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Original Article

Serotype, Shiga Toxin (Stx) Type, and Antimicrobial Resistance of Stx-Producing *Escherichia coli* Isolated from Humans in Shizuoka Prefecture, Japan (2003–2007)

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SUMMARY: The serotype, Shiga toxin (Stx) type, and antimicrobial resistance patterns of 138 Stx-producing *Escherichia coli* (STEC) strains isolated from humans between 2003 and 2007 in Shizuoka Prefecture, Japan were characterized. The predominant O serogroups of the STEC isolates were O157, O26, and O111. Antimicrobial susceptibility testing of the STEC isolates showed that 31 of the 138 isolates (22.5%) were resistant to antibiotics. Compared to the results reported in the previous studies, a higher rate of STEC O157 isolates were susceptible to all the antimicrobial agents used in this study. However, antimicrobial susceptibility data from this study showed that antimicrobial resistance patterns have increased by 6 compared to the survey performed by Masuda et al. between 1987 and 2002 (Jpn. J. Food Microbiol., 21, 44–51, 2004). This indicates that STEC isolates have evolved to show a variety of antimicrobial resistance patterns. It is important to consider the population of isolates showing decreased susceptibility to clinically relevant drugs such as ciprofloxacin (CPFX) and fosfomycin (FOM). All the 3 STEC isolates resistant to nalidixic acid showed low susceptibility to CPFX (MIC, 0.25–0.5 μg/ml). In addition, a decreased susceptibility to FOM was clearly observed in the *E. coli* O26 isolates. Our findings also showed that 1 STEC O26 strain could possibly be a chromosomal AmpC β-lactamase hyperproducer. These results suggest that antimicrobial therapy may be less effective in patients with non-O157 STEC infections than in those with STEC O157 infections.

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC), an important foodborne pathogen, can cause mild to severe bloody diarrhea that is sometimes followed by life-threatening complications such as the hemolytic uremic syndrome (HUS) (1). The *E. coli* O157:H7 infectious dose may be less than 1,000 cells (2,3). An extraordinarily low infectious dose of less than 45 cells was reported in an outbreak of *E. coli* O157 infection (4). Elderly and pediatric patients are at an increased risk of developing *E. coli* O157:H7-associated conditions such as diarrhea, HUS, