Basic Medical Research

Genotypic pattern of fluoroquinolone resistance among extended-spectrum beta-lactamase-producing *Escherichia coli*

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ABSTRACT

BACKGROUND Fluoroquinolone (FQ) is one of the therapeutic options for treating extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (ESBL-Ec) infection, but its use could increase the resistance level of ESBL-Ec. This study aimed to analyze the resistant genes responsible for ESBL production and FQ resistance among the ESBL-Ec isolated from healthy humans and communal wastewater.

METHODS This was an observational study using stored isolates and laboratory data. Genome sequencing was done on 43 *E. coli* DNA isolates before resistance genes, mutations, and high-risk clones were examined through bioinformatic data analysis.

RESULTS The analysis of 39 ESBL-Ec isolates showed ESBL genes, including $bla_{\text{CTX-M-55}}$ (56%), $bla_{\text{CTX-M-15}}$ (31%), and $bla_{\text{CTX-M-27}}$ (8%). ESBL-Ec isolates exhibited mutations in *gyr*A (54%), *gyr*B (0%), *par*C (28%), and *par*E (10%). Plasmid-mediated quinolone resistance genes detected included *qnr*S1 (54%), *qnr*S13 (13%), *qnr*B2 (3%), *aac(6')-Ib-cr5* (3%), *qep*A (0%), and *oqx*AB (0%). ESBL and FQ resistance genes were simultaneously detected in 33 ESBL-Ec isolates, with high-risk clones identified as ST155, ST10, ST23, ST38, ST131, and ST69 Cplx.

CONCLUSIONS ESBL and FQ resistance genes were simultaneously detected in ESBL-Ec isolated from healthy humans and communal wastewater.

KEYWORDS *Escherichia coli*, fluoroquinolone, resistance

Transmission of antimicrobial-resistant bacteria poses a serious threat to global public health. Therefore, surveillance is needed to track and mitigate the transmission of resistant bacteria with varying levels of colonization in all sectors, including humans, animals, and the environment. The World Health Organization guidelines on integrated disease surveillance for 2021 indicates *Escherichia coli*, particularly extended-spectrum beta-lactamase (ESBL)-producing *E. coli* (ESBL-Ec) strains, as a bacterial resistance surveillance indicator.[1,2]

In Asia, especially Thailand, the incidence of ESBL-Ec was 5.4 per 100,000 people in 2008, which increased to 12.8 in 2014.[3] In Indonesia, the prevalence of phenotypically confirmed ESBL-Ec was 40% in healthy humans and 12.8% in aquatic environment.[4] The high prevalence of ESBL-Ec in Indonesia may be attributable to the ESBL genes typically found in *E. coli*, such as $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}. Nevertheless, other rarely observed ESBL genes found in *E. coli*, including *blaGES*, bla_{PER}, bla_{VEB}, bla_{OXA}, bla_{CMT}, bla_{BEL}, bla_{TLA}, bla_{SFO}, and bla_{OXY}, may also be present.[5–7]

ESBL can hydrolyze the beta-lactam ring in the penicillin group, third-generation cephalosporins, and aztreonam. This results in the detachment of antibiotics from the penicillin-binding protein receptor in the bacterial cell wall, rendering them inactive and leading to resistance. This enzymatic activity limits

the treatment options for infections caused by ESBL-producing bacteria.[1,8]

The Infectious Diseases Society of America 2023 guidelines on the treatment of antimicrobial-resistant gram-negative infections recommend fluoroquinolone (FQ) as the preferred antibiotic for treating urinary tract infections (UTIs), including uncomplicated cystitis, pyelonephritis, and complicated UTI caused by ESBL-producing Enterobacterales.[9] These antibiotics are broad-spectrum and inhibit the replication and transcription of bacterial DNA.[10]

In Indonesia, ciprofloxacin is a widely used type of FQ in primary healthcare, ranking third among antibiotics.[11] This high frequency in FQ use can lead to ESBL-Ec being resistant to the drug, as the *E. coli* genome can quickly acquire various resistance mechanisms.[1,12] Such FQ resistance is usually caused by mutation accumulations in the quinolone resistance-determining region (QRDR) and plasmid-mediated quinolone resistance (PMQR) transmission.[13–15] Notably, plasmid-mediated FQ-resistance genes such as *qnr* and *aac(6')-Ib-cr* are usually present on the same plasmids as ESBL genes in *E. coli.*[14] This study aimed to analyze the genotype responsible for ESBL production and FQ resistance among ESBL-Ec isolated from healthy humans and communal wastewater.

METHODS

This observational study used stored isolates and laboratory data collected from the same area and during the same period in Jakarta, Indonesia's capital city, based on a previous study sampling method.[4] Ethical approval has been obtained from the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: KET-612/UN2.F1/ETIK/PPM.00.02/2023). This study is a part of Working Package 4: Molecular Characterization, within the Tricycle Project (pilot) in Indonesia and has obtained ethical approval from the Health Research Ethics Commission, National Institute of Health Research and Development (KEPK-BPPK), with approval number LB.02.01/2/KE.704/2021.

The inclusion criteria were isolates collected from healthy human commensal and communal wastewater within proximity of both areas and sampling time in Jatinegara, East Jakarta. A total of 43 archived *E. coli* isolates and their laboratory data were used, comprising 39 ESBL-Ec and 4 non-ESBL-Ec isolates. The exclusion criteria were isolates identified as non-*E. coli* based on the results of bioinformatics analysis using the Type (Strain) Genome Server (TYGS) (Leibniz Institute, Germany).

