Characteristics and Molecular Epidemiology of Cefotaxime Resistant *Enterobacteriaceae* Isolated from Healthy College Students

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1. Introduction

Several antimicrobial resistant bacteria are spreading throughout the world. It is difficult to treat a patient infected with an immunity to antibiotics and to develope a novel antimicrobial agent. Antimicrobial resistant bacteria have typically been isolated from hospitals (inpatients, environments and medical staff). In 1969, moreover, “the Swann Report” drew attention to the potential for antibiotic-resistant bacteria to spread from treated animals to humans through consumption. Nevertheless, there was little response to this finding until the detection of vancomycin-resistant enterococci (VRE), a related glycopeptide avoparcin, in animals fed the enhanced feed [1]. Recently, antimicrobial resistant bacteria such as extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* and community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) have been pervaded in healthy humans [2]. It has been increasingly considered that ESBL producing strains are spreading to healthy humans through the consumption of livestock, processed with the use of a broiler like in the case of VRE [3].

Although *Enterobacteriaceae* normally exists in the intestinal tracts of humans, it is also frequently caused not only various intestinal diseases but also extraintestinal diseases such as urinary tract infection, bacteremia, and pneumonia. Broad-spectrum oxyminocephalosporins, such as cefotaxime, ceftazidime and ceftriaxone, have potent activity against *Enterobacteriaceae*, which is one of clinical isolates possessing resistance to narrow-spectrum β-lactam agents. On the other hand, ESBL is produced by *Enterobacteriaceae* effectively hydrolyses the third generation cephalosporins but not cephamycins (cefmetazole, cefoxitin) or carbapenems (imipenem, meropenem) [4]. Most ESBLs can be classified into three main groups: TEM, SHV, and CTX-M families. The number of natural variants found within these families has increased; 170, 130, and 100 types respectively had been identified by 2010, respectively [5]. The CTX-M types of ESBL that can efficiently hydrolyze cefotaxime and ceftriaxone are spreading in Japan [6]. Moreover, international attention is brought to the fact that a

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strain of CTX-M-15 type ESBL producing bacteria could hydrolyze ceftazidime and is not susceptible to other drugs such as fluoroquinolone [7) 8)]. Other β-lactam agent resistant mechanisms are associated with by upregulation of the chromosomal *AmpC* gene, plasmid mediated *AmpC*, efflux pumps, decreased in the permeability of the outer membrane and a mutation of penicillin binding protein (PBP). The aim of this research is to investigate the characteristics and molecular epidemiology of cefotaxime resistant *Enterobacteriaceae* isolated from healthy college students.

2. Materials and Methods

2.1 Bacterial Isolates

One hundred and fory-five stool swab samples were collected from healthy college and nursing school students. Stool samples were cultured for 18–24 hours in BHI culture media (BD, Japan) with 2 mg/mL cefotaxime (Sigma, Japan). Growth samples were isolated MacConkey ager (Eiken Chemical, Japan) including 2 mg/mL cefotaxime and incubated at 37 ℃ for 18–24 hours. Colonies were chosen and sub-cultured by the BTB lactose ager (Kokuto-Seiyaku, Japan) and identified by Api20E (biomerieux).

2.2 Antimicrobial Susceptibility Tests

Antimicrobial susceptibility was evaluated by ager plate dilution methods with ampicillin (Wako, Japan), cefazolin (Sigma, Japan), cefmetazole (Sigma, Japan), ceftazidime (Sigma, Japan), cefotaxime (Sigma, Japan), gentamycin (Wako, Japan), amikacin (Wako, Japan), and ciprofloxacin (Wako, Japan). Susceptibilities to amoxicillin/clavulanate, ceftriaxone, imipenem, and meropenem were evaluated using Etest (biomerieux). Breakpoints were determined according to the recommendations of the CLSI [9)]. Intermediate susceptibility to each antibiotic was defined as resistance. An ESBL confirming test was performed by double disk synergy test (DDST) used with cefotaxime (BD, Japan), cefotaxime/clavulanate (Eiken Chemical, Japan), ceftazidime (BD, Japan), and ceftazidime/clavulanate (Eiken Chemical, Japan).

2.3 PCR & DNA Direct Sequence Analysis

2.3.1 β-Lactamase Identification

ESBL and plasmid-mediated AmpC β-lactamase (pAmpC) genes were detected by PCR amplification of $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV} genes and six main groups (*ACCM*, *CITM*, *DHAM*, *EBCM*, *FOXM*, *MOXM*) of pAmpC-type genes, as described previously [10) 11) 12)]. The PCR was performed with Takara *Ex taq* (Takara Bio, Japan). All PCR amplicons were verified by gel electrophoresis on a 1.8 % SeaKem LE agarose (Takara Bio, Japan).

