JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2009, p. 791–795 Vol. 47, No. 3
0095-1137/09/$08.00+0 doi:10.1128/JCM.01740-08

Genetic Profiles of Fluoroquinolone-Resistant *Escherichia coli* Isolates Obtained from Patients with Cystitis: Phylogeny, Virulence Factors, PAI*usp* Subtypes, and Mutation Patterns[▽]

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Received 9 September 2008/Returned for modification 10 November 2008/Accepted 13 January 2009

The low virulence of quinolone- and fluoroquinolone-resistant *Escherichia coli* strains is known, although the reasons for this remain unclear. We surveyed the mutation patterns of quinolone resistance determining regions (QRDRs), phylogenetic distribution, prevalence of 18 urovirulence genes, and PAI*usp* subtypes in 89 fluoroquinolone-resistant *E. coli* (FQREC) isolates obtained from patients with cystitis and compared them with those of their fluoroquinolone-susceptible counterparts (FQSEC). Phylogenetic group B2 was significantly less prevalent in FQREC than in FQSEC (49% versus 78%; $P = 0.013$8), but it still dominated, followed by phylogroup D (35%), in FQREC. When the prevalences of virulence factor (VF) genes were compared between FQREC and FQSEC, *sfa*/*foc*, *cnf1*, *hly*, *kpsMT*, *ompT*, *ibeA*, *usp*, and *iroN* showed significantly lower prevalences in FQREC than in FQSEC (1.1% versus 24% [$P < 0.0001$], 0% versus 29% [$P < 0.0001$], 7.9% versus 33% [$P < 0.0001$], 74% versus 90% [$P = 0.01$], 71% versus 87% [$P = 0.017$], 5.6% versus 37% [$P < 0.0001$], 54% versus 82% [$P < 0.0001$], and 7.9% versus 32% [$P = 0.0001$], respectively), whereas *aer*, *iha*, and *ETTT* showed significantly higher prevalences in FQREC (85% versus 36% [$P < 0.0001$], 66% versus 29% [$P < 0.0001$], and 53% versus 16% [$P < 0.0001$], respectively). Furthermore, a similar difference in prevalences of uropathogenic VF genes was seen between FQREC and FQSEC in phylogroup B2. This indicated that the low virulence in FQREC was intimately correlated with a lesser distribution of VFs in phylogroup B2, which dominated in FQREC and FQSEC. It was interesting that the mutation pattern of Ser83Leu and Asp87Asn encoded in *gyrA* and Ser80Ile and Glu84Val encoded in *parC* was frequently found in FQREC isolates that belonged to phylogroup B2 and that most of these isolates showed PAI*usp* subtype 2a. PAI*usp* subtypes 1a, 1b, and 2b, which were frequently seen in FQSEC, were rarely found in FQREC. These results suggested that the acquisition of fluoroquinolone resistance, e.g., mutations in QRDRs, might be a specific event in limited strains, such as those that possess PAI*usp* subtype 2a in phylogroup B2.

Escherichia coli is the microorganism most commonly isolated from patients with urinary tract infections (UTIs). Its distinct characteristics for colonization of the urinary tract, such as uropathogenic virulence factors (VFs), have been investigated. Since the emergence of *E. coli* strains resistant to most first-line antimicrobial agents, especially to fluoroquinolones (FQs), has become a major problem in the management of UTIs (7, 10, 12, 20), several studies have focused on the unique characteristics of FQ-resistant *E. coli* (FQREC). In terms of uropathogenic VFs, several studies have shown the decreased prevalence of some major VFs, including adhesins and toxins, and an increased prevalence of VFs related to the iron uptake system in quinolone- or FQ-resistant *E. coli* isolates (5, 8, 9, 14, 15, 19).

