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Characterization of CTX-M-Type Extended-Spectrum Beta-Lactamase-Producing Diarrheagenic *Escherichia coli* Isolates in the Republic of Korea During 2008–2011

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To characterize the extended-spectrum beta-lactamases (ESBLs) in diarrheagenic *Escherichia coli* from Korea in 2008–2011, we screened seven enterotoxigenic *E. coli* (ETEC) and one enteroaggregative *E. coli* (EAEC) that produce ESBLs from a nationwide survey. All eight isolates produced CTX-M-type ESBLs, including CTX-M-12 (n = 4), CTX-M-14 (n = 2), and CTX-M-15 (n = 2). PCR-based replicon typing indicated that the $bla_{CTX\text{-}M\text{-}12}$ genes of four ETEC isolates were carried on a conjugative IncF plasmid, whereas the $bla_{CTX\text{-}M\text{-}14}$ of one EAEC was located on an IncK plasmid. This is the first report of the occurrence of $bla_{CTX\text{-}M}$ genes in clinical isolates of EAEC in Korea. The ESBL-producing isolates were shown to be different based on pulsed-field gel electrophoresis and multilocus sequence typing, whereas the four isolates with CTX-M-12 were clonally related. These observations raise an alarm for the spread of plasmid-mediated resistance to ESBL among diarrheagenic *E. coli*.

Keywords: ESBL, CTX-M, IncF, ETEC, EAEC

Introduction

Most *Escherichia coli* strains are commensal microbiota in the mammalian gastrointestinal gut, yet some strains can cause severe diarrheal illnesses in humans [12]. These *E. coli* can be classified into six major types based on the presence of specific virulence traits: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC). ETEC is a major etiologic pathogen of diarrhea in children from developing countries and in travelers from developed countries to the developing world [20]. It has been observed that ETEC infections usually occur as a result of the ingestion of contaminated food or water [20]. EAEC has been implicated as a causal agent of diarrheal disease in both developing and developed countries [17] and has been associated with acute and persistent diarrhea in children, adults, and HIV/AIDS patients [9].

Third-generation cephalosporins and fluoroquinolones are generally used for the treatment of enteric pathogens. However, the development of resistance to extended-spectrum beta-lactamases (ESBLs) in *Enterobacteriaceae* is becoming a great concern in developing countries. During the last decade, CTX-M beta-lactamases have spread rapidly among diarrheagenic *E. coli* strains, and the dominant types of CTX-M have been distributed worldwide [4]. In Korea, the prevalence of CTX-M-producing bacteria has increased since it was first reported in retrospective studies [15, 19]. The CTX-M-14 and CTX-M-15 beta-lactamases have been the dominant types in various enteric bacteria [16, 22, 24], and a CTX-M-12 beta-lactamase was also recently reported [2]. However, EAEC strains have not previously been considered as ESBL-producers. Moreover, few data are available on the prevalence of ESBLs in ETEC or EAEC strains from patients with diarrhea. Here, we characterized diarrheagenic *E. coli* isolates with ESBL production in Korea.

Materials and Methods

Bacterial Strains

From 2008 to 2011, the National Public Health Network collected consecutive, non-duplicated *E. coli* strains that were isolated from patients with a clinical history of diarrhea. Bacteria were plated or incubated on MacConkey agar or Mueller-Hinton agar containing cefotaxime (10 μg/ml) when required. All isolates were identified by VITEK-2 system (bioMérieux, France) and serotyped by slide agglutination with O antigen-specific antisera [18]. Pathotypes of *E. coli* strains were determined by PCR analysis with specific primers for the following virulence genes: enterotoxins LT and STh for ETEC, and pCVD432 plasmid for EAEC [21]. The single colonies positive for pCVD432 were examined for *aggR*, *aggA*, *aafA*, *aap*, and *astA* genes as described previously [10]. The 111 ETEC strains from 2008 to 2010 and 141 EAEC strains from 2010 to 2011 were selected for this study and further characterized.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of different antibiotics were analyzed using VITEK-2 AST-N160 cards (bioMérieux), and were confirmed by a broth microdilution method using Sensititre ESB1F plates (Trek Diagnostic Systems, USA). All susceptibility results were interpreted using the breakpoints of the Clinical and Laboratory Standards Institute (CLSI) guidelines [6]. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control organisms in antimicrobial susceptibility experiments. Of these isolates, we selected seven ETEC and one EAEC with high-level resistance to third-generation cephalosporins.