Sample collection and phenotypic test

We used stored *E. coli* isolates from the Tricycle Project (pilot) collection at the National Reference Laboratory, Prof. Dr. Sri Oemijati, Health Development Policy Agency. In this study, 39 ESBL-Ec isolates were obtained from 27 healthy humans and 12 communal wastewater isolates. The 39 ESBL-Ec isolates were identified using the automatic VITEK®2 COMPACT (bioMérieux, France). Four non-ESBL-Ec isolates—three isolates from healthy humans and one from communal wastewater—were also used for comparison. A phenotypic antimicrobial susceptibility test was performed on 29 of 43 isolates using VITEK®2 COMPACT (bioMérieux) based on data correlation between sampling time and previous FQ phenotypic resistance result (R/I/S).

All isolates were cultured and purified using MacConkey agar medium, according to the Koch method, and incubated for 24 hours at 37°C. The suspected *E. coli* colonies were indicated by the morphology of round colonies, ranging from light to dark pink from fermenting lactose, dry, with a slight indentation in the middle, and surrounded by a dark pink area owing to bile salt precipitation. Morphological colonies and Gram staining were conducted after selecting individual colonies. Pure isolates were prepared for DNA isolation by creating bacterial suspensions using 1,000 µl nuclease-free water in a cryotube.

DNA isolation

DNA isolation was performed using the QiAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Quality and quantity of DNA were measured using NanoDrop Spectrophotometers (Thermo Scientific, USA) and Qubit (Thermo Scientific). DNA was stored at –20°C before being used for library preparation.

Library preparation and whole-genome sequencing

Library preparation was performed according to the Illumina DNA Prep Workflow (Illumina Inc., USA), involving several stages including genomic DNA fragmentation, post-fragmentation and clean-up, fragmented DNA amplification, library clean-up

and pool libraries, and running the sample through whole-genome sequencing using the Illumina MiSeq Instrument (Illumina Inc.).

Bioinformatics analysis

Primary bioinformatics analysis, including quality control, trimming, and *de novo* assembly processes, was performed using the CLC Genomic Workbench version 21.0.4 (QIAGEN). Genomic bacterial strain identification is using the TYGS (Leibniz Institute). Resistance gene, sequence type (ST), clonal cluster, and virulence factor identification were conducted using Ridom SeqSphere+ (Ridom GmbH, Germany). The resistance genes included ESBL and FQ-resistance genes (QRDR mutation and PMQR).

RESULTS

ESBL genes in ESBL-Ec isolates

Based on resistance gene analysis using Ridom SeqSphere+ (Ridom GmbH), a bla_{CTX-M} ESBL gene variant was the most commonly detected variant among the 39 ESBL-Ec isolates from healthy humans and communal wastewater. Major ESBL variants of bla_{SHV} and $bla_{TEM,}$ and other minor ESBL gene types (bla_{GES}, bla_{PER}, bla_{VEB}, bla_{CMT}, bla_{BEL}, bla_{TLA}, bla_{SFO}, and bla_{OXY}) were not detected in the genome sequences analyzed in this study. The most frequently identified bla_{CTX-M} variant was $bla_{CTX-M-55}$ (n = 22; 56%), detected in 13 healthy humans and nine communal wastewater isolates. The $bla_{CTX-M-15}$ (n = 12; 31%) and $bla_{CTX-M-55}$ were found in ESBL-Ec isolates from both sources, whereas $bla_{CTX-M-27}$ (n = 3; 8%) was found only in ESBL-Ec isolates from healthy humans.

In addition to the ESBL gene, other *beta-lactamase* genes were detected in healthy humans and communal wastewater isolates. In the bla_{TEM} type, only bla_{TEM-1} and $bla_{TEM-176}$ variants were detected; the type of bla_{OXA} obtained was bla_{OXA-1}. The genes bla_{TEM-1}, $bla_{TEM-176}$, and bla_{OXA-1} are included in the Ambler class A group and belong to a narrow-spectrum; therefore, they are not included in the ESBL genes but are grouped with other beta-lactamase genes. From the results, bla_{CMY-2} was also detected, which included the *AmpC beta-lactamase* gene group.

Isolates from both sites carried bla_{CMY-2} (n = 2; 5%) and other beta-lactamase genes including bla_{TEM-1} (n = 8; 21%), $bla_{TEM-176}$ (n = 1; 3%), and bla_{OXA-1} (n = 1; 3%). In total, 28 isolates carried an ESBL gene and other beta-lactamase genes, with $bla_{CTX-M-55}$+bla_{TEM-1} (n = 7, 18%) as the most identified combination.

FQ-resistance genes in ESBL-Ec isolates

Among the 39 ESBL-Ec isolates, FQ resistance from healthy humans was primarily associated with PMQR, whereas isolates from communal wastewater often exhibited QRDR mutations simultaneously with PMQR acquisition. However, two ESBL-Ec isolates from healthy humans and one from communal wastewater had no QRDR mutations, and no PMQR genes were detected (n = 3; 8%).

Several ESBL-Ec isolates from healthy humans and communal wastewater harbored QRDR mutations in varying combinations, found in *gyr*A (n = 21, 54%), *gyr*B (n = 0, 0%), *par*C (n = 11, 28%), and *par*E (n = 4, 10%). These isolates also carried PMQR genes, including *qnr*S1 (n = 21; 54%), *qnr*S13 (n = 5; 13%), *qnr*B2 (n = 1; 3%), and *aac(6')-Ib-cr5* (n = 1; 3%).

QRDR mutations were predominantly observed in *gyr*A, with the amino acid substitution S83L (n = 10; 26%) and double mutation *gyr*A_S83L/D87N (n = 9; 23%). Double mutations in *gyr*A were commonly accompanied by the amino acid change S80I in parC (n = 8, 21%) (Table 1). Among the 39 ESBL-Ec isolates, *gyr*A mutations were detected in six healthy humans and three communal wastewater isolates, whereas *gyr*A+*par*C mutations were observed in three healthy humans and five communal wastewater isolates. One isolate from a healthy human harbored *gyr*A+*par*E mutations (3%), whereas the other three isolates harbored *gyr*A+*par*C+*par*E mutations (8%). No *par*E mutations were identified in the isolates from communal wastewater. Overall, no mutations were detected in gyrB (0/39; 0%) in any of the isolates.

The PMQR gene with the highest proportion, *qnr*S1 (57%), was identified in ESBL-Ec isolates from both healthy humans (n = 12) and communal wastewater (n = 9). *qnr*B2 was detected concurrently with *qnr*S13 in one isolate from a healthy human (CM46). Another rarely identified gene, *aac(6')-Ib-cr5*, was found in an ESBL-Ec isolate from a healthy human (CM84), accompanied by double mutations *gyr*A_D87N/S83L, *par*C_S80I mutation, and *par*E_S458T mutation (Table 1).

ESBL-Ec resistance to FQs

We compared phenotypic data on ESBL-Ec resistance to third-generation cephalosporins and

Table 1. Mutations in QRDR and PMQR detection in ESBL-Ec from healthy human and communal wastewater

ESBL-Ec	PMQR	QRDR mutation			
		*gyr*A	*gyr*B	*par*C	*par*E
CM3	-	S83L	-	S80I	-
CM4	*qnr*S13	-	-	-	-
CM7	*qnr*S13	D87G	-	-	-
CM9	*qnr*S1	-	-	-	-
CM12	*qnr*S1	-	-	-	-
CM23	-	S83V	-	-	-
CM27	*qnr*S1	-	-	-	-
CM28	*qnr*S1	-	-	-	-
CM32	-	-	-	-	-
CM39	-	D87N/S83L	-	E84G/S80I	-
CM46	*qnr*B2, *qnr*S13	S83L	-	-	-
CM60	*qnr*S1	S83L	-	-	I355T
CM64	-	S83L	-	-	-
CM67	*qnr*S1	-	-	-	-
CM68	*qnr*S1	-	-	-	-
CM69	*qnr*S1	-	-	-	-
CM76	-	D87N/S83L	-	S80I	S458A
CM77	-	D87N/S83L	-	E84V/S80I	I529L
CM78	-	-	-	-	-
CM81	*qnr*S1	-	-	-	-
CM82	*qnr*S1	-	-	-	-
CM83	-	S83L	-	-	-
CM84	*aac(6')-Ib-cr5*	D87N/S83L	-	S80I	S458T
CM95	-	D87N/S83L	-	S80I	-
CM97	-	S83L	-	-	-
CM100	qnrS1	-	-	-	-
CM101	qnrS1	-	-	-	-
CMW92	*qnr*S1	D87N/S83L	-	S80R	-
CMW96	*qnr*S1	S83L	-	-	-
CMW104	*qnr*S13	S83L	-	-	-
CMW206	-	-	-	-	-
CMW328	*qnr*S13	-	-	-	-
CMW343	*qnr*S1	S83L	-	S80R	-
CMW450	*qnr*S1	-	-	-	-
CMW580	*qnr*S1	-	-	-	-
CMW684	*qnr*S1	S83L	-	-	-
CMW692	*qnr*S1	D87N/S83L	-	S80I	-
CMW696	*qnr*S1	D87N/S83L	-	S80I	-
CMW698	*qnr*S1	D87N/S83L	-	E84K	-

A=alanine; D=aspartic acid; E=glutamic acid; ESBL-Ec=extended spectrum beta-lactamase-producing *E. coli*; G=glycine; I=isoleucine; K=lysine; L=leucine; N=asparagine; PMQR=plasmid-mediated quinolone resistance; QRDR=quinolone-resistance determining region; R=arginine; S=serine; V=valine; T=threonine
CM: healthy human isolates; CMW: communal wastewater isolates

FQs with ESBL and FQ-resistance gene detection results. Antimicrobial susceptibility testing on 39 ESBL-Ec isolates from healthy humans and communal wastewater identified 67% (n = 26) of the isolates as phenotypically resistant to ceftriaxone (third-generation cephalosporin), aztreonam (monobactam), and ciprofloxacin (FQ) (Figure 1).

ST, high-risk clone, and virulence factor

Subtyping was conducted using the cgMLST scheme of Ridom SeqSphere+ (Ridom GmbH), and 29 STs were detected among the selected isolates. STs were assigned according to the Warwick scheme based on variations in specific gene loci in each genome. Based on the similarities between the STs obtained, several clusters were detected, including the high-risk clones ST10 Cplx, ST131 Cplx, ST155Cplx, ST23 Cplx, ST38 Cplx, and ST69 Cplx (Table 2).

Six ESBL-Ec isolates (two from healthy humans and four from communal wastewater) were identified as members of the high-risk ST10 Cplx cluster. Some high-risk clones were detected only in healthy human isolates: ST131 Cplx, ST23 Cplx, ST38 Cplx, and ST69 Cplx. The most common high-risk clones detected were ESBL-Ec isolates that were resistant to FQs. There was a 5% no strain match in the Ridom SeqSphere+ database (n = 2), representing a novel ST from Indonesia that was never found in other countries. In addition to having resistance genes, all ST isolates included in the high-risk clone have virulence factors of adherence, invasion, iron uptake, effector delivery systems that support transmission, and their ability to persist for a long time in the host (Table 3).

DISCUSSION

This study analyzed the genotype responsible for ESBL production and FQ resistance among ESBL-Ec isolated from healthy humans and communal wastewater. We found $bla_{CTX\text{-}M\text{-}55}$ as the most frequently identified ESBL gene in both isolates. $bla_{CTX\text{-}M}$ is a dominant ESBL gene that has been documented since 2000 and has been identified globally in bacteria in humans, animals, and the environment.[6,16,17] This $bla_{CTX\text{-}M}$ gene group was first identified in patients in Germany, Italy, and South America.[6,17] The $bla_{CTX\text{-}M}$ gene, especially $bla_{CTX\text{-}M\text{-}15}$, is commonly found in clinically derived isolates, although it can also be detected in healthy human samples with UTI symptoms.[18] The current variations

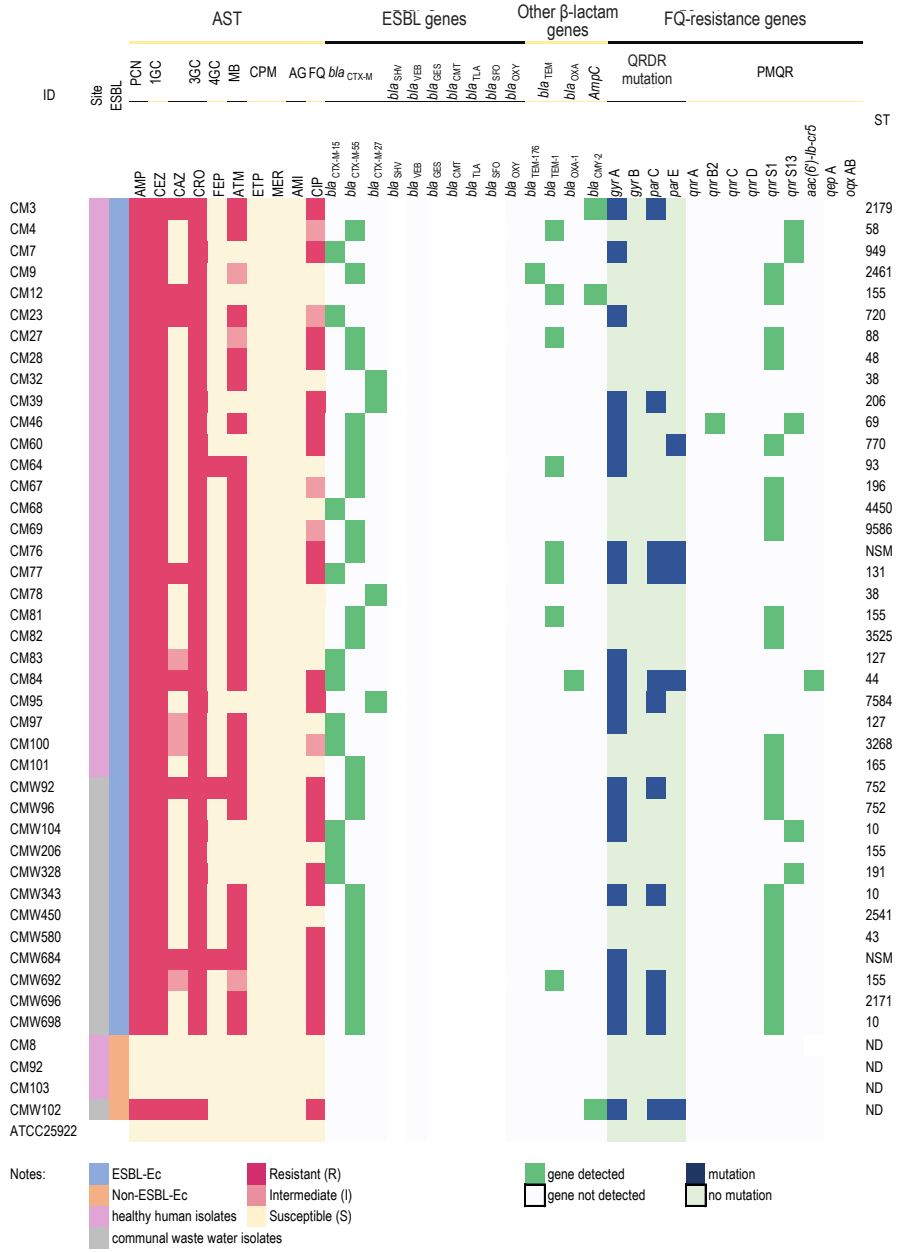

Figure 1. Heat map of antimicrobial susceptibility test results and third generation cephalosporin-FQ resistance genes. CM: healthy human isolates; CMW: communal wastewater isolates; and ATCC: *Escherichia coli* ATCC 25922. AG=aminoglycoside; AMI=amikacin; AMP=ampicillin; AST=antimicrobial susceptibility testing; ATM=aztreonam; CAZ=ceftazidime; CEZ=cefazolin; CIP=ciprofloxacin; CPM=carbapenem; CRO=ceftriaxone; ESBL=extended-spectrum beta-lactamase; ESBL-Ec=extended-spectrum beta-lactamase producing *Escherichia coli*; ETP=ertapenem; FEP=cefepime; FQ=fluoroquinolone; MB=monobactam; MER=meropenem; ND=not defined; NSM=no strain match in the database; PCN=penicillin; PMQR=plasmid-mediated quinolone resistance; QRDR=quinolone resistance-determining region; ST=sequence type; 1GC=first-generation cephalosporins; 3GC=third-generation cephalosporins; 4GC=fourth-generation cephalosporins

in bla_{CTX-M} are more diverse than those of the bla_{TEM} and bla_{SHV} genes, which dominated earlier.[6,17] The prevalence of $bla_{CTX-M-15}$ in healthy human-sourced *E. coli* identified in healthy travelers to South Asia has reached up to 75% of ESBL-producing strains, significantly higher than in Europe and North America.[19] Following $bla_{CTX-M-15}$, the highest prevalence is observed with $bla_{CTX-M-14}$ and $bla_{CTX-M-27}$, which have been detected in multiple countries. However, the prevalence of ESBL genes varies among geographical locations.[6]