Several other studies have demonstrated that phylogenetic prevalence in FQREC differs from that in FQ-susceptible *E. coli* (FQSEC), in which phylogroup B2 dominates. For example, Moreno et al. have shown that out of 18 FQREC isolates from cystitis, pyelonephritis, and blood from UTI-associated bacteremia, 56% belonged to phylogroup A and only 11% to phylogroup B2, whereas 79% of 132 FQSEC isolates belonged to phylogroup B2 (14). Johnson et al. have reported that 87% of 15 extraintestinal FQREC isolates belonged to phylogroup D and the rest to phylogroup B2 whereas 83% of 46 FQSEC isolates belonged to phylogroup B2 and the rest to phylogroup D (9). Although the dominant group in FQREC varies between studies, these results suggest that the minor prevalence of phylogroup B2 might account for the decreased number of VFs in FQREC. However, Piatti et al. have analyzed 123 FQREC isolates from UTIs and have reported that phylogroup B2 isolates were significantly fewer in FQREC than in FQSEC but were still dominant in FQREC (50%) (15). They have demonstrated that in phylogroup B2, some VF genes, including *papC*, *hlyA*, and *cnf*, were less frequent in FQREC than in FQSEC isolates. This indicates that the mutation that confers FQ resistance may require a particular genetic background, not strictly correlated with a phylogenetic group.

In 89 FQREC isolates and 89 FQSEC isolates from complicated or uncomplicated cystitis, we determined the prevalences

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▽ Published ahead of print on 21 January 2009.

TABLE 1. Mutation patterns of QRDRs and CIP MICs in FQREC and FQSEC isolates

Resistance group	Mutated amino acid encoded in:								Total no. of isolates	No. of isolates for which the ciprofloxacin MIC (μg/ml) was:													
	gyrA		*parC*			*parE*				0.008	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	>128
	Ser83	Asp87	Ser80	Glu84	Ala108	Leu445	Ser458	Asp475																	
FQREC	Leu	Asn	Ile						26										1	9	7	4	4		1
	Leu	His	Ile						1											1					
	Leu	Asn	Ile	Val					37											1	3	19	7	4	3
	Leu	Asn	Ile	Gly					6											1		3	1	1	
	Val	Asn	Ile	Lys					1															1	
	Leu	Tyr	Arg	Val					1															1	
	Leu	Asn	Ile		Thr				3												2		1		
	Leu	Asn	Ile			His			2														1	1	
	Leu	Asn	Ile				Ala		8													1	3	3	1
	Leu	Asn	Ile				Trp		1															1	
	Leu	Tyr	Ile				Ala		1																1
	Leu	Asn	Ile	Val		His			2													1	1		
FQSEC									73	15	37	17	1	1	2									
	Leu								10	1			2		5	2									
		Gly							2				2												
								Glu	2	1	1														
	Leu							Glu	1					1											
		Gly						Glu	1				1												

of 18 VF genes, phylogenetic groups, and PAI*usp* subtypes, which have been shown to be closely associated with several VF genes (11). To further clarify the genetic backgrounds in UTI-derived FQREC, the mutational patterns in quinolone resistance determining regions (QRDRs) including *gyrA*, *gyrB*, *parC*, and *parE* were also revealed in this collection and were compared with the prevalences of phylogenetic groups and PAI*usp* subtypes.

MIC determination. Antimicrobial susceptibility testing for *E. coli* was performed by the twofold serial agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (4). Isolates with MICs for ciprofloxacin of ≥4 μg/ml were defined as FQREC and those with lower MICs as FQSEC.

Bacterial isolates and DNA preparation. We collected 595 *E. coli* strains from 335 patients with uncomplicated cystitis and 260 patients with complicated cystitis at hospitals that participated in the Japanese Research Group for UTI between 2005 and 2007. By determination of the MIC for ciprofloxacin, 89 (15%) FQREC isolates, including 38 (11%) from patients with uncomplicated cystitis and 51 (20%) from patients with complicated cystitis, were obtained. For each FQREC isolate, a matched susceptible counterpart was selected from the collection of FQSEC, which had similarity in terms of patient background, such as source (uncomplicated or complicated), age, and institution. Cystitis was diagnosed as previously reported (17). The DNA templates were prepared as previously described (17).