Genetic Characterization of *bla* Genes

All isolates with an ESBL phenotype were tested by multiplex PCR for *bla* genes of the TEM, SHV, CMY, OXA, DHA, and CTX-M types, and the $bla_{CTX\text{-}M}$ genes were characterized by sequencing analysis of the amplicons [14]. For strains with a positive result for $bla_{CTX\text{-}M}$, the physical linkages between an IS*Ecp1*-like element and the $bla_{CTX\text{-}M\text{-}12}$ or $bla_{CTX\text{-}M\text{-}14}$ genes were determined by PCR and sequencing analysis [1].

Transfer of the *bla* Genes

The transmission capacity of the *bla* genes in ETEC or EAEC isolates was examined by conjugation experiments using azide-resistant *E. coli* J53 as the recipient strain. Transconjugants were selected on MacConkey agar plates (Difco, USA) supplemented with cefotaxime (1 μg/ml) and sodium azide (100 μg/ml). The acquisition of the *bla* gene was confirmed by PCR and sequencing analysis as described above.

PCR-Based Replicon Typing of Plasmids

Replicon typing of plasmids carrying the $bla_{CTX\text{-}M}$ gene was performed using a previously described PCR-based method that identifies 18 major plasmid types in *Enterobacteriaceae* [5].

Pulsed-Field Gel Electrophoresis

Genotyping of the ESBL-positive isolates was performed by pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol (http://www.pulsenetinternational.org/protocols/). Agarose-embedded genomic DNA was digested with *Xba*I and separated by PFGE using a CHEF-Mapper system (Bio-Rad Laboratories,

Table 1. Antimicrobial susceptibility of ESBL-producing *E. coli* isolates.

Antimicrobial agent	Breakpoints (resistant)	ETEC (n = 111)		EAEC (n = 141)	
		n[a]	%[b]	n	%
Ampicillin	≥32	32	28.8	99	70.2
Amoxicillin/Clavulanic acid	≥32	3	2.7	8	5.7
Ampicillin/Sulbactam	≥32	15	13.5	45	31.9
Cephalothin	≥32	16	14.4	52	36.9
Ceftriaxone	≥4	8	7.2	1	0.7
Cefotaxime	≥4	8	7.2	1	0.7
Cefoxitin	≥32	2	1.8	8	5.7
Imipenem	≥4	0	0	0	0
Amikacin	≥64	0	0	0	0
Gentamicin	≥16	1	0.9	48	34.0
Nalidixic acid	≥32	40	36	86	61.0
Ciprofloxacin	≥4	2	1.8	5	3.5
Trimethoprim/Sulfamethoxazole	≥4	19	17.1	75	53.2
Chloramphenicol	≥32	2	1.8	24	17.0
Tetracycline	≥16	17	15.3	76	53.9

[a]Number of resistant isolates.

[b]Approximate percentage of resistant isolates.

USA) with initial and final switch times of 2.16 to 54.17 sec at 6 V/cm for 18 h at 14°C. Genomic DNA of *Salmonella enterica* serotype Braenderup H9812 (ATCC BAA-664) restricted with *Xba*I was used as a size standard. The PFGE patterns were analyzed with BioNumerics software ver. 5.1 (Applied Maths, Belgium) using the Dice similarity coefficient with a 1.5% position tolerance.

Multilocus Sequence Typing

For seven ESBL-producing ETEC strains, multilocus sequence typing (MLST) was performed using seven conserved housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*) [11]. The internal fragments of all loci were sequenced, and the corresponding sequence types of the isolates were designated in accordance with the MLST database for pathogenic *E. coli* (http://www.shigatox.net/ecmlst). The novel *fadD* allele (fadD131) identified in this study was released under GenBank Accession No. KJ190942.