2.3.2 Phylogenetic Groups of *Escherichia coli*

E. coli isolates were analyzed to determine their phylogenetic groups (A, B1, B2, and D) using the triplex PCR assay of *chuA*, *yjaA*, and *tspE4.C2*, as described previously [13)].

2.3.3 Fluoroquinolone Resistant Mechanisms

The quinolone resistance determining regions (QRDRs) of *gyrA* and *parC* genes were sequenced, and the correlating amino acids were compared with the corresponding regions of *E. coli* strain (GeneBank No. AF052244, AB083821). PCR was performed in a final volume of 25 μL. Each reaction contained 2.0 mM MgCl, 0.2 mM dNTPs, 0.4 μM of each primer, and 1.0 U of *Ex taq*. The primers for PCR were used as described previously [14) 15)]. The PCR program consisted of an initial denaturation step at 95 ℃ for 5 minutes, followed by 30 cycles of DNA denaturation at 95 ℃ for 15 seconds, annealing at 56 ℃ for 20 seconds, and extension at 72 ℃ for 1 minute. After the last cycle, a final extension step at 72 ℃ for 5 minutes was added. PCR products were purified by DNA RNA

Table 1. All strains isolated and susceptibility tests

Strain No.	Identification	MIC (μg/mL)											
		ABPC	AMO/CVA *	CEZ	CMZ	CTX	CAZ	CTRX *	IPM *	MEPM *	CPFX	GM	AMK
24OT016-1	*Enterobacter cloacae*	>=256	64	>=32	>=32	>=32	>=32	>=32	0.38	0.064	<=0.5	<=0.5	<=4
24OT021-1	*Enterobacter cloacae*	>=256	>=256	>=32	>=32	16	16	>=32	0.5	0.064	<=0.5	<=0.5	<=4
24OT029-1	*Citrobacter braakii*	>=256	32	>=32	>=32	>=32	>=32	>=32	0.75	0.125	<=0.5	<=0.5	<=4
24OT031-2	*Escherichia coli*	>=256	4	>=32	<=0.5	>=32	<=0.5	12	0.125	0.023	<=0.5	<=0.5	<=4
24OT036-3	*Escherichia coli*	>=256	4	>=32	<=0.5	>=32	<=0.5	32	0.125	0.23	<=0.5	1	8
24OT040-1	*Escherichia coli*	>=256	8	>=32	2	16	1	>=32	0.19	0.023	<=0.5	1	<=4
24OT041-1	*Citrobacter braakii*	>=256	128	>=32	>=32	16	>=32	>=32	0.5	0.047	<=0.5	<=0.5	<=4
24OT042-1	*Enterobacter cloacae*	>=256	48	>=32	>=32	16	>=32	>=32	0.5	0.125	<=0.5	<=0.5	<=4
25KC005-1	*Escherichia coli*	>=256	6	>=32	2	>=32	<=0.5	>=32	0.19	0.032	<=0.5	>=32	<=4
25KC005-2	*Escherichia coli*	>=256	6	>=32	2	>=32	1	>=32	0.19	0.032	<=0.5	<=0.5	<=4
25KC011-2	*Escherichia coli*	>=256	6	>=32	1	>=32	8	>=32	0.38	0.064	<=0.5	<=0.5	<=4
25KC012-1	*Hafnia alvei*	>=256	48	>=32	>=32	8	>=32	12	0.5	0.064	<=0.5	<=0.5	<=4
25KC016-2	*Escherichia coli*	>=256	12	>=32	1	>=32	1	>=32	0.19	0.047	>=32	1	<=4
25KC017-1	*Escherichia coli*	>=256	12	>=32	1	4	<=0.5	>=32	0.19	0.047	>=32	<=0.5	<=4
25KC017-2	*Escherichia coli*	>=256	6	>=32	1	>=32	<=0.5	12	0.19	0.032	>=32	<=0.5	<=4
25KC019-2	*Escherichia coli*	>=256	8	>=32	2	16	<=0.5	>=32	0.19	0.047	>=32	1	<=4
25KC055-1	*Escherichia coli*	>=256	3	>=32	<=0.5	8	<=0.5	>=32	0.19	0.012	16	16	<=4
25KC057-1	*Escherichia coli*	>=256	6	>=32	1	>=32	4	>=32	0.19	0.023	>=32	1	<=4
25KC057-2	*Escherichia coli*	>=256	4	>=32	2	>=32	16	>=32	0.125	0.012	>=32	>=32	<=4
25KC065-1	*Enterobacter aerogenes*	>=256	32	>=32	>=32	>=32	8	>=32	0.25	0.047	<=0.5	<=0.5	<=4
25KC071-1	*Klebsiella oxytoca*	>=256	4	>=32	2	>=32	8	>=32	0.125	0.016	>=32	<=0.5	<=4
25KC071-2	*Klebsiella oxytoca*	>=256	8	>=32	2	>=32	4	>=32	0.25	0.032	8	<=0.5	<=4
26ST003-1	*Escherichia coli*	>=256	4	>=32	1	16	<=0.5	12	0.5	0.032	8	<=0.5	<=4
26ST010-1	*Escherichia coli*	>=256	4	>=32	1	>=32	>=32	>=32	0.094	0.032	<=0.5	<=0.5	<=4
26ST013-1	*Escherichia coli*	>=256	6	>=32	1	2	2	1.5	0.19	0.023	4	1	<=4