Mutational analysis of QRDRs in *gyrA*, *gyrB*, *parC*, and *parE*. The QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were amplified by PCR using previously published primers (6, 16, 18). The PCR was performed in a 20-μl reaction mixture that contained 2 μl DNA template, 4 pmol (each) primer, 0.2 mM (each) deoxynucleoside triphosphate, 2 μl 10× PCR buffer with 15 mM $MgCl_2$, and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The reaction was carried out in the PCR thermal cycler GeneAmp PCR System 9700 (Applied Biosystems) with the following schedule: preheating at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and certificated under UV transillumination. The amplified PCR products were processed by using the BigDye Terminator v3.1 cycle sequencing kit, purified by using the BigDye XTerminator kit, and analyzed in an automatic DNA sequencer, GA 3130xl (Applied Biosystems), according to the manufacturer's protocol. Predicted polypeptide products were analyzed for amino acid changes by comparison with sequences for wild-type *E. coli gyrA* (GenBank accession number X06373), *gyrB* (accession no. X04341), *parC* (accession no. M58408), and *parE* (accession no. AE000385).

VF profiling, phylogenetic analysis, and PAI*usp* subtyping by PCR. Eighteen VF genes, *aer* (aerobactin), *afa* (afimbrial adhesin), *cnf1* (cytotoxic necrotizing factor 1), *cvaC* (colicin V), *ETTT* (type III secretion system), *fimH* (type 1 fimbria adhesin), *fyuA* (yersiniabactin receptor for ferric yersiniabactin uptake), *hly* (alpha hemolysin), *ibeA* (invasion of brain endothelium), *iroN* (catecholate siderophore receptor), *iha* (iron-regulated gene A homologue adhesin), *kpsMT* (group 2 capsule), *ompT* (outer membrane protease T), *PAI* (pathogenic island marker of CFT073), *pap* (P fimbriae), *sfa/foc* (S/F1C fimbriae), *traT* (serum resistance associates), and *usp* (uropathogenic specific protein), were determined as described previously (17). Phylogenetic grouping was done by use of a dichotomous decision tree, as previously reported by Clermont et al., using the results of PCR amplification of *chuA*, *yjaA*, and TspE4.C2 (3). Briefly, *chuA*-positive/*yjaA*-positive, *chuA*-positive/*yjaA*-negative, *chuA*-negative/TspE4.C2-positive, and *chuA*-negative/TspE4.C2-negative strains were classified as B2, D, B1, and A. In *usp*-positive strains, the mosaic variation of PAI*usp* subtypes, 1a, 1b, 2a, and 2b, was also determined by PCR as described previously (11). Briefly, three sets of primer combinations, USP 81f and ORFU1r, USP 81f and ORFU2r, and USP 81f and ORFU3r, were developed to amplify the three open reading frames (ORFs), *orfU1*, *orfU2*, and *orfU3*, and to reveal the mosaic patterns according to the presence or absence and sequence of the three ORFs.

Statistical analysis. Comparisons of proportions were tested using the χ^2 test or Fisher's exact test. *P* values of <0.05 was considered to indicate statistical significance.

Mutations in QRDRs. All FQREC isolates possessed at least triple points of mutation at Ser83 and Asp87 encoded in *gyrA* and at Ser80 encoded in *parC*. In addition, some isolates that possessed one or two more mutation points at Glu84 or Ala108 encoded in *parC* and/or at Leu445 or Ser458 encoded in *parE* showed relatively higher MICs (Table 1). Among the FQSEC isolates, only 16 (18%) possessed a single point mutation at Ser83 or Asp87 encoded in *gyrA*, with/without mutation at Asp475 encoded in *parE*. The MICs of isolates that possessed a single point mutation in *gyrA* were relatively higher but remained at 0.5 μg/ml or lower (Table 1). No mutation was found in *gyrB* in these strains.

VF gene distributions in FQREC and FQSEC. Previously we examined the genetic characters of *E. coli* isolated from patients with uncomplicated cystitis, complicated cystitis, and complicated asymptomatic bacteriuria and showed similar distribution among the three categories (17). As shown in Table 2, when the prevalences of VF genes was compared between FQSEC isolates from patients with uncomplicated and complicated cystitis, few significant differences were found (Table 2). As well, when the distribution of VF genes was compared between FQREC isolates from patients with uncomplicated and complicated cystitis, few significant differences were noted (Table 2). These results indicated that the VF genes were similarly prevalent, regardless of uncomplicated or complicated cystitis, in FQREC and in FQSEC isolates.