Results and Discussion

Description of Diarrheagenic *E. coli* Isolates

Among *E. coli* isolates collected during 2008−2011, a total of 111 isolates positive for the presence of a heat-labile toxin (LT), heat-stable toxin (ST), or both genes were considered as ETEC. One hundred and forty-one isolates positive for the pCVD432 plasmid were identified as EAEC. On observing the susceptibility testing of these isolates, ETEC and EAEC showed 7.2% (8/111) and 0.7% (1/141) antimicrobial resistance to ceftriaxone and cefotaxime, respectively (Table 1). Based on screening by CLSI ESBL phenotypic confirmatory tests, seven ETEC and one EAEC isolates were phenotypically confirmed to be ESBL producers. They showed 8-fold increase in the MICs for either ceftazidime or cefotaxime tested in combination with clavulanic acid compared with the MIC obtained when tested alone (data not shown). Among the seven ETEC isolates, 2 and 3 strains were isolated in 2008 and 2009, respectively, from Gyeonggi province. Two strains, ET2010003 and ET2010011 were isolated from travelers returning to Korea from China and India, respectively, and were classified as imported cases. One ESBL-producing EAEC strain (EA2011016), the first detected in Korea, was isolated from Gyeonggi province in 2011 (Table 2).

Molecular Characterization of ESBL Genes

Multiplex PCR and sequencing analysis showed that all

Table 2. Strain typing, antibiotic susceptibility, ESBL types, and plasmid replicon types in ESBL-positive *E. coli* isolates.

Strain	Origin[a]	Toxin type[b]	ST toxin type[b]	O serotype	MLST	MIC (μg/ml)[c]				ESBL type	Plasmid replicons
						AMP	FOT	AXO	SXT		
ETEC											
ET2008002	Gyeonggi	ST	STh	O20	980	1,024	64	64	320	CTX-M-12	F, K, Y
ET2008005	Gyeonggi	ST	STh	O20	979	1,024	64	64	2	CTX-M-12	F, K, Y
ET2009003	Gyeonggi	ST	STh	O20	981	1,024	32	32	2	CTX-M-12	F, K, Y
ET2009004	Gyeonggi	ST	STh	O20	86	1,024	64	64	320	CTX-M-12	F, K, Y
ET2009011	Gyeonggi	ST	STh	O51	618	1,024	64	64	2	CTX-M-15	N
ET2010003	Imported (China)	ST	STh	O153	134	1,024	64	64	2	CTX-M-14	F
ET2010011	Imported (India)	LT, ST	STh	O6	273	1,024	32	32	320	CTX-M-15	I1
EAEC											
EA2011016	Gyeonggi	*astA*	STh	O175	-	1,024	16	64	2	CTX-M-14	F, K
E. coli J53											
ET2008005tc	-	-	-	-	-	1,024	32	64	2	CTX-M-12	F
ET2009004tc	-	-	-	-	-	1,024	32	64	2	CTX-M-12	F
ET2010003tc	-	-	-	-	-	1,024	32	64	2	CTX-M-14	UT[d]
EA2011016tc	-	-	-	-	-	1,024	32	64	2	CTX-M-14	K

[a]Imported, the isolate was recovered from a traveler returning from China or India.

[b]ST, heat-stable toxin; LT, heat-labile toxin; STh, ST toxin from human origin.

[c]AMP, ampicillin; FOT, cefotaxime; AXO, ceftriaxone; SXT, sulfamethoxazole/trimethoprim.

[d]UT, untypeable.

the ESBL types belonged to the CTX-M family: four of eight ESBL-positive isolates carried a $bla_{CTX\text{-}M\text{-}12}$ gene, two harbored a $bla_{CTX\text{-}M\text{-}14}$ gene, and two had a $bla_{CTX\text{-}M\text{-}15}$ gene (Table 2). CTX-M-12 ESBL differs from CTX-M-3 by three amino acid substitutions and has a high level of hydrolytic activity against cefotaxime compared with ceftazidime [13]. Since CTX-M-12 was first reported in *K. pneumoniae* isolates from Kenya in 2001 [13], and subsequently in Colombia in 2002 [26], it has been detected in clinical isolates from *E. coli*, *K. pneumoniae*, and *Proteus mirabilis* in Korea [2, 3, 23, 24]. All of the isolates in this present study concomitantly produced TEM-1 beta-lactamase. PCR-based screenings for SHV-, DHA-, CMY-2-, and OXA-type ESBL genes were negative in all strains.

Transferability and Replicon Types of Plasmids Encoding the $bla_{CTX\text{-}M}$ Genes

Despite repeated attempts using different methods, the $bla_{CTX\text{-}M}$-carrying plasmids were only successfully transferred to the recipient *E. coli* strain J53 Azi^R by conjugation for four (three ETEC and one EAEC) of the eight isolates (Table 2). Plasmid replicon typing analysis found that all four ETEC isolates with $bla_{CTX\text{-}M\text{-}12}$ (ET2008002, ET2008005, ET2009003, and ET2009004) contained the same replicons: F, K, and Y (Table 2). However, the F replicon was only identified in two transconjugant strains, ET2008005-tc and ET2009004-tc, indicating that the $bla_{CTX\text{-}M\text{-}12}$ genes were carried on a transferrable IncF plasmid. The CTX-M-15-producing ETEC isolates ET2009001 and ET2010001 carried two replicons of F in combination with N or I1, respectively. The F replicon was also found in the ET2010003 isolate, whereas the plasmid replicon of ET2010003-tc was not typeable in this study. In one EAEC isolate (EA2011016), a $bla_{CTX\text{-}M\text{-}14}$ gene was located in an IncK plasmid (Table 2).

The mobile insertion sequence IS*Ecp1* element was located 48 bp upstream of the $bla_{CTX\text{-}M\text{-}12}$ in all four ETEC isolates and showed 100% nucleotide identity with plasmid pSME0403 (GenBank Accession No. DQ658220) that was previously reported in Korea [2].

The $bla_{CTX\text{-}M\text{-}14}$ gene in EA2011016 was carried on the IncK plasmid that was successfully transferred by conjugation. In Spain, the spread of $bla_{CTX\text{-}M\text{-}14}$ is associated with a specific IncK plasmid, including plasmid pCT, which was isolated from calves with diarrhea [7], and the complete nucleotide sequence of the plasmid was recently determined (GenBank Accession No. FN868832) [25]. In the IncK plasmid of EA2011016, an intergenic region of 42 bp was identified between the IS*Ecp1* element and $bla_{CTX\text{-}M\text{-}14}$, which is identical to the corresponding region of the IncK plasmid pCT. However, an IS903 mobile element was not detected downstream of the $bla_{CTX\text{-}M\text{-}14}$ gene by PCR, despite the use of a several different primer sets. Further studies are needed to determine the similarity between the IncK plasmid in EA2011016 and pCT and to elucidate the worldwide dissemination of ESBL-producing IncK plasmids [8].

MLST and PFGE Patterns of *E. coli* Isolates

The genetic relatedness of the ESBL-producing ETEC isolates was investigated by PFGE. Among the seven ESBL-producing ETEC isolates, we identified five different PFGE

