates positive): *papC/EF/G*, P fimbriae assembly (23%), tip pilins (24%), and adhesin (21%), respectively; *papA* alleles F7-2 (2%), F9 (1%), F10 (1%), F11 (1%), F12 (5%), F13 (6%), F16 (1%), and F48 (5%); *papG* allele I, P adhesin variant (1%); *focG*, F1C fimbriae (12%); *sfaS*, S fimbriae (6%); *afa/draBC*, Dr-binding adhesins (2%); *afaE8*, afimbrial adhesin variant (5%); *iha*, adhesin-siderophore receptor (7%); *hra*, heat-resistant agglutinin (30%); *bmaE*, M fimbriae (5%); *fimH*, type 1 fimbriae (95%); *hlyF*, variant hemolysin (10%); *cdtB*, cytolethal distending toxin (5%); *sat*, secreted autotransporter toxin (7%); *astA*, enteroaggregative *E. coli* toxin (14%); *fyuA*, yersiniabactin receptor (60%); *ireA*, siderophore receptor (11%); K1, group 2 capsule variant (10%); K2, group 2 capsule variant (1%); *kfiC*, K5 group 2 capsule variant (10%); *kpsM* III, group 3 capsule (16%); *rfc*, O4 lipopolysaccharide (4%); *cvaC*, microcin V (5%); *iss*, increased serum survival (10%).

[c] Accessory traits sought but not detected: *papA* alleles F7-1, F8, F14, and F15; *gafD* (G fimbriae); F17 fimbriae (mannose-resistant adhesin); *clpG* (mannose-resistant adhesin), K15 (group 2 capsule variant), H7 *fliC* (flagellin variant).

The FQ-R isolates likewise exhibited a significantly lower prevalence of 15 ExPEC-associated virulence genes, including *papA, papG* allele III (P adhesin variant), *sfa/focDE, hlyD* (hemolysin), *cnf1* (cytotoxic necrotizing factor), *pic* (serine protease), *clbB* and *clbN* (colibactin-associated), *vat* (vacuolating toxin), *iroN* (siderophore receptor), *kpsM* II, *usp* (uropathogenic specific protein), *ibeA* (invasion of brain endothelium), *ompT* (outer membrane protease), and *malX* (pathogenicity island marker), and a numerically lower prevalence of most of the remaining virulence genes (Table 1). However, the FQ-R isolates did exhibit a significantly higher prevalence of two virulence genes, *iutA* and *traT* (serum resistance-associated), which commonly occur on certain large conjugative resistance plasmids (Johnson et al., 2002a, 2006b; Valvano et al., 1986), and a numerically higher prevalence of *papG* allele II (P fimbrial adhesin variant), *cvaC* (microcin V), and *iss* (increased serum survival), the latter 2 of which also commonly occur on certain resistance plasmids (Johnson et al., 2006c). Accordingly, aggregate virulence scores were significantly lower among FQ-R isolates than FQ-S isolates (median score, 4.5 [range, 0–14], vs. 8.0 [range, 1–13]: $P < .001$). Likewise, a numerically lower proportion of FQ-R isolates qualified as ExPEC (10/38 [26%] for FQ-R, vs. 27/62 [44%] for FQ-S).

3.2. ExPEC isolates

In a similar analysis limited to the 37 ExPEC-qualifying isolates, analogous patterns were observed. That is, compared with the 27 FQ-S ExPEC isolates, the 10 FQ-R ExPEC isolates were significantly depleted for group B2 (20% vs. 81%: $P = .001$) and enriched for group D (60% vs. 15%: $P = .01$). They also exhibited a significantly lower prevalence of eight virulence genes, i.e. *sfa/focDE* (20% vs. 70%), *hra* (20% vs. 63%), *vat* (20% vs. 85%), *clbB* (20% vs. 67%), *clbN* (20% vs. 74%), *fyuA* (40% vs. 89%), *usp* (60% vs. 93%), *ompT* (20% vs. 85%), and *malX* (40% vs. 85%), and a numerically lower prevalence of most of the remaining genes (not shown). However, they exhibited a significantly higher prevalence of *papG* allele II (40% vs. 7%) and *iutA* (80% vs. 26%). Accordingly, aggregate virulence scores were significantly lower among the FQ-R isolates as compared with the FQ-S isolates (median score, 8.5 [range, 5–14], vs. 11 [range, 4.5–13]: $P = .002$).

Table 2
Stepwise multivariable logistic regression analysis for correlates of enrofloxacin resistance among 100 *Escherichia coli* urine isolates from dogs with urinary tract infection.