However, when the prevalences of VF genes were compared between FQREC and FQSEC, *sfa*/*foc*, *cnf1*, *hly*, *kpsMT*, *ompT*, *ibeA*, *usp*, and *iroN* showed significantly lower prevalences in FQREC than in FQSEC (1.1% versus 24% [$P < 0.0001$], 0% versus 29% [$P < 0.0001$], 7.9% versus 33% [$P < 0.0001$], 74% versus 90% [$P = 0.01$], 71% versus 87% [$P = 0.017$], 5.6% versus 37% [$P < 0.0001$], 54% versus 82% [$P < 0.0001$], and 7.9% versus 32% [$P = 0.0001$], respectively), whereas *aer*, *iha*, and *ETTT* showed significantly higher prevalences in FQREC than in FQSEC (85% versus 36% [$P < 0.0001$], 66% versus 29% [$P < 0.0001$], and 53% versus 16% [$P < 0.0001$], respectively).

Phylogenetic groups and VF gene distribution. Phylogenetic group B2 was significantly less prevalent in FQREC than in FQSEC (49% versus 78%; $P = 0.0138$), but it was still dominant, followed by phylogroup D (35%) in FQREC (Tables 3 and 4). When the prevalences of VF genes were compared between FQREC and FQSEC in each phylogenetic group, most VF genes were similarly distributed in phylogroups A and B1, but several significant differences were noted in phylogroups B2 and D. In phylogroup B2, FQREC showed lower prevalences of *sfa*/*foc*, *cnf1*, *hly*, *kpsMT*, *ibeA*, and *iroN* (2.3% versus 30% [$P = 0.0001$], 0% versus 38% [$P < 0.0001$], 9.1% versus 42% [$P = 0.0001$], 82% versus 99% [$P = 0.0022$], 4.5% versus 42% [$P < 0.0001$], and 0% versus 35% [$P < 0.0001$], respectively) and higher prevalences of *aer*, *iha*, and *ETTT* (95% versus 43% [$P < 0.0001$], 91% versus 36% [$P < 0.0001$], and 20% versus 0% [$P < 0.0001$], respectively) than FQSEC (Table 3). In phylogroup D, FQREC showed a low prevalence of *iroN* (3.2% versus 27%; $P = 0.0486$) compared with results for FQSEC (Table 3).

TABLE 2. Prevalences of VF genes in FQREC and FQSEC

Resistance group and case type[a] (n[b])	No. (%) of isolates with indicated gene[c]																	
	Adhesin gene				Iron uptake gene			Toxin gene		Cell protection gene			Other					
	afa	*fimH*	*iha*	*pap*	*sfa*/*foc*	*aer*	*fyuA*	*iroN*	*cnf1*	*hly*	*cvaC*	*kpsMT*	*ompT*	*traT*	*ibeA*	PAI	*usp*	*ETTT*
FQREC																		
UC (38)	6 (16)	38 (100)	32 (84)	12 (32)	0	33 (87)	34 (89)	2 (5.3)	0	4 (11)	0	31 (82)	32 (84)	28 (74)	2 (5.3)	25 (66)	21 (55)	21 (55)
CC (51)	2 (3.9)	49 (96)	27 (53)	10 (20)	1 (2.0)	43 (84)	42 (82)	5 (9.8)	0	3 (5.9)	4 (7.8)	35 (69)	31 (61)	38 (75)	3 (5.9)	34 (67)	27 (53)	26 (51)
Total (89)	8 (9.0)	87 (98)	59 (66) a	22 (25)	1 (1.1) a	76 (85) a	76 (85)	7 (7.9) b	0 a	7 (7.9) a	4 (4.5)	66 (74) d	63 (71) d	66 (74)	5 (5.6) a	59 (66)	48 (54) a	47 (53) a
FQSEC																		
UC (38)	4 (11)	37 (97)	12 (32)	8 (21)	0	15 (40)	37 (97)	11 (29)	12 (32)	12 (32)	5 (13)	36 (95)	34 (90)	33 (87)	17 (45)	30 (79)	32 (84)	5 (13)
CC (51)	4 (7.8)	50 (98)	14 (28)	13 (25)	17 (33)	44 (86)	17 (33)	14 (27)	17 (33)	4 (7.8)	44 (86)	43 (84)	35 (69)	16 (31)	40 (78)	41 (80)	9 (18)	
Total (89)	8 (9.0)	87 (98)	26 (29)	21 (24)	32 (36)	81 (91)	28 (32)	26 (29)	29 (33)	9 (10)	80 (90)	77 (87)	68 (76)	33 (37)	70 (79)	73 (82)	14 (16)	

[a] UC, uncomplicated cystitis; CC, complicated cystitis.
[b] *n*, no. of isolates.
[c] Letters indicate *P* values for results for FQREC versus those for FQSEC: a, $P < 0.0001$; b, $P < 0.001$; c, $P < 0.01$; d, $P < 0.05$.

TABLE 3. Prevalences of VF genes for each phylogenetic group

Phylogroup	Resistance group (n[a])	No. (%) of isolates with indicated gene[b]											
		Adhesin gene			Iron uptake system gene		Toxin gene		Cell protection gene		Other		
		iha	*pap*	*sfa/foc*	*aer*	*iroN*	*cnf1*	*hly*	*cvaC*	*kpsMT*	*ibeA*	*usp*	*ETTT*
A	FQREC (5)	2 (40)	0	0	5 (100) d	1 (20)	0	0	1 (20)	3 (60)	0	0	3 (60)
	FQSEC (5)	1 (20)	0	0	1 (20)	0	0	0	0	1 (20)	0	0	4 (80)
B1	FQREC (9)	0	0	0	4 (44)	5 (56)	0	0	2 (22)	1 (11)	0	0	8 (89)
	FQSEC (4)	0	0	0	0	1 (25)	0	0	0	0	0	0	3 (75)
B2	FQREC (44)	40 (91)	6 (14) b	1 (2.3) b	42 (95) a	0 a	0 a	4 (9.1) b	0 d	36 (82) c	2 (4.5) a	43 (98)	9 (20) a
	FQSEC (69)	25 (36)	31 (45)	21 (30)	30 (43)	24 (35)	26 (38)	29 (42)	7 (10)	68 (99)	29 (42)	69 (100)	0
D	FQREC (31)	17 (55) b	16 (52) d	0	25 (81) a	1 (3.2) d	0	3 (9.7)	1 (3.2)	26 (84)	3 (9.7)	6 (19)	27 (87)
	FQSEC (11)	0	1 (9.1)	0	1 (9.1)	3 (27)	0	0	2 (18)	11 (100)	4 (36)	4 (36)	7 (64)

[a] n, no. of isolates.
[b] Letters indicate P values for results for FQREC versus those for FQSEC: a, $P < 0.0001$; b, $P < 0.001$; c, $P < 0.01$; d, $P < 0.05$.

PAI*usp* subtypes and mutations in QRDRs. In both FQREC and FQSEC, *usp* was frequently found in phylogroup B2 but rarely seen in other phylogroups (Table 3). In terms of PAI*usp* subtyping, 2a was predominantly found (36/44) but 1a and 1b were rarely seen in phylogroup B2 of FQREC, whereas 1a, 1b, 2a, and 2b were found in 17, 8, 29, and 13 isolates, respectively, in phylogroup B2 of FQSEC (Table 4). Interestingly, in FQREC, 36 (82%) of 44 phylogroup B2 isolates possessed the same mutations of Ser83Leu and Asp87Asn encoded in *gyrA* and Ser80Ile and Glu84Val encoded in *parC* whereas 44 (98%) of 45 isolates that belonged to phylogroups A, B1, and D possessed other patterns of mutation in QRDRs (Table 4).

Implications from our results. The current study clarified several distinct characteristics and some de novo epidemiological divergences that accorded with the QRDR mutation patterns of FQREC isolated from cystitis.

The relationship between quinolone resistance and mutations in the QRDRs of *gyrA*, *parC*, and *parE* has been established (1, 2). When the mutation patterns for QRDRs of four genes, *gyrA*, *gyrB*, *parC*, and *parE*, were determined, no mutation was detected in *gyrB* in any of the isolates. This was similar to earlier reports (1, 6, 13). Two mutations at Ser83 (mostly to Leu) and Asp87 (mostly to Asn) encoded in *gyrA* were simultaneously present in all FQREC isolates, thus supporting the suggestion that these mutations constitute the most important factor for *E. coli* acquisition of FQ resistance. Furthermore, mutations at Ser80 (mostly to Ile) encoded in *parC*, homologous to Ser83 encoded in *gyrA*, in all FQREC isolates and several additional mutations in *parC* and *parE* in some FQREC isolates were also found, which suggests that these mutations cause the increase in MICs for FQs.

In terms of uropathogenic VFs, the lower prevalence of several urovirulence genes and higher prevalence of *aer* or *iha* in FQREC than in FQSEC were noted, which supports the results of previous studies (5, 8, 9, 14, 15, 19). As for the phylogenetic distribution, phylogoup B2 was shown to be less prevalent in FQREC than in FQSEC but still was dominant among the four phylogroups in FQREC. This result was similar to that in work by Piatti et al. but different from those in work by Moreno et al. and Johnson et al. (9, 14, 15). The controversy among these reports may be interpreted as being the result of the number of samples, geographical variation, or various clinical sources.

In the present study, to assess whether the low virulence of

TABLE 4. Mutation patterns of QRDRs by phylogroup and PAI*usp* subtype[a]

Phylogroup	PAI*usp* subtype	No. of FQREC isolates						No. of FQSEC isolates			
		Total	Carrying *gyrA*(*S83L D87N*) and *parC*(*S80I*) with:				With other pattern	Total	With no mutation	With *gyrA*(*S83L*)	With other pattern
			No mutation	*parC*(*E84V*)	*parC*(*E84G*)	*parE*(*S458A*)					
A	None	5	4				1	5	5		
B1	None	9	3		1	5		4	3	1	
B2	1a	2	1				1	17	12	5	
	1b	0						8	6	1	1
	2a	36	3	30			3	29	27	1	1
	2b	5		5				13	12	1	
	NT[b]	0						2	2		
	None	1		1				0			
D	1a	0						4			4
	2a	5	2		1		2	0			
	None	26	13	1	4	3	5	7	6	1	

[a] Eighty-nine FQREC isolates and 89 FQSEC isolates were used.
[b] NT, nontypeable.

FQ-resistant isolates was primarily correlated with the phylogenetic distribution, e.g., fewer phylogroup B2 isolates, the prevalence of VF genes in FQREC and FQSEC in each phylogroup was compared. In phylogroups A and B1, originally having low virulence, FQREC and FQSEC showed similar distributions of VF genes. It was notable that in phylogroup B2, the most virulent among the four phylogroups, there were decreased prevalences of several VF genes and increased prevalences of *aer* and *iha* in FQREC. In phylogroup D, the second most virulent group, a similar difference between FQREC and FQSEC isolates was found for *aer* and *iroN* but not for *pap*. Overall, our results showed that the low virulence of FQREC was very closely correlated with the lower distribution of VF genes in phylogroup B2, which was still dominant in FQREC.

It was also very interesting that the mutation pattern of Ser83Leu and Asp87Asn encoded in *gyrA* and Ser80Ile and Glu84Val encoded in *parC* was frequently found in FQREC isolates that belonged to phylogroup B2 and that most of these isolates showed PAI*usp* subtype 2a. PAI*usp* subtypes 1a, 1b, and 2b, which were frequently seen in FQSEC, were rarely found in FQREC. These results suggested that the acquisition of FQ resistance, e.g., mutations in QRDRs, may be a relatively specific event in limited strains, such as those possessing PAI*usp* subtype 2a in phylogroup B2. However, for other phylogroups, A, B1, and D, another interpretation is required in terms of acquisition of FQ resistance, since these phylogroups rarely possessed *usp* and showed different mutation patterns in ORDRs from those of phylogroup B2.

In summary, the lower urovirulence of FQREC than of FQSEC may be very closely correlated with the lower distribution of VF genes in phylogroup B2, which was still dominant in FQREC. Furthermore, the same mutation pattern for *gyrA* and *parC* was frequently found in FQREC isolates belonging to phylogroup B2, with most of these isolates showing PAI*usp* subtype 2a, suggesting that mutations in QRDRs may be specific in limited strains. Our results shed light on why low-virulence isolates are so frequent in FQREC isolated from patients with UTIs, although further investigation will be required to clarify the mechanism by which the mutations in QRDRs are acquired by FQREC.

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