The identification of $bla_{CTX-M-55}$ as the most dominant bla_{CTX-M} type in this study differed from that reported by Bevan et al,[17] Castanheira et al,[6] and Yang et al[16]. These studies suggest that global trends in CTX-M beta-lactamase genes describe a predominance of $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$, especially in Asian countries, notably Japan and Korea.[6,16,19] $bla_{CTX-M-15}$ has also been detected in low-income countries, such as Ethiopia, with a frequency as high as 88.4%. This gene is typically identified in strains isolated from patients with

suspected pneumonia, diarrhea, wound infections, and UTIs.[20,21] Negeri et al[21] reported that $bla_{CTX\text{-}M\text{-}15}$ is transmitted by plasmids, which were detected in 75% of clinical isolates from hospitals in Ethiopia. The $bla_{CTX\text{-}M\text{-}55}$ in ESBL-Ec has been frequently reported in vegetables in Thailand, food and pets in Hong Kong and China, and poultry in China.[16,22,23] A study in Japan[24] also detected $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, and $bla_{CTX\text{-}M\text{-}55}$ in ESBL-Ec isolated from river water. The high rate of $bla_{CTX\text{-}M\text{-}55}$ detection in this study may be due to the transmission of bacteria from poultry products or other foods in the market to healthy humans and communal wastewater isolated from that area. Further investigations of $bla_{CTX\text{-}M}$ variants in poultry products are required.

In addition to $bla_{CTX\text{-}M}$, we also detected the *AmpC beta-lactamase* gene and other beta-lactamase

Table 2. Distribution of high-risk clones in ESBL-Ec isolates from healthy human and communal wastewater

	CC Warwick	ID	
		Healthy human isolates	Communal wastewater isolates
High-risk clone	ST10 Cplx	CM28*, CM84*	CMW104*, CMW343*, CMW580*, CMW698*
	ST131 Cplx	CM77*	
	ST155 Cplx	CM4*, CM12*, CM81*	CMW206, CMW692*
	ST23 Cplx	CM27*	
	ST38 Cplx	CM32, CM78, CM100*	
	ST69 Cplx	CM46*	

CC=clonal cluster; Cplx=complex; ESBL-Ec=extended-spectrum beta-lactamase producing *Escherichia coli*
*ESBL-Ec resistant to fluoroquinolone (FQ)

Table 3. Virulence factor genes of high-risk clone detected

CC	ID/ST	Virulence factor				
		Adherence	Invasion	Iron uptake	Toxin	Effector delivery system
ST10 Cplx	CM28 (ST48)	*aslA, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fiml, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE,* and *iroN*		*espL1, espL4, espX4,* and *espX5*
	CM84 (CM44)	*aslA, afaA, afaB-I, afaC-I, afaC-III, afaD, afaE-V, daaA, daaC, daaD, daaF, draA, draB, draC, daaD, draP, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH,fiml, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE,fepA, fepB, fepC, fepD, fepG, fes, iucA, iucB,* and *iucC*		*espL1, espL4, espX1, espX4,* and *espX5*
	CMW104 (ST10)	*aslA, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA, papB, and papI*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*		*espL1, espL4, espX4,* and *espX5*
	CMW343 (ST10)	*aslA, astA, fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*	*east1*	*espL1, espL4, espX1, espX4,* and *espX5*
	CMW580 (ST43)	*aslA, astA, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG,* and *fes*	*east1*	*espL1, espL4, espX1, espX4,* and *espX5*
	CMW698 (ST10)	*aslA, astA, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE,* and *iroN*	*east1*	*espL1, espL4, espX4,* and *espX5*

Table continued on next page

Table 3. (Continued)

CC	ID/ST	Virulence factor				
		Adherence	Invasion	Iron uptake	Toxin	Effector delivery system
ST131 Cplx	CM77 (ST131)	*aap/aspU, afaA, afaB-I, afaC-I, afaD, afaF-III, aslA, astA, fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM, ompA, papB, papI,* and *papX*	*chuA, chuS, chuT, chuU, chuW, chuX, chuY, entB, entC, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iucA, iucB,* and *iucC*	*east1 and sat*	
ST155 Cplx	CM4 (ST58)	*fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE,* and *iroN*		*espL1, espX1, espX4,* and *espX5*
	CM12 (ST155)	*fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE,* and *iroN*		*espL1, espX1, espX4,* and *espX5*
	CM81 (ST155)	*fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*		*espL1, espX1, espX4,* and *espX5*
	CMW206 (ST155)	*fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM,* and *ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE, iroN, iucA, iucB,* and *iucC*		*espL1, espX1, espX4,* and *espX5*
	CMW692 (155)	*fdeC, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*		*espL1, espX1, espX4,* and *espX5*
ST23 Cplx	CM27 (ST88)	*fdeC,fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fiml, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*ompA*	*fepA, fepB, fepC, fepD, fepG, fes, entB, entC, entD, entE, entS, iroB, iroC, iroD, iroE, iroN, iucA, iucB,* and *iucC*		*espL1, espX1, espX4,* and *espX5,*
ST38 Cplx	CM32 (ST38)	*aap/aspU, afaA, afaB-I, afaC-I, afaD, afaF-III, aslA, astA, daaA, daaC, daaD, daaF, draA, draB, draC, daaD, draP, faeF, faeI, faeJ, fdeC, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM, ompA,* and *papX*	*chuA, chuS, chuT, chuU, chuW, chuX, chuY, entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*	*east1*	*espL1, espL4, espR1, espX1, espX4, espX5, espY1, espY2, espY3,* and *espY4*
	CM78 (ST38)	*aap/aspU, afaA, afaB-I, afaC-I, afaD, afaF-III, aslA, astA, daaA, daaC, daaD, daaF, draA, draB, draC, draP, faeF, faeI, faeJ, fdeC, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM, ompA,* and *papX*	*chuA, chuS, chuT, chuU, chuW, chuX, chuY, entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*	*east1*	*espL1, espL4, espR1, espX1, espX4, espX5, espY1, espY2, espY3,* and *espY4*
	CM100 (ST3268)	*aslA, astA, fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fiml, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM,* and *ompA*	*chuA, chuS, chuT, chuU,chuW, chuX, chuY, entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG,* and *fes*	*east1*	*espL1, espL4, espR1, espR3, espR4, espX1, espX4, espX5, espY1, espY3,* and *espY4*
ST69 Cplx	CM46 (ST69)	*aslA, astA, fdeC, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA,* and *ykgK/ecpR*	*kpsD, kpsM, ompA,* and *papX*	*chuA, chuS, chuT, chuU, chuW, chuX, chuY, entB, entC, entD, entE, entS, fepA, fepB, fepC, fepD, fepG, fes, iroB, iroC, iroD, iroE, iroN, iucA, iucB,* and *iucC*		*espL1, espL4, espR1, espX1, espX4, espX5, espY3,* and *espY4*