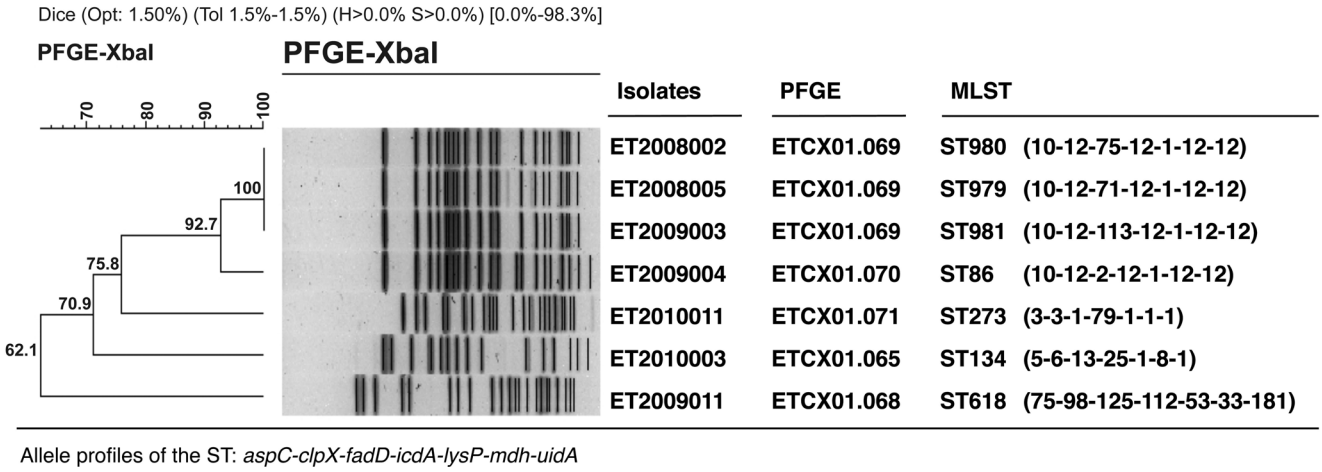

Fig. 1. *Xba*I PFGE dendrogram with the corresponding MLST sequence types of the ESBL-producing ETEC isolates.
Based on the UPGMA algorithm, the dendrogram revealed five different PFGE patterns. Four CTX-M-12-producing isolates with >90% similarity were considered as genetically related.

patterns (Fig. 1). Three CTX-M-12-producing isolates (ET2008002, ET2008005, and ET2009003) showed the same ETCX01.069 pattern; this pattern showed a genetic similarity of 92.7% with the ETCX01.070 PFGE pattern of one isolate (ET2009004) that also carried $bla_{CTX\text{-}M\text{-}12}$. The remaining three isolates each displayed a different PFGE pattern (ETCX01.065, ETCX01.068, and ETCX01.071). The MLST study of the genotypic diversity of the ETEC isolates identified seven unique sequence types (STs). Three STs (ST979, ST980, and ST981) of the CTX-M-12-producing isolates were newly described and subsequently registered in the EcMLST database. These new STs represent new combinations of previously described alleles ST86 found in ET2009004 (10, 12, X, 12, 1, 12, and 12; where X is *fadD*). Although three isolates with the ETCX01.069 PFGE pattern had multiple STs, these STs, except for one, had identical allele numbers at each locus, indicating that these isolates may be genetically related strains (*i.e.*, the same ST complex; ST86 complex). However, *E. coli* strains within the ST86 complex were not previously described as CTX-M ESBL-producing strains.

In this study, we screened and characterized ESBL-producing diarrheagenic *E. coli* strains isolated in 2008-2011 in the Republic of Korea. Our study showed that the IncF plasmid carrying the $bla_{CTX\text{-}M\text{-}12}$ gene is responsible for the resistance to cephalosporin antibiotics in ETEC isolates, suggesting that the IncF plasmid might have contributed to the dissemination of CTX-M-12 in Korea, especially in Gyeonggi province during 2008 and 2009. In addition, we reported, to our knowledge, the first occurrence of $bla_{CTX\text{-}M\text{-}14}$ in a diarrheagenic EAEC isolate from humans in Korea.

CTX-M has been the predominant ESBL type among *Enterobacteriaceae*, and the $bla_{CTX\text{-}M}$ genes in conjunction with mobile element IS*Ecp1* can be readily disseminated to other bacteria. We found a low incidence of ESBL production (less than 3.2%) in the collected diarrheagenic *E. coli* isolates. However, the progressive increase in CTX-M-producing diarrheagenic *E. coli* could become a risk factor for the spread of antimicrobial resistance to other pathogenic bacteria, which may narrow the choice of effective antibiotics and lead to clinical treatment failure. Therefore, additional active surveillance and effective infection control measures are needed to minimize the spread of cefotaxime resistance among diarrheagenic *E. coli*.

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