High Prevalence of Cross-Resistance to Aminoglycosides in Fluoroquinolone-Resistant *Escherichia coli* Clinical Isolates

Naoyuki Tsukamoto^{a,b} Yasuo Ohkoshi^{a,b} Torahiko Okubo^d Toyotaka Sato^d
Osamu Kuwahara^c Nobuhiro Fujii^a Yutaka Tamura^d Shin-ichi Yokota^a

^aDepartment of Microbiology, Sapporo Medical University School of Medicine, ^bHokkaido Laboratory, SRL Inc., and ^cSapporo Clinical Laboratories Inc., Sapporo, and ^dLaboratory of Food Microbiology and Food Safety, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Japan

Key Words

Escherichia coli · Fluoroquinolones · Aminoglycosides · Extended-spectrum β -lactamases · Antibiotic resistance

Abstract

Background: Multidrug-resistant *Escherichia coli*, especially a lineage of O25b:H4-ST131, has increased and spread worldwide. The surveillance of cross-resistance of *E. coli* is necessary. **Methods:** Cross-resistance to fluoroquinolones (FQs) and aminoglycosides (AGs) was examined in *E. coli* isolated in Hokkaido Prefecture, Japan, between 2008 and 2009. **Results:** Gentamicin (GEN) resistance was more common in FQ-resistant isolates (30/112 strains; 26.8%) than in FQ-susceptible isolates (2/100 strains; 2%). The frequency of GEN resistance was similar in two groups of FQ-resistant strains, O25b:H4-ST131 genotype (22/87 strains; 25.3%) and a group of other FQ-resistant genotypes (8/25 strains; 32.0%). The main AG resistance gene was *aac(3)-II* (87.5% of GEN-resistant strains). The only amikacin-resistant strain which was FQ resistant carried the *aac(6')-Ib-cr* gene. CTX-M type extended-spectrum β -lactamase (ESBL) genes were also found in FQ-resistant strains at a high frequency. However, the number of strains with both ESBL and AG-modifying enzyme genes was relatively low (8 strains). **Conclusion:** All FQ-resis-

tant strains, not only O25b:H4-ST131, appeared to preferentially acquire ESBL genes and/or genes encoding AG-modifying enzymes; however, the acquisitions of these genes seemed to occur independently.

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Introduction

Escherichia coli is a commensal bacterium found in the intestines of most animals, including humans. However, a small proportion of *E. coli* strains that share specific pathogenic factors are highly enteropathogenic [1]. Commensal strains of *E. coli* that have colonized the intestines may cause extraintestinal diseases, such as urinary tract infections as a primary infection [2, 3]. Pneumonia and septicemia, which result in considerable morbidity and mortality, may be caused as opportunistic infections. *E. coli* is the most common causative agent of urinary tract infections, accounting for approximately 80% of uncomplicated community-acquired urinary tract infections [4, 5]. Fluoroquinolones (FQs) are often used as first-choice

N.T. and Y.O. contributed equally to this work.

antibiotics in the treatment of urinary tract infections; however, FQ-resistant *E. coli* strains have become a problem in urology. In addition, multidrug-resistant strains of *E. coli* producing extended-spectrum β -lactamases (ESBLs) and metallo- β -lactamases, namely carbapenemases, have become more common [6–9]. A lineage of the multilocus sequence type ST131, belonging to O25b:H4, has recently spread worldwide [10–13]. This lineage is FQ resistant and often acquires ESBL genes, particularly the CTX-M-15 of group 1. In contrast, other ESBL genes, particularly CTX-M groups 9 (CTX-M-14 and 27) and 2 (CTX-M-2), are dominant in Japanese O25b:H4-ST131 strains [14, 15]. However, CTX-M-15-producing O25b:H4-ST131 strains have been found in recent years in Japan [16].

In *E. coli*, resistance to other antibiotics, such as carbapenems and aminoglycosides (AGs), has become a concern [17]. These antibiotics are occasionally used to treat severe systemic infections, such as sepsis. The mechanism underlying resistance to FQs involves somatic point mutations in the target genes of topoisomerase IV and DNA gyrase [18], whereas that underlying resistance to β -lactams is usually the acquisition of β -lactamase genes in Gram-negative bacteria [19]. Resistance to AGs mainly occurs through the acquisition of genes encoding AG-modifying enzymes, such as acetyltransferase (*aac*), phosphotransferase (*aph*) and nucleotidyltransferase (*ant*) genes [20–23]. Numerous types of AG-modifying enzymes have been identified and each provides resistance to specific AGs according to their substrate specificity. On the other hand, acquisition of 16S rRNA methylase genes (*rtmA*, *rtmB* and *armA*), point mutations in rRNA and increase of the activity of efflux pumps are also involved in the molecular mechanisms of AG resistance [20, 23–25]. These mechanisms provide resistance to various types of AGs. In this study, we screened for AG resistance among FQ-resistant and FQ-susceptible *E. coli* clinical isolates obtained from patients in Hokkaido Prefecture, Japan.

Materials and Methods

Bacterial Isolates

E. coli strains ($n = 478$) isolated from various human clinical specimens during 2008 and 2009 were collected, identified and stocked in Sapporo Clinical Laboratory Inc. (Sapporo, Japan) as described previously [15]. The strains were randomly selected and strains isolated from the same patients were omitted. These laboratory samples were obtained from the entire area of Hokkaido Prefecture, Japan. Identification was performed using the MicroScan WalkAway 40 system (Siemens Healthcare Diagnostics, Tokyo, Japan). This study was approved by the review boards of the rele-

Table 1. PCR primer sets used to detect genes encoding AG-modifying enzymes

Target		Sequence	Ref.
<i>aac(3)-I</i>	F	5'-ACC TAC TCC CAA CAT CAG CC-3'	[28]
	R	5'-ATA TAG ATC TCA CTA CGC GC-3'	
<i>aac(3)-II</i>	F	5'-TGA AAC GCT GAC GGA GCC TC-3'	[29]
	R	5'-GTC GAA CAG GTA GCA CTG AG-3'	
<i>aac(3)-III</i>	F	5'-CAC AAG AAC GTG GTC CGC TA-3'	[28]
	R	5'-AAC AGG TAA GCA TCC GCA TC-3'	
<i>aac(3)-IV</i>	F	5'-GTG TGC TGC TGG TCC ACA GC-3'	[29]
	R	5'-AGT TGA CCC AGG GCT GTC GC-3'	
<i>ant(2'')-I</i>	F	5'-GGG CGC GTC ATG GAG GAG TT-3'	[29]
	R	5'-TAT CGC GAC CTG AAA GCG GC-3'	
<i>aac(6')-Ib</i>	F	5'-TTGCGATGCTCTATGAGTGGCTA-3'	[31]
	R	5'-CTCGAATGCCTGGCGTGT-3'	
<i>aac(6')-II</i>	F	5'-CGACCATTTCATGTCC-3'	[30]
	R	5'-GAAGGCTTGTCGTGTTT-3'	

vant institutions. Of these strains, 112 FQ-resistant and 100 FQ-susceptible strains were examined further. The strains were isolated from the following clinical specimens: urine (87; 41.0%), catheter urine (76; 35.8%), sputum (15; 7.1%), stool (7; 3.3%), vaginal secretion (6; 2.8%), pus (3; 1.4%), aspiration tube (3; 1.4%), drainage tube (2; 0.9%), intravenous hyperalimentation catheter tube (2; 0.9%), rhinorrhea (2; 0.9%), ascites (1; 0.5%), anal gland fluid (1; 0.5%), decubitus (1; 0.5%), injury site (1; 0.5%), intestinal juice (1; 0.5%), stoma (1; 0.5%), PEG insertion site (1; 0.5%), pharynx fluid (1; 0.5%) and synovial fluid (1; 0.5%).

Antibiotic Susceptibility

Susceptibility data for FQs were taken from our previous paper [15]. Gentamicin (GEN), amikacin (AMK) and tobramycin (TOB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The susceptibility was determined by minimum inhibitory concentration determined by the microdilution method, and disc diffusion method using Sensi-Disc (Beckton Dickinson, Franklin Lakes, N.J., USA). Breakpoints were according to the recommendations of the Clinical and Laboratory Standards Institute [26]. Screening for the presence of ESBLs was performed with Sensi-Disc of cefpodoxime, ceftazidime and clavulanic acid-ceftazidime according to the recommendations of the Clinical and Laboratory Standards Institute [27].

Genetic Analysis

Genomic DNA, which was used as the template for PCR, was isolated from bacterial cells using the QuickGene DNA tissue kit S (Fuji Film, Tokyo, Japan) and the QuickGene 800 system (Fuji Film). Genes encoding AG-modifying enzymes were detected by PCR using HotStarTaq polymerase (Qiagen, Hilden, Germany). The PCR primers [28–31] are listed in table 1. The PCR product for *aac(6')-Ib* was sequenced with primer 5'-CGTCACTCCATA

Table 2. AG resistance and genes encoding AG-modifying enzymes in FQ-resistant and susceptible *E. coli* strains