CC=colonal cluster; Cplx=complex; ST=sequence type

resistance-related genes. Five healthy human ESBL-Ec isolates (CM4, CM27, CM64, CM76, and CM81) were detected using bla$_{CTX-M-55}$ and *bla*$_{TEM-1}$, similar to those in one communal wastewater isolate (CMW692). This finding is consistent with that of Yang et al,[16] who reported that *bla*$_{CTX-M-55}$ is commonly found concurrently with *bla*$_{TEM-1}$ in ESBL-EC isolates. In the isolate CM9, *bla*$_{CTX-M-55}$ was detected along with *bla*$_{TEM-176}$ in the same isolate. Moreover, Barguigua et al[25] found 49 ESBL-Ec isolates from patients with UTI in Morocco. Until now, *bla*$_{TEM-176}$ is still considered a new variant that requires further characterization and is classified under the *bla*$_{TEM-1}$ beta-lactamase gene in databases (SeqSphere+ and ResFinder 4.0).[26]

Furthermore, *bla*$_{OXA-1}$ was detected in an isolate carrying *bla*$_{CTX-M-15}$ (CM84). According to Livermore et al,[27] OXA-1 belongs to the penicillinase group, and *bla*$_{OXA-1}$ is commonly associated with *bla*$_{CTX-M-15}$ in ESBL-Ec. In a study in London, 50.9% of ESBL-Ec isolates carried *bla*$_{CTX-M-15}$+*bla*$_{OXA-1}$ in patients with bloodstream infections.[27] In addition to other beta-lactamase genes, we found *bla*$_{CMY-2}$ alone in isolate CM3, and both *bla*$_{CMY-2}$ and *bla*$_{TEM-1}$ in isolate CM12 without ESBL genes. The presence of ESBL genes and *bla*$_{CMY-2}$ depends upon the genotypic backgrounds of the isolates. Deng et al[28] reported that *bla*$_{CMY-2}$ could be horizontally transferred between different bacterial species and strains. Generally, *bla*$_{CMY-2}$ is detected at high rates in animals, such as pigs and poultry, with detection rates ranging from 0% to 80%, typically without ESBL genes.[28]

FQ resistance is strongly influenced by mutations in QRDR. We detected various mutations in these regions, including single mutations in one QRDR and double mutations in several QRDRs. *gyr*A mutations with amino acid changes, particularly S83L, were the predominant mutation type identified in ESBL-Ec isolates from both sources.

The mutation rates in *gyr*A (20.5–23.1%) obtained in this study were consistent with those reported by Johnning et al,[29] who identified double mutations *gyr*A_S83L/D87N in *E. coli* isolated from community water environments, with a 32% S83L and 31% D87N amino acid change. Regarding mutations in *par*C, Hamzah et al[30] stated that substituting serine with isoleucine is a frequently detected QRDR mutation in *par*C in *E. coli*, with a prevalence of 81.2%. In a recent study, mutations in *par*C were consistently associated with mutations in other QRDRs such as *gyr*A. Conversely, no *gyr*B mutations were found in ESBL-Ec isolates from either site. The *gyr*B mutations are less associated with FQ resistance in clinical cases than *gyr*A mutations. Feng et al[31] mention that *gyr*B mutations occur more frequently in *Pseudomonas aeruginosa* than in *E. coli*. In *P. aeruginosa*, a single mutation in *gyr*B increases resistance to FQs by more than two mutations in *gyr*A and *gyr*B.

PMQR was detected in healthy humans working with poultry, the poultry, and their surrounding environments were dominated by *qnr*S1, *qnr*B19, and *aac(6')-Ib-cr*.[32] In contrast, *qnr* detected in this study included *qnr*S1, *qnr*S13, *qnr*S13+*qnr*B2, and *aac(6')-Ib-cr5*. In ESBL-Ec isolates from healthy humans, PMQR mutations (44%) were detected more commonly than QRDR mutations (33%). The high prevalence of PMQR in healthy human-derived strains is noteworthy, considering that Kotb et al[33] detected *qnr* in up to 57.1% of Enterobacteriaceae isolates from humans with UTI. Although the contribution of *qnr* to FQ resistance is relatively low, it can increase chromosomal mutations in *gyr*A and *par*C, which may contribute to the increase in FQ resistance in *E. coli*.[34]