* Examined by Etest

Table 2. Resistant rate and MIC parameter

	Resistant rate (%)	MIC Range		MIC50	MIC90
		Min.	Max.		
Ampicillin	100	>=256	>=256	>=256	>=256
Amoxicillin/ Clavulanate	36	3	>=256	4	64
Cefazolin	100	>=32	>=32	>=32	>=32
Cefmetazole	28	<=0.5	>=32	2	>=32
Cefotaxime	96	2	>=32	>=32	>=32
Ceftazidime	32	<=0.5	>=32	4	>=32
Ceftriaxone	100	1.5	>=32	>=32	>=32
Imipenem	0	0.094	0.75	0.19	0.5
Meropenem	0	0.012	0.23	0.032	0.125
Ciprofloxacin	44	<=0.5	>=32	<=0.5	>=32
Gentamycin	12	<=0.5	>=32	<=0.5	16
Amikacin	0	<=4	8	<=4	<=4

and Protein Purification Kit (Macherey- Nagel, Germany). After PCR purification, all of them were subjected to direct sequencing by the dye-terminator method by using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Science, USA) with the ABI Prism 3100 Genetic Analyzer (ThermoFisher Science, USA).

3. Results

3.1 Bacterial Isolates and Antimicrobial Susceptibility Profile

Cefotaxime resistant *Enterobacteriaceae* was isolated from 22 samples (15.2 %) and gathered 25 strains of the 16 isolates of *Escherichia coli*, 3 isolates of *Enterobacter cloacae*, 2 isolates of *Citrobacter braakii*, 2 isolates of *Klebsiella oxytoca*, 1 isolate of *Enterobacter aerogenes*, and 1 isolate of *Hafnia alvei*. These antibiotic susceptibility are listed in ***Table 1***. The resistant rate and MIC parameter is shown in ***Table 2***. Amoxicillin/clavulanate, ceftriaxone and ciprofloxacin were less active with resistance rates of 36, 100, and 44 %. Ceftazidime, cefmetazole, and gentamycin had moderate activity, and their resistance rates were 32, 28, and 12 %, respectively. All the strains were susceptible to imipenem, meropenem, and amikacin. As shawn in Table 3, moreover 18 strains (16 *E. coli* and 2 *K. oxytoca*) were positive of ESBL screening DDST.

3.2 Molecular Characteristic of Antimicrobial Resistance *Enterobacteriaceae*

All of the ESBL-producing *E. coli* and *K. oytoca* were examined by PCR for the determination of $bla_{CTX\text{-}M}$ genes. Five *E. coli* and one *K. oxytoca* carried bla_{TEM} combined with $bla_{CTX\text{-}M}$. One isolate for which the cefotaxime MIC value was 2 mg/mL harbored bla_{SHV}. Additionally, *ACCM* was positive in *H. alvei*. None of the other types of *AmpC* were detected (***Table 3***). Among 18 CTX-M-positive isolates, 4 isolates carried CTX-M-1 group ESBL genes, and 13 isolates carried CTX-M-2 group ESBL genes. Isolated *E. coli* of phylogenetic subgroup D was predominant, followed by subgroup B2, A, and B1 (***Table 4***). The *qnrA* and *B* of fluoroquinolone binding protein was not detected in any strain (data not shown).

Table 3. Characteristic of β-lactamase

Species	ESBL DDST	ESBL genes			plasmid mediated *AmpC* gene
		CTX-M	*TEM*	*SHV*	
Escherichia coli (16)	16	15	5	1	0
Enterobacter cloacae (3)	0	0	0	0	0
Klebsiella oxytoca (2)	2	2	1	0	0
Citrobacter braakii (2)	0	0	0	0	0
Hafnia alvei (1)	0	0	0	0	*ACCM* (1)
Enterobacter aerogenes (1)	0	0	0	0	0

Table 4. Relationship between CTX-M types and phylogenetic group of *E. coli*

Phylogen. group	Group of CTX-M types			Total
	CTX-M-1	CTX-M-2	Other	
A	0	1	0	1
B1	0	1	0	1
B2	1	2	0	3
D	3	7	1	11

4. Discussion

In this study, prevalent rates of the third generation cephalosporins resistance *Enterobacteriaceae* in healthy college students were investigated. The prevalent rate was 15.9 %, much higher than that in the previous study reported by Luvsansharav UO in Japan [16]. One of the reasons may be that the fecal samples were collected from the students studying medical and nursing techniques in a college. Among the 25 isolated strains, 16 strains (64.0 %) were *E. coli* producing ESBLs. ESBL is produced by not only *E. coli* by but also *K. oxytoca*, which was susceptible to cefmetazole, ceftazidime, and carbapenems. Additionally, SH Choi reported that cefepime was susceptible to ESBL producing strain and could still treat their infections [17]. Carbapenem is the most commonly used antimicrobial agent, but it contributes to the initial selection of multi-drug resistant organisms.