	Strains, n	GEN	TOB	AMK	AG-modifying enzyme gene	GEN	TOB	Phylogenetic group	Serogroup
FQ-resistant O25b:H4-ST131	87	R (22), I (0), S (65)	R (20), I (2), S (65)	R (0), I (0), S (85)	<i>aac(3)-II</i> (21) <i>ant(2'')-I</i> (1)	R (21) R (1)	R (19), I (2) R (1)	B2	O25b
FQ-resistant other genotypes	25	R (8), I (0), S (17)	R (6), I (3), S (16)	R (1), I (0), S (24)	<i>aac(3)-II</i> (5) <i>ant(2'')-I</i> (1) <i>aac(3)-IV</i> (1) <i>aac(6')-Ib-cr</i> (1) Not detected (1)	R (5) R (1) R (1) S (1) R (1)	R (4), I (1) R (1) I (1) R (1) I (1)	D (4), B2 (1) D (1) A (1) D (1) D (1)	O1 (3), n.d. ¹ (2) n.d. (1) n.d. (1) O1 (1) O153 (1)
FQ-susceptible	100	R (2), I (0), S (98)	R (2), I (0), S (98)	R (0), I (0), S (100)	<i>aac(3)-II</i> (2)			D (1) A (1)	n.d. (2)

Values in parentheses indicate the number of strains. GEN = Gentamicin; TOB = tobramycin; AMK = amikacin; R = resistant; I = intermediate; S = susceptible.

¹ n.d. – Serogroup could not be determined using the serogrouping antisera kit and multiplex PCR.

CATTGCAA-3' to identify the variant of *aac(6')-Ib-cr* [31]. ESBL genes were detected and identified as described previously [32]. Multilocus sequence type and phylogenetic groups were determined as described previously [15, 33]. Pulse-field gel electrophoresis was performed as described previously [15, 32]. Serogroup determination of the O-serogroup was determined using *E. coli* antisera 'SEIKEN' Set 1 (DENKA Seiken, Tokyo, Japan) and multiplex PCR [34].

Results

E. coli isolates were classified into the following 3 groups: FQ-resistant O25b:H4-ST131 strains (n = 87), FQ-resistant strains other than O25b:H4-ST131 (n = 25), and FQ-susceptible strains (n = 100). We screened the AG resistance of these strains (table 2). The frequencies of GEN resistance were 25.3, 32.0 and 2.0%, and those of TOB resistance were 23.0, 24.0 and 2.0% in the 3 groups, respectively. Only one isolate (an FQ-resistant strain other than O25b:H4-ST131) was resistant to AMK. The genes encoding AG-modifying enzymes were screened by PCR. Of the O25b:H4-ST131 strains, *aac(3)-II* and *ant(2'')-I* were found in 21 strains and 1 strain, respectively. All GEN-resistant O25b:H4-ST131 strains harbored one of them. Two *aac(3)-II*-carrying strains of them demonstrated intermediate resistance to TOB. Of the FQ-resistant strains other than O25b:H4-ST131, the genes of *aac(3)-II* (5 strains), *ant(2'')-I* (1 strain), *aac(3)-IV* (1 strain) and *aac(6')-Ib* (1 strain) were found. The strains harboring these genes, except the isolate harboring *aac(6')-Ib* gene, were resistant to GEN. However, the

genes encoding AG-modifying enzymes so far tested were not detected in 1 strain that was resistant to GEN and intermediate to TOB. One strain (O1-ST648) carrying the *aac(6')-Ib-cr* was resistant to AMK and TOB. The *cr* variant of *aac(6')-Ib* modifies not only AG, such as AMK, but also quinolones [35]. This is known as a plasmid-mediated quinolone resistance gene. Two FQ-susceptible strains that were resistant to GEN and TOB contained the *aac(3)-II* gene. The 16S rRNA methylase genes (*rtmA*, *rtmB*, and *armA*) were not detected in any strains (data not shown).

In our previous study [15], the presence of ESBL genes was examined in each of these *E. coli* strains. CTX-M-type ESBL genes were frequently found in FQ-resistant strains (32.2% of O25b:H4-ST131 strains and 32.0% of strains of other genotypes), but have not been detected in FQ-susceptible strains. The acquisition frequencies are similar to those of genes encoding AG-modifying enzymes. However, only 8 strains harbored both ESBL genes and genes encoding AG-modifying enzymes (table 3; fig. 1). All 8 strains were FQ resistant. The low frequency of the simultaneous occurrence of these two types of resistance genes indicated that they were acquired independently. The 8 strains comprised 4 O25b:H4-ST131 strains, 3 O1-ST648 strains and 1 ST167 strain. Strains SRE76 and SRE77 (O25b:H4-ST131) and strains SRE30 and SRE109 (O1-ST648) had a similarity index of more than 90% of each (fig. 1). Although these pairs shared the same resistance genes, their QRDR mutation patterns differed (table 3), indicating that several strains showed a strong clonal relationship, but a varied genetic status.

Fig. 1. Pulse-field gel electrophoresis patterns (*Xba*I digestion) and phylogenetic tree of *E. coli* isolates with both genes encoding AG-modifying enzymes and ESBL genes.

Table 3. Genotypes and resistance mechanisms of *E. coli* strains harboring both genes encoding AG-modifying enzymes and ESBL genes

Strain	Genotype	AG-modifying enzyme gene	ESBL gene ¹	FQ resistance mutations other than GyrA ²	
				ParC	ParE
SRE54	B2-O25b:H4-ST131	<i>aac(3)-II</i>	CTX-M-14, TEM	Ser80Ile, Glu84Val	–
SRE76	B2-O25b:H4-ST131	<i>aac(3)-II</i>	CTX-M-2, TEM	Ser80Ile, Glu84Val	–
SRE77	B2-O25b:H4-ST131	<i>aac(3)-II</i>	CTX-M-2, TEM	Ser80Ile, Glu84Val	Leu445His
SRE90	B2-O25b:H4-ST131	<i>aac(3)-II</i>	CTX-M-27, TEM	Ser80Ile, Glu84Val	–
SRE109	D-O1-ST648	<i>aac(3)-II</i>	CTX-M-14	Ser80Ile, Glu84Val	–
SRE30	D-O1-ST648	<i>aac(3)-II</i>	CTX-M-14	Ser80Ile, Glu84Gly	–
SRE59	D-O1-ST648	<i>aac(3)-II</i>	CTX-M-14, TEM	Ser80Ile	–
SRE62	A-ST167	<i>aac(3)-IV</i>	CTX-M-2	Ser80Ile	Glu460Val

¹ CTX-M-14 and CTX-M-27 are group 9, and CTX-M-2 is group 2.

² Mutations in *gyrA* (Ser83Leu and Asp87Asn) were the same in all strains.

Discussion

Multidrug-resistant Gram-negative bacteria, particularly those responsible for opportunistic infections, are a serious problem [7–9]. One example is the FQ-resistant *E. coli* O25b:H4-ST131 that has spread globally [10, 11, 13], and frequently acquires CTX-M-type ESBL genes. Our recent findings [15] show that CTX-M genes were frequently found not only in O25b:H4-ST131, but also in other genotypes of FQ-resistant *E. coli* isolated from the Hokkaido Prefecture, Japan. In contrast, CTX-M genes were rarely found in FQ-susceptible strains. There is great concern that strains that are resistant to particular antibiotics may acquire resistance to other antibiotics more frequently than non-resistant strains, generating multidrug-

resistant strains of *E. coli*. A recent nationwide survey in the urology field in Japan indicated that, of the isolates examined, less than 10% were resistant to GEN and none were resistant to AMK [5]. Our present study showed a similar frequency of GEN resistance in all *E. coli* strains. On the other hand, the incidence of GEN resistance and genes encoding AG-modifying enzymes were much higher in the FQ-resistant strains, including O25b:H4-ST131 and other genotypes, than in FQ-susceptible strains. The simultaneous occurrence of genes encoding AG-modifying enzymes and ESBL CTX-M genes was relatively low (fig. 2), suggesting they were acquired independently.

E. coli strains harboring both types of resistance genes are B2-O25b:H4-ST131, D-O1-ST648 and A-ST167. The latter two ST types are frequently identified as ESBL-car-

Fig. 2. Schematic representation of antimicrobial resistance in *E. coli* clinical isolates.

rying *E. coli* isolates not only in humans, but also in veterinary fields [32, 33, 36–38]. Notably, of 5 D1-O1-ST648 strains in 212 strains examined in the present study, 3 strains harbored both *aac(3)-II* and CTX-M-type ESBL genes (table 3), and another D1-O1-ST648 strain harbored the *aac(6')-Ib-cr* gene and was resistant to AMK. Our findings identified two pairs of clonally related strains with the same foreign genes (fig. 1; table 3); however, their

QRDR mutation patterns were different. These results suggest that these resistant clones are continuing to evolve by acquiring further resistance. The main mechanism of FQ resistance is somatic mutations in topoisomerase IV and DNA gyrase, which should be independent of the acquisition of foreign genes, such as genes encoding β -lactamases and AG-modifying enzymes. It is unclear why FQ-resistant *E. coli* preferentially acquired these resistance genes. One explanation is that bacteria resistant to one antibiotic may be frequently exposed to other antibiotics. Particularly, bacteria causing chronic and repetitive infections, especially opportunistic infections, may receive numerous antibiotic pressures and selection.

In conclusion, in comparison to strains that were FQ-susceptible, FQ-resistant strains of *E. coli* were frequently resistant to other antibiotics, such as AGs and cepheims, including not only O25b:H4-ST131, but also other genotypes.

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