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Extended-Spectrum β-Lactamase-Producing Shiga Toxin Gene (stx_1)-Positive *Escherichia coli* O26:H11: a New Concern

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***Escherichia coli* strain TUM2139 was isolated from a stool sample from a 9-year-old girl on 16 June 2004. This strain was categorized as Shiga toxin-producing *Escherichia coli* (STEC) because the Shiga-like toxin gene stx_1 was detected by immunochromatography and PCR assay. The strain was highly resistant to cefotaxime (256 μg/ml) and was also resistant to cefepime, cefpodoxime, ceftriaxone, and aztreonam. In the presence of 4 μg of clavulanic acid per ml, the MIC of cefotaxime decreased to ≤0.12 μg/ml, indicating that this strain was an extended-spectrum β-lactamase (ESBL) producer. Cefotaxime resistance was transferred to *E. coli* C600 by conjugation at a frequency of 3.0×10^{-6}. A PCR assay was performed with primer sets specific for TEM-type and SHV-type ESBLs and for the CTX-M-2 (Toho-1), CTX-M-3, and CTX-M-9 groups of ESBLs. A specific signal was observed with the primer set specific for the CTX-M-9 group of β-lactamases. This β-lactamase was confirmed to be the ESBL CTX-M-18 by DNA sequencing. This is the first report of an ESBL-producing STEC isolate.**

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an important cause of waterborne and food-borne illnesses. STEC is ingested most commonly with undercooked ground beef (22). Human infection with STEC is potentially fatal and may be associated with serious complications such as hemolytic-uremic syndrome (HUS) and hemorrhagic colitis (21). The production of Stx is the unifying feature of all STEC strains. Various types of Stxs are produced, but they fall into two main types: Stx1 and Stx2 (4, 5, 6, 8). The clinical significance of four serological or biological variants of Stx2 (Stx2c, Stx2d, Stx2e, and Stx2f) is unknown (17). The majority of Stx genes are encoded by bacteriophages. The most severe or important pathogen among STEC strains is *E. coli* O157:H7. The cases of HUS in approximately 90% of children in the developed part of the world are associated with infections caused by Stx-producing bacteria; among these, 70% are caused by *E. coli* O157:H7 (21). Epidemiologic data suggest that isolates that produce Stx2 alone are more likely to cause severe disease than those that produce only Stx1 or a combination of Stx1 and Stx2 (20).

Recently, Schroeder et al. (23) reported on the antimicrobial resistance of 752 STEC strains from animals and humans. They found that 50% of *E. coli* O26, O103, O111, O128, and O145 strains from humans were resistant to ampicillin, cephalothin, tetracycline, streptomycin, or sulfamethoxazole but that no strain was resistant to expanded-spectrum β-lactams, including cefotaxime, cefpodoxime, or aztreonam. A recent study suggested that antibiotic therapy for the early stage of STEC infection is able to prevent progression of the disease to HUS (24). However, antimicrobial therapy for STEC infection is still regarded as controversial, because antibiotics induce increased levels of Stx production in vivo (18). On the other hand, many patients with diarrhea receive empirical antibiotic therapy (21).

E. coli TUM2139 was isolated from a clinical stool specimen at Toho University Ohashi Hospital. This strain was resistant to cefotaxime. On the other hand, the O-antigen type of this strain was determined to be O26. In this study, the resistance of *E. coli* TUM2139 to β-lactam antibiotics and the presence of virulence factors in this strain were confirmed by PCR assay and the direct sequencing technique.

MATERIALS AND METHODS

Bacterial strain, media, and culture. On 16 June 2004, *E. coli* TUM2139 was isolated from a stool sample from a 9-year-old female patient at Toho University Ohashi Hospital. She had diarrhea, vomiting, and a low-grade fever. The strain was identified with the Phoenix system (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan). *E. coli* C600 (F^- *lacY1 leuB6 supE44 thi-1 thr-1 tonA21* nalidixic acid resistant) was used as the recipient strain for the conjugation experiments. The bacterial strains were stored at −70°C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol. Subsequently, the bacterial strains were inoculated onto Mueller-Hinton agar plates and incubated overnight at 35°C.

Serotyping. *E. coli* TUM2139 was screened for Vero toxin (Stx) with a Capillia O157 immunochromatography assay kit for Vero toxin (Nippon Becton Dickinson Company, Ltd.). The O- and H-antigen types of the strain were determined with neutralizing antisera. A total of 181 types of antisera against the O antigen (O1 to O181) and also 56 different kinds of antisera against the H antigen were used. These antisera were made by the National Institute of Infectious Diseases, Tokyo, Japan, which is the typing center for *E. coli* in Japan.