Ceftazidime resistant isolates were only *Enterobacter* sp., *Citrobacter* sp., and *Hafnia* sp., which were characterized by chromosomally, encoded *AmpC* β-lactamases. In these species, upregulation of chromosomal *AmpC* develop resistance to ceftazidime, cefmetazole, and other third and forth generation cepharosporins. Pattarachai K. found that 17 % of non-*Escherichia* and non-*Klebsiella Enterobacteriaceae* had harboring p*AmpC* genes and that almost all isolates had a coexistence of ESBL genes in tertiary care university hospital patients in Thailand [18]. This study suggested that p*AmpC* harboring *Enterobacteriaceae* did not emerge in healthy adults yet. On the other hand, Amber class C (ACC) β- lactamase which *H. alvei* has on its chromosome was detected by *AmpC* multiplex PCR. David N reported that the transfer of the plasmid by ACC β-lactamase gene of *H. alvei* and the receipt of plasmids by *K. pneumoniae* occurred during the outbreak in their hospital [19]. Therefore, the colonization

Table 5. Fluoroquinorone resistant stains

Strain No.	Identification	Phylogen. group	β-lactamase	QRDR mutations of amino acid	
				gyrA	parC
25KC016-2	*Escherichia coli*	D	*CTM-M2* *TEM*	S83L D87N	S80I E84G
25KC017-1	*Escherichia coli*	D	*CTM-M2*	S83L D87N	
25KC017-2	*Escherichia coli*	D	*CTM-M1*	ND	
25KC019-2	*Escherichia coli*	D	*CTM-M2*	S83L D87N	S80I
25KC055-1	*Escherichia coli*	A	*CTM-M2* *TEM*	S83L D87N	
25KC057-1	*Escherichia coli*	B2	*CTM-M2*	S83L D87N	S80I E84V
25KC057-2	*Escherichia coli*	D	*CTM-M1*	S83L D87N	
25KC071-1	*Klebsiella oxytoca*	–	*CTM-M2*	S83L D87N	S80I E84V
25KC071-2	*Klebsiella oxytoca*	–	*CTM-M2* *TEM*	S83L D87N	S80I
26ST003-1	*Escherichia coli*	D	*CTM-M1* *TEM*	NT	
26ST013-1	*Escherichia coli*	D	*SHV*	NT	

of *AmpC* overproducing strains is a risk factor for spreading plasmid to other species when the host initial flora is affected by antimicrobial selection pressure.

The most prevalent ESBL type is the CTX-M-2 group at cefotaxime resistant strains in healthy adults. The CTX-M types are categorized into four groups; CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9 groups. The CTX-M-2 group ESBLs are dominant in hospital patients in Japan [20]. In a current study, CTX-M-15 type, which belongs to CTX-M-1 group of ESBL producing *E. coli* B2-O25:H4-ST131, developed a resistance to another ceftazidime and was found to be emerging in European countries [8]. Furthermore, a current study suggested that the CTX-M-15 harboring *E. coli* B2-O25:H4-ST131 strain emerge in Japan [7] and that the strain could have multiple reservoirs from which they spread to different $bla_{CTX\text{-}M}$ types [21]. Nevertheless, this potential strain was not isolated in this study because of the susceptibility of CTX-M type producing strains to ceftazidime. These strains are resistance to fluoroquinolones, aminoglycosides, and other antibiotics. JANIS, a medical surveillance system in Japan, reported a drastic increase of fluoroquinolone resistance *E. coli* as the result of isolation at medical facilities. However, this study suggests that fluoroquinolone resistant *Enterobaceriaceae* is found in ESBL producing strains in healthy adults, and this finding may be due to previous abuse of antibiotics. The high rates of resistance to fluoroquinolone is probably caused by chromosome-mediated resistance such as point mutations in the gyrase and topoisomerase IV genes (***Table 5***). Quinolones are frequently used to treat infections among outpatients in many countries including Japan. But there is concern that these drugs will not be effective and will apply selection pressure to these isolates. Continuous surveillance of antibiotic resistance is essential to detect the emergence and spread of broad-spectrum β-lactamase producing strains in *Enterobacteriaceae*.

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