This study found that 50% of ESBL-Ec isolates from communal wastewater had QRDR mutations and PMQR detection simultaneously. The accumulation of FQ-resistance genes in these communal wastewater isolates may have increased their resistance to these antibiotics. The gene related to co-resistance to FQs and aminoglycosides, *aa-Ib-cr5*, was found in only 3% (1/39) of healthy human ESBL-Ec isolates and detected along with *bla*$_{CTX-M-15}$ in isolate CM84, which was the least frequently detected co-resistance gene in this study. This is consistent with Bodendoerfer et al,[35] who also found *aa-Ib-cr* in the lowest proportion (11.8%; 52/3358) of ESBL-Ec isolates collected in the Zurich region of Switzerland. In that study, *aa-Ib-cr* was associated with *bla*$_{CTX-M-15}$/*bla*$_{OXA-1}$. In other studies conducted in the United States and South Korea,[29,30] several reasons for the limited detection of *aa-Ib-cr gene* have been identified. These include low resistance rates, which are often found in combination with other FQ-resistance genes, leading to the assumption that other FQ-resistance genes play a more significant role in conferring resistance. To date, studies on this gene have been limited to certain countries.[29–33,36]

Most of the ESBL-Ec isolates in this study were FQ-resistant based on phenotypic results and detected resistance genes. The isolates were phenotypically (n = 26) and genotypically (n = 33) resistant to third-

generation cephalosporins and FQs. According to Corona and Martinez,[37] phenotypic and genotypic differences can occur owing to low gene expression in the genomes of isolates. Low expression of these genes can occur due to weak regulatory gene complexes or promoters caused by mutations.[37]

Most ESBL-Ec isolates in this study exhibited phenotypic FQ resistance and resistance-conferring genes in the two isolates (CM3 and CM12) that transitioned phenotypically from ESBL-Ec to non-ESBL-Ec. However, both isolates carried bla_{CMY-2} (AmpC beta-lactamase gene), bla_{TEM-1}, *gyr*A+*par*C, and *qnr*. Therefore, it is presumed that the resistance mechanism of these isolates to third-generation cephalosporins is also influenced by the presence of bla_{CMY-2}. According to Thomson et al,[38] *AmpC* beta-lactamase production in an isolate will affect the detection of ESBL in testing using VITEK.

ESBL-Ec exhibits multidrug resistance, particularly against FQs.[5,25] This phenomenon may be influenced partly by the increased use of FQs among healthy humans, such as tourists.[39] FQs are also widely used for patients with UTI at health centers,[11] which may select for and promote transmission of ESBL-FQ-resistance genes and resistant microbes in the community and the environment, turning healthy humans into carriers and acting as a reservoir that is the source of clinical resistance occurred.[40,41]

Compared to data from 2005, FQ-resistant *E. coli* from populations visiting health centers in Indonesia has increased. The total population tested in the 2005 study was 3,996 humans, with only 2% of the *E. coli* strains phenotypically resistant to FQ.[42] However, in this study, phenotypic results showed very high levels of FQ resistance, with 59% (n = 16/27) in healthy humans and 83% (n = 10/12) in the communal wastewater of 39 ESBL-Ec isolates.

Based on Expert Rules v.32 for Enterobacterales, *E. coli* is an indicator microorganism for ciprofloxacin. Therefore, if resistance in Enterobacterales (except *Salmonella* spp.) is phenotypically found to be resistant to ciprofloxacin, resistance to all FQs must be reported. Thus, the entire FQ group is affected, and FQ is not recommended for use in therapy. Phenotypic testing should be accompanied by at least two target mutations in the QRDR (double mutations in gyrA, or mutations in gyrA accompanied by mutations in parC).[43]

If an isolate detected *aac(6')-Ib-cr* before the phenotypic results, there is a possibility that FQ-resistant isolates become false negatives, indicating that they could not be detected due to enzyme inactivation.[43] In contrast to this study, isolates with the *aac(6')-Ib-cr gene* (CM84) were phenotypically resistant to FQs. This is likely due to the inactivation of the enzyme, which only causes low levels of resistance in isolates.[36,44,45] There are also double mutations in *gyr*A_D87N/S83L as well as in *par*C_S80I and *par*E_S458T, which play a greater role in FQ resistance.

Mutations at different genetic loci in a single pathogen and the presence of transfer-resistant genes can be difficult to treat because the pathogen is more resistant and causes infection. If this condition is supported by virulence factors that can spread extensively, the pathogen could be a high-risk clone that can transmit globally and cause an outbreak.[46] High-risk clones are defined as globally distributed clones, have several determinants of antimicrobial resistance and high pathogenicity related to their virulence factors, and are capable of colonizing and surviving in the host for more than 6 months, transmitting effectively between hosts, causing severe and recurrent infections. The most frequently reported high-risk clones are ST131, ST69, ST10, ST23, ST405, ST38, ST95, ST648, ST73, and ST1193.[39,47]

Of all the isolates, the largest ST proportions detected were ST155 and ST10, contrary to those in a study by Castanheira et al,[7] who reported that ST131 dominates the global ESBL-Ec population. In this study, among FQ-resistant ESBL-Ec isolates, ST131, ST69, and ST58 were detected from healthy human isolates, whereas ST10 was more dominant in communal wastewater. Only a small proportion of high-risk clones was detected among the FQ-susceptible ESBl-Ec isolates.

We identified a high-risk clone, ST131 Cplx, in a healthy human isolate (CM77). Antimicrobial susceptibility test results revealed that the ESBL-Ec isolates were phenotypically resistant to third-generation cephalosporins, aztreonam, and ciprofloxacin. Resistance genes detected in these isolates included $bla_{CTX-M-15}$ and QRDR mutations (*gyr*A_D87N/S83L, *par*C_E84V/S80I, and *par*E_I529L). These findings align with those of a previous study, which found that *E. coli* ST131, an ESBL-producing bacterium, is highly correlated with FQ resistance, commonly an ESBL-producing bacteria.[6,48]

The proportion of ST131 in this study was smaller (3%) compared to Kudinha and Kong's[48] results, which reported ST131 in healthy women (5%) and children in

Australia (3%). Finn et al[49] detected ST131 in communal wastewater (1.86%), while our study found none. This result indicates that variations in ST131 distribution in communal wastewater could be influenced by factors such as geographical location, sampling methods, and study period.

ST131 was the only gene with a secreted autotransporter toxin gene (*sat*). This toxin has cytotoxic activity on the epithelial cells of the kidney, bladder, urinary tract, and endothelial cells that form the lining of blood vessels.[50,51] Another toxin virulence factor gene detected in this CM77 isolate (ST131 Cplx) is enteroaggregative heat-stable enterotoxin 1 (*east1*), which can cause diarrhea in humans and several animal species, including cattle and pigs.[51] The possible risk from ST131 is that it can colonize the human digestive tract, move and infect the bladder, and be found in the bloodstream. ST131 colonization in the digestive tract persists over a long period and is promoted by particular adhesin virulence factors.[52]

Other high-risk clones detected only in the isolates from healthy humans were ST23 Cplx, ST38 Cplx, and ST69 Cplx. Information regarding the ST23 and ST38 clusters in healthy humans is still limited, but these clones may be related to strain transmission from poultry production sites. Kromann et al[53] detected ST23 during a deadly colibacillosis outbreak in poultry in Denmark.

The second highest-risk clone found globally is ST69. This cluster is known for its ability to produce biofilms and cause outbreaks, commonly harboring virulence factors that promote attachment to epithelial cells and the production of bacteriocins.[39,54] Neumann et al[54] reported that the virulence gene associated with biofilm production in ST69 is *air* as an adhesion virulence factor and its regulator *eil*A. In this study, neither *air* nor *eil*A genes were detected in ST69 Cplx. However, another gene associated with biofilm production, *sfaX*, was obtained from the CM77 (ST131 Cplx), CM32, and CM78 (ST38 Cplx) isolates. According to Ballén et al,[55] the most studied virulence factors related to biofilm formation are type I fimbriae, flagella, and curli fimbriae. The *fim*A gene, which encodes the major subunit of type I fimbriae, is known to be involved in the initial stage of biofilm formation.[55] Most of the ST obtained in this study harbored the *fim*A gene, except the isolates CM28, CM84, and CMW580 (ST10), CM32, and CM78 (ST38 Cplx). The isolates with *sfaX* did not have the *fim* gene in the genome. According to Laconi et al,[56] this is because *sfaX* is part of the S fimbriae gene cluster, which promotes fimbrial expression to increase biofilm production in uropathogenic *E. coli* and causes UTI on biotic surfaces. Meanwhile, *fim* is associated with biofilm formation and adhesion to abiotic surfaces.[57]

In this study, two clusters of high-risk clones were detected at both sites (ST10 Cplx and ST155Cplx). The ST10 Cplx cluster is the third highest-risk clone found globally.[39] Fuga et al[58] explained that *E. coli* ST10 is predominantly found in environmental samples from Asian and European countries, while it has been detected only in Brazil since 1989, and carries $bla_{CTX\text{-}M}$. In contrast, our study not only obtained ST10 from communal wastewater, but also from two isolates from healthy humans included in ST10 Cplx. All isolates included in the ST10 Cplx in this study were *E. coli* resistant to FQs.

We also obtained *E. coli* ST155 from both sources. The only isolate that was not resistant to FQs was CMW206. This aligns with the data showing no QRDR mutation or PMQR acquisition; however, the genome of the isolate contained $bla_{CTX\text{-}M\text{-}15}$ and was phenotypically resistant to ceftriaxone. Other isolates belonging to ST155 Cplx did not have mutations while carrying the *qnr* gene. According to Neumann et al,[54] *E. coli* ST155 is known as a foodborne pathogen with zoonotic infection potential, and its resistance genes may be horizontally transferred by plasmids. These bacteria belong to phylogenetic group D and have been identified in humans and animals. The prevalence of *E. coli* ST155 has not been determined, but *E. coli* ST155 was found in Indian wastewater.[54,59]

To our knowledge, this study is the first to obtain evidence related to FQ-resistant ESBL-Ec isolates. However, this study also has some limitations. During the examination of stored isolates, some changes in the phenotypic characteristics of the bacteria in the two *E. coli* isolates occurred, and they were no longer detected as ESBL producers, possibly because of changes in the stability of ESBL gene expression, loss of plasmids carrying ESBL genes in stored isolates, and the influence of colony selection purification results after it could not be distinguished between susceptible and resistant colonies.

In conclusion, the resistance genes responsible for ESBL production detected in this study in both ESBL-Ec isolates from healthy humans and communal wastewater were $bla_{CTX\text{-}M\text{-}55}$, $bla_{CTX\text{-}M\text{-}15}$, and $bla_{CTX\text{-}M\text{-}27}$. In

addition to the ESBL genes, narrow-spectrum beta-lactamase and AmpC beta-lactamase genes were detected. The QRDR mutations detected in the isolates were in *gyr*A, *par*C, and *par*E, whereas no mutations were detected in *gyr*B. PMQR was detected in the isolates, including *qnr*S1, *qnr*S13, *qnr*B2, and *aac(6')-Ib-cr5*, but *qep*A and *oqx*AB were not detected. Resistance genes responsible for ESBL production and FQ-resistance genes were detected simultaneously in the genomes of ESBL-Ec isolates from healthy humans and communal wastewater, and most were high-risk clones. The high level of FQ resistance in ESBL-Ec isolates from healthy people and communal wastewater limits the treatment options for infections caused by ESBL-producing bacteria.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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