Independent variable[a]	Step of entry	*P* value	Odds ratio	95% confidence interval
ompT	1	<.001	0.028	0.005–0.16
iutA	2	<.001	15.22	3.76–61.64
rfc	3	.003	59.14	4.19–834.07
iha	4	.015	0.07	0.008–0.59

Note: Candidate independent variables included all individual virulence genes (plus *papA* and *papG* alleles) and phylogenetic groups (A, B1, B2, and D). For the final model as shown, Nagelkerke $r^2 = 0.60$.

[a] Definitions of variables: *ompT*, outer membrane protein (protease) T; *iutA*, aerobactin receptor; *rfc*, O4 lipopolysaccharide synthesis; *iha*, adhesin-siderophore receptor.

3.3. Multivariable logistic regression analysis

To identify bacterial traits independently associated with fluoroquinolone resistance, a stepwise multivariable logistic regression model was constructed, using as candidate independent variables all the individual virulence genes and the four phylogenetic groups. In the resulting model, four virulence genes emerged as significant correlates of fluoroquinolone resistance (Table 2), including *iutA* and *rfc* (O4 lipopolysaccharide) (positive associations), and *ompT* (outer membrane protease) and *iha* (adhesin-siderophore receptor) (negative associations). The strongest associations ($P < .001$) involved *iutA* and *ompT*, both of which (in contrast to *iha* and *rfc*) were statistically significant also in the univariate analyses. The final model, which did not include any phylogenetic group, accounted for 60% of total fluoroquinolone resistance-specific variance.

3.4. Correspondence analysis

To better understand the relationships amongst the variables, correspondence analysis was applied to the total dataset. The first 2 factors of this analysis, F1 and F2, accounted for only 16.1% and 10.9%, respectively, of total variance (27.1% overall). When the individual variables were plotted according to their coordinates on the F1–F2 plane, most of the virulence genes clustered near and to the right of the origin, surrounding group B2 and in the vicinity of Susceptible (Fig. 1). In contrast, several (including *iutA, iss*, and *cvaC*) clustered in the lower left quadrant near Resistant and group B1. Outlier variables included group A (extreme left) and *afaE8*/*bmaE* (afimbrial adhesin variant and M fimbriae) (high, left of center).

3.5. Principal coordinates analysis

To clarify the relationship between the FQ-R isolates and FQ-S isolates with all the variables considered simultaneously, PCoA was applied to the total dataset. The first three principle coordinates explained 52.7%, 14.7%, and 10.9% of total variance, respectively. Each of the first two principle coordinates significantly differentiated the FQ-R and FQ-S populations (for axis 1, $P < .001$; for axis 2, $P = .017$), accounting for 17.5% and 4.7% of FQ-R vs. FQ-S variance, respectively. When the isolates were plotted on the (axis 1-axis 2) plane, a large fraction of the FQ-S isolates could be separated from the FQ-R isolates by an (arbitrarily drawn) diagonal extending from lower left to upper right (Fig. 2).

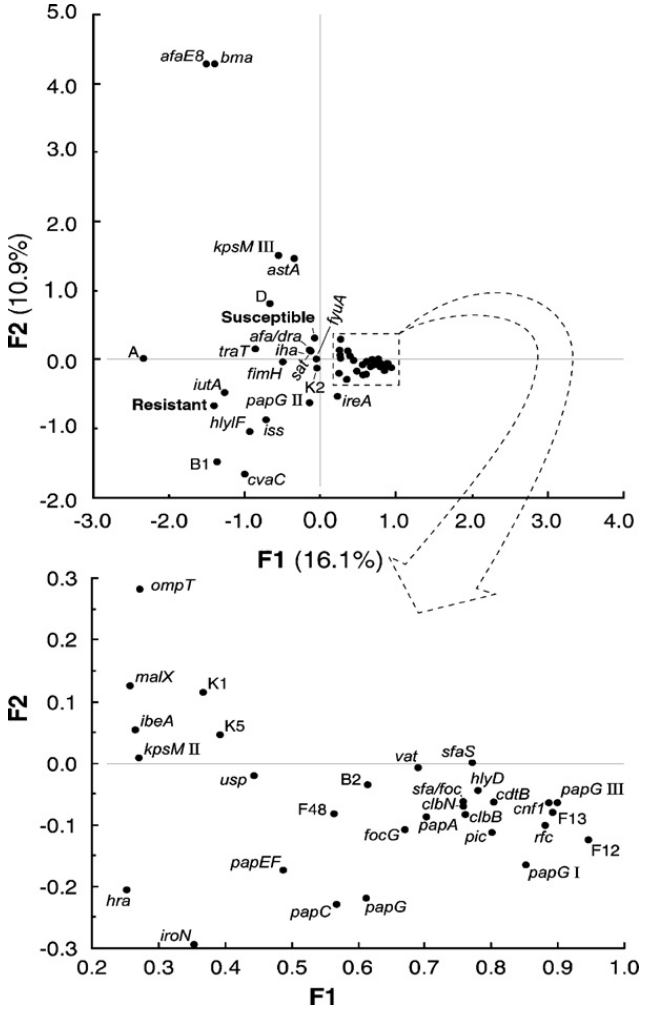

Fig. 1. Correspondence analysis. Relationships among bacterial characteristics, including enrofloxacin-resistant vs. susceptible status, for 100 *Escherichia coli* isolates from dogs with urinary tract infection, according to factors F1 and F2 from a correspondence analysis. Susceptible was placed close to the origin, near a dense cluster of group B2-associated traits (inset), whereas Resistant was remote from this cluster, nearer to groups A and B1 and to several non-B2-associated traits, e.g. *hlyF*, *iss*, *cvaC*, and *iutA*. Percent values indicate proportion of total variance accounted for by factors F1 (16.1%) and F2 (10.9%), respectively.

4. Discussion

The results of this molecular-epidemiological comparison of 100 FQ-R and FQ-S *E. coli* isolates from dogs with UTI provide novel insights into both the origins of canine-source FQ-R *E. coli* and the predicted virulence potential of such strains for dogs and humans. Specifically, we found that, as a group, the FQ-R isolates differed substantially from the FQ-S isolates according to virulence profiles and phylogenetic background. The observed patterns were consistent with a different (and possibly non-canine)

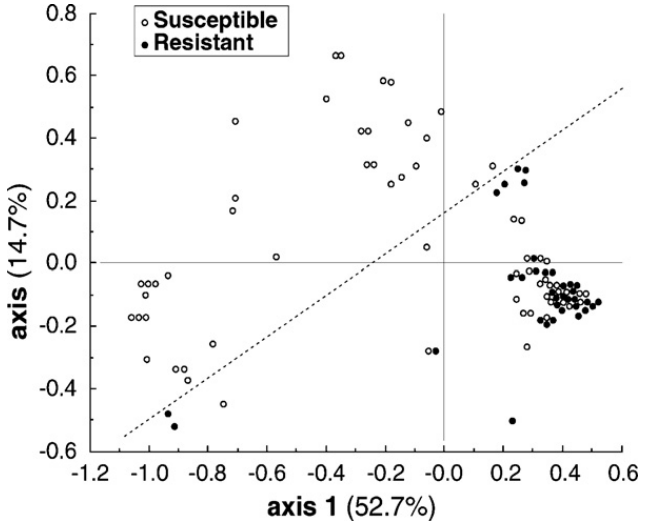

Fig. 2. Principle coordinates analysis. Position of 100 *Escherichia coli* isolates from dogs with urinary tract infection on the axis 1–axis 2 plane from a principle coordinates analysis based on all the bacterial variables in the dataset. Enrofloxacin-resistant isolates (solid circles) segregate to the lower right of the field, Enrofloxacin-susceptible isolates (open circles) largely to the upper left. Percent values indicate proportion of total variance accounted for by axis 1 (52.7%) and axis 2 (14.6%), respectively.

source for the FQ-R isolates, which appeared less virulent overall than FQ-S isolates, but more likely to contain certain non-dog-associated virulence genes. Nonetheless, an appreciable minority of FQ-R isolates represented ExPEC, and thus appeared to possibly pose the dual threat of extraintestinal virulence and fluoroquinolone resistance.

The FQ-R and FQ-S isolates differed in patterns consistent with those previously observed among antimicrobial-resistant vs. susceptible clinical and fecal isolates from humans (Johnson et al., 2004, 2003b; Moreno et al., 2006b; Vila et al., 2002). That is, the FQ-R isolates exhibited lower prevalences for most of the virulence genes and phylogenetic group B2 and lower aggregate virulence scores, whereas they were enriched for (typically low-virulence) phylogenetic groups A and B1. Moreover, FQ-R isolates were significantly less likely to qualify as ExPEC, and even those that did so qualify exhibited significantly lower aggregate virulence scores, and were shifted toward group D, compared with FQ-S ExPEC isolates. Only two virulence genes, *iutA* and *papG* allele II, were significantly more prevalent among FQ-R than FQ-S ExPEC isolates.

These observations could have several possible explanations. The first and most obvious possibility is that enrofloxacin use in dogs selects for conversion to fluoroquinolone resistance among FQ-S *E. coli* within the canine gut flora. If so, the observed differences between the FQ-R and FQ-S populations could have arisen if ease of conversion to fluoroquinolone resistance varies by phylogenetic group, with non-group B2 *E. coli* (which tend to lack the studied virulence genes, except for [group D-associated] *iutA* and *papG* allele II) able to become FQ-R more readily than group B2 strains. However, the available experimental and observational evidence does not support this hypothesis (Johnson et al., 2005b, 2006a). Alternatively, the FQ-R and FQ-S strains might derive from distinct groups of canine hosts, with the FQ-R isolates perhaps coming preferentially from compromised hosts that are predisposed to UTI, and that therefore are both more heavily fluoroquinolone-exposed and more likely to experience UTI due to low-virulence strains, analogous to what has been described among humans (Johnson et al., 1994, 2002b). However, the present FQ-R isolates' shifts away from group B2 and paucity of virulence genes seem extreme even for canine fecal isolates (Johnson et al., 2001; Yuri et al., 1998), let alone canine UTI isolates, which casts doubt also on this hypothesis.

A second, less obvious possibility is that the FQ-R isolates arose initially in an extra-canine selection environment where low-virulence, non-B2 *E. coli* predominate, then were imported into dogs. Even if so, their subsequent persistence and expansion in dogs could have been favored by the administration of enrofloxacin or other co-selecting antimicrobial agents to their new canine hosts. Food-source antimicrobial-resistant *E. coli*, including chicken-source FQ-R strains, commonly exhibit a phylogenetic group distribution and paucity of virulence genes similar to that observed among the present canine FQ-R UTI isolates, which suggests one possible external source for these strains (Johnson et al., 2005a, 2006a, 2005d). Likewise, fluoroquinolone-resistant *E. coli* from humans typically exhibit similar characteristics, suggesting another possible source (Horcajada et al., 2005; Johnson et al., 2005c, 2002b; Kuntaman et al., 2005; Moreno et al., 2006a).

Whatever the origins of the dog-associated FQ-R *E. coli*, these strains' subsequent fate also warrants consideration. Dogs shed large quantities of fecal *E. coli* into the environment (Johnson et al., 2001). Humans conceivably may acquire such organisms by fecal-oral transmission directly, or via fomites, environmental surfaces, or vectors such as the dogs themselves, whose fur, paws, or mouths may become contaminated with their own feces or that of other dogs. In dogs with *E. coli* UTI the urine strain is usually the host's predominant fecal strain, which implies antecedent intestinal colonization, and likely environmental contamination, with the UTI strain prior to or during the dog's UTI episode (Johnson et al., 2003a). We found here that an appreciable minority of FQ-R canine UTI isolates represented ExPEC and exhibited virulence traits suggesting human virulence potential. This suggests that even if the source canine hosts served only as a secondary amplification system for the resistant clones (rather than having been the original selection reservoir), such dogs may still pose a health threat to humans and, conceivably, to other dogs.

Limitations of the study include the small sample size, the undefined demographic and clinical characteristics of the dogs, and the isolates' limited geographical range and pre-2000 origin. Regarding the latter, especially considering the ongoing use of fluoroquinolones in human and veterinary medicine, it would be of interest to update the study using recent isolates, to determine whether these findings remain current. An additional limitation, the use of multiple comparisons, increased the chance of a Type I

error, i.e. of falsely identifying as significant an association that occurred by chance alone. We guarded against this both by considering the strength and number of observed significant univariable associations in relation to the number of comparisons made (which consistently was much greater than would occur by chance) and by using virulence score analysis and PCoA to collapse the multi-dimensional dataset for simplified between-group comparisons. Study strengths include the incorporation of a concurrent FQ-S control group, attention to phylogenetic background, breadth and depth of virulence genotyping, and use of multiple complementary statistical analysis approaches.

5. Conclusion

This molecular-epidemiological comparison of FQ-R and FQ-S *E. coli* from dogs with UTI showed that the FQ-R isolates differed substantially from FQ-S isolates according to virulence profiles and phylogenetic background, in patterns consistent with a different (and possibly extra-canine) source. Additionally, although overall the FQ-R isolates appeared less virulent than the FQ-S isolates, some FQ-R isolates nonetheless represented ExPEC, which therefore may pose the dual threat of virulence and fluoroquinolone resistance. These findings, especially if confirmed among current isolates, support additional efforts to define the sources of the FQ-R *E. coli* encountered in dogs, and attention to dogs as a possible reservoir of FQ-R ExPEC for transmission to other pets and humans.

Conflict of interest statement

The authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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