PCR assay for stx_1, stx_2, *eaeA*, and *hlyA*. The use of multiplex PCR for the detection of stx_1, stx_2, *eaeA*, and enterohemorrhagic *E. coli hlyA* was reported by Fagan et al. (18). DNA amplification was performed by a PCR method directly with the colonies (26). A small amount of the test organism was picked up with a toothpick and transferred directly to 50 μl of the PCR mixture. The primers and the predicted sizes of the PCR amplicons are listed in Table 1. PCR assays were performed with EX*taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) and a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, Conn.). The PCR

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TABLE 1. Primer sequences and predicted lengths of PCR amplification products

Primer specificity	Direction	Primer sequence (5′ to 3′)	Fragment size (bp)	Reference or source
Virulence factors[a]				
stx_1	Sense	ACGATGTGGTTTATTCTGGA	165	8
	Antisense	CTTCACGTGACCATACATAT		
stx_2	Sense	ACACTGGATGATCTCAGTGG	614	10
	Antisense	CTGAATCCCCCTCCATTATG		
EHEC *hlyA*	Sense	CCATGACAACGGACAGCAGTT	779	10
	Antisense	CCTGTCAACTGAGCAGCACTTTG		
eaeA	Sense	GTGGCGAATACTGGCGAGACT	890	9
	Antisense	CCCCATCTTTTTCACCGTCG		
β-Lactamases[a]				
CTX-M-2	Sense	GCGAACAGCGTGCAACAGCAGCTGG	521	This study
	Antisense	GCCAGCGCTTTACCCAGCGTCAG		
CTX-M-3	Sense	GAGCATATGGTTAAAAAATCACTGCGTCAGTTC	891	This study
	Antisense	CAGGGATCCTTACAAACCGTCGGTGACGATTTTAGCC		
CTX-M-9	Sense	GTTTGAGCATATGGTGACAAAGAGAGGCAACGG	895	11
	Antisense	CAGGGATCCTTACAGCCCTTCGGCGATG		
TEM	Sense	GGGGAGCTCATAAAATTCTTGAAGAC	1,199	23
	Antisense	GGGGGATCCTTACCAATGCTTAATCA		
SHV	Sense	GTTCATATGCGTTATATTCGCCTGTG	876	This study
	Antisense	ATAGGATCCTTAGCGTTGCCAGTGCT		
CTX-M-18[b]				11
	Sense	AGAGAGTGCAACGGATGATGTT		
	Sense	GTTGCAGTACAGCGACAATACC		
	Sense	GCTGGTTCTGGTGACCTATTTTAC		
	Antisense	GCCATAACTTTACTGGTACTGCAC		
	Antisense	CTGGGTAAAATAGGTCACCAGAAC		

[a] Primers for PCR.
[b] Primers for sequencing. DNA template amplified by primers for CTX-M-9 was used for sequencing.

conditions were as follows: incubation 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s, with a final cycle of 72°C for 7 min. The amplified DNA fragments of the target genes were identified by electrophoresis on a 2% agarose gel. After electrophoresis, the gels were stained with 0.5 mg of ethidium bromide per ml and visualized with UV illumination.

Antimicrobial susceptibility testing. MICs were determined by a broth microdilution method with cation-adjusted Mueller-Hinton broth. The dilution ranges of the antibiotic agents were 0.25 to 512 μg/ml. Quality control was done by using *E. coli* ATCC 25922 as a reference strain for antibiotic susceptibility. All procedures were done and the results were interpreted as described by the National Committee for Clinical Laboratory Standards (15, 16). The organisms were inoculated at about 5×10^5 cells per well by using a MIC2000 inoculation device (Dynatech, McLean, Va.). The MIC was defined as the lowest concentration that prevented visible growth after incubation for 18 h at 35°C.

Antibiotics. The following agents, all with known potencies, were used in this study: piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan); cephalothin (Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan); cefoxitin, imipenem, and ciprofloxacin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan); ceftazidime and clavulanic acid (Glaxo Smith Kline Ltd., Tokyo, Japan); cefotaxime (Aventis Japan Ltd., Tokyo, Japan); aztreonam (Eisai Co., Ltd., Tokyo, Japan); tazobactam (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan); cefepime (Bristol Pharmaceutical Co., Ltd., Tokyo, Japan); cefotetan (Yamanouchi Pharmaceuticals Co. Ltd., Japan); cefmetazole and cefpodoxime (Sankyo Co., Ltd., Tokyo, Japan); ceftriaxone (Roche Japan K.K., Tokyo, Japan); faropenem (Suntory Ltd., Tokyo, Japan); gentamicin (Nihon Shering K.K. Osaka, Japan); kanamycin (Meiji Seika Ltd., Tokyo, Japan); minocycline (Wyeth K.K. Tokyo, Japan); and nalidixic acid (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan).

Conjugation experiment. Conjugation experiments were performed by the broth method (11). A nalidixic acid-resistant marker was added to recipient cells (*E. coli* C600). Then, the recipient cells and the donor cells (*E. coli* TUM2139) were mixed in a ratio of 1:9. The same volume of fresh LB broth was added to the cell mixture and the mixture was incubated for 90 min at 35°C. After 90 min, the cells were plated onto LB agar plates containing 5 μg of cefotaxime per ml and 25 μg of nalidixic acid per ml and incubated at 35°C overnight. Donor cells were also plated on LB agar in the absence of antibiotics. After incubation, the colonies were counted and the frequency of conjugation of a plasmid was calculated.

PCR assay for ESBLs and ESBL DNA sequence analysis. The total DNA template was obtained and the amplification products were identified by the same procedure used for the PCR assay of the bla_{TEM} type, bla_{SHV} type, bla_{Toho-1} group, $bla_{CTX-M-3}$ group, and $bla_{CTX-M-9}$ group of extended-spectrum β-lactamases (ESBLs). The primers used for PCR, the predicted sizes of the PCR amplicons, and the primers used for sequencing are listed in Table 1. PCR conditions were as follows: incubation at 94°C for 3 min and 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min), with a final extension at 72°C for 7 min.

Both strands of the PCR products obtained were sequenced by using the same set of primers used to obtain the PCR amplification products and then by using primers synthesized by primer walking. All primers used for PCR and DNA sequencing are listed in Table 1. DNA sequencing was carried out with a BigDye (version 3.1) Terminator Cycle Sequencing kit and a model 310 DNA sequencer (Applied Bio-

TABLE 2. Antimicrobial susceptibilities of donor, recipient, and conjugant strains

Antibiotic	MIC (μg/ml)		
	E. coli TUM2319	Recipient	Conjugant
Piperacillin	128	≤0.25	64
Piperacillin-tazobactam	2/4	≤0.25/4	1/4
Cephalothin	512	0.5	512
Cefoxitin	4	≤0.25	2
Cefmetazole	1	≤0.25	1
Cefotaxime	256	≤0.25	32
Cefotaxime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Ceftazidime	2	≤0.25	2
Ceftazidime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Cefpodoxime	256	≤0.25	128
Ceftriaxone	256	≤0.25	128
Cefotetan	≤0.25	≤0.25	≤0.25
Cefepime	8	≤0.25	4
Aztreonam	8	≤0.25	8
Faropenem	≤0.25	≤0.25	≤0.25
Imipenem	≤0.25	≤0.25	≤0.25
Gentamicin	≤0.25	≤0.25	≤0.25
Kanamycin	2	≤0.25	≤0.25
Minocyclin	≤0.25	≤0.25	≤0.25
Nalidixic acid	4	64	64
Ciprofloxacin	≤0.25	≤0.25	≤0.25

systems, Foster City, Calif.). The deduced amino acid sequences were examined by using the BLAST program at the DNA Data Bank of Japan (Shizuoka, Japan).

RESULTS

Characterization of *E. coli* TUM2319. *E. coli* TUM2319 was confirmed to be a O26:H11 strain with antisera. The Capillia O157 immunochromatography assay kit was used to determine whether the Vero toxin (Stx) produced by this strain is Vero toxin 1 (VT1; Stx1) and/or VT2 (Stx2). After this screening, the strain was investigated for the presence of the stx_1 and stx_2 genes by the PCR assay directly with the bacterial colonies. Only the stx_1 gene was amplified. The stx_2 gene was not detected in this strain. The *eaeA* and enterohemorrhagic *E. coli* (EHEC) *hlyA* genes were also detected by PCR. From these results, *E. coli* TUM2319 was confirmed to be an STEC strain.

Antimicrobial susceptibility testing of *E. coli* TUM2319. The antimicrobial susceptibility testing results for *E. coli* TUM2319 are presented in Table 2. *E. coli* TUM2319 was resistant to piperacillin, cephalothin, cefotaxime, ceftriaxone, and cefpodoxime but was susceptible to all other agents tested. The MIC of cefotaxime was reduced from 256 to ≤ 0.25 μg/ml in the presence of 4 μg of clavulanic acid per ml.

Conjugation experiments. Conjugation experiments showed that conjugants grew on LB agar plates in the presence of 25 μg of nalidixic acid per ml and 5 μg of cefotaxime per ml. The frequency of conjugation was 3.0×10^{-6}. The MICs of piperacillin, cephalothin, cefotaxime, cefpodoxime, and ceftriaxone for the conjugants were significantly increased compared with those for *E. coli* C600, the recipient strain (Table 2). These results show that this resistance marker could move from a resistant strain to a sensitive strain by conjugation. *E. coli* TUM2319 and its conjugants harbored a plasmid of the same size, approximately 80 kbp (data not shown).

Type of ESBL. A class A β-lactamase gene of the CTX-M-9 group was detected by PCR in *E. coli* TUM2319; but genes for ESBLs of the TEM type, SHV type, Toho-1 (CTX-M-2) group, and CTX-M-3 group were not detected. DNA sequencing confirmed that the enzyme of the CTX-M-9 group was the CTX-M-18 ESBL. This β-lactamase gene was also detected on an 80-kbp plasmid in the conjugant. Moreover, the EHEC *hlyA* gene was detected in the conjugants.

DISCUSSION

E. coli strain TUM2319 was confirmed to be an O26:H11 strain and an stx_1-, *eaeA*-, and EHEC *hlyA*-positive but stx_2-negative STEC strain by serological tests and PCR assays. *E. coli* O157:H7 infection in humans sometimes leads to HUS; however, almost all *E. coli* O26:H11 infections are mild (5).

Antibiotic treatment for STEC infections is not recommended (18). However, chemotherapy might be initiated before the diagnosis of an STEC infection. It is most widely accepted, however, that empirical therapy with antibiotics be started for children with acute diarrhea. β-Lactam antibiotics, especially expanded-spectrum β-lactams, such as cefotaxime, ceftazidime, ceftriaxone, and cefoperazone, are the most useful clinically because they combine safety with high potency against gram-negative bacteria, such as members of the family *Enterobacteriaceae*, including *E. coli*. Accordingly, expanded-spectrum β-lactams are one of the groups of antibiotics recommended for the treatment of serious *E. coli* infections (21).

Expanded-spectrum β-lactams are very stable to class A, class C, and class D β-lactamases of the Ambler classification (2); however, ESBLs can easily hydrolyze this group of β-lactam antibiotics, such as cefpodoxime, ceftriaxone, cefotaxime, and ceftazidime (7). In Japan, CTX-M-type β-lactamase-producing *E. coli* strains, such as cefotaxime-resistant *E. coli*, are often isolated from clinical specimens, with the CTX-M-2 or CTX-M-18 β-lactamases becoming the most commonly encountered ESBLs (11, 14, 25, 28, 29). The CTX-M-18 enzyme, which was initially reported to be Toho-3 (GenBank accession number AB038771) when the sequence was electronically published in the DNA database on 23 February 2000, does not hydrolyze ceftazidime. CTX-M-19 is a derivative of CTX-M-18 that can hydrolyze ceftazidime (19). If a single amino acid residue change occurs in the CTX-M-18 enzyme, this mutant (CTX-M-19) can acquire the capability to hydrolyze ceftazidime (12, 19). Accordingly, we believe that CTX-M-18 poses a higher risk when it is encountered in clinical isolates due to this potential ability to mutate into a ceftazidime-hydrolyzing enzyme.

ESBL-producing *Salmonella* and *Shigella* strains have been reported worldwide. Some 0.8 to 3.4% of clinical isolates of *Salmonella* recovered from 1997 to 1999 expressed the ESBL phenotype (29). Recently, investigators have also reported on CTX-M-3 (3), TEM-3 (1), and SHV-2a (13) ESBL-producing *Salmonella* isolates. However, to our knowledge, no ESBL-producing STEC or EHEC isolate has been described, until now.

In conclusion, this is the first report of an ESBL-producing STEC isolate. The possibility that this isolate may be an unsuspected reservoir of CTX-M-18 capable of spreading the plasmid carrying this ESBL to other bacteria might be worth

considering. It is necessary to extend antimicrobial resistance surveillance programs for *E. coli*, including STEC, not only to the clinical field but also to the agricultural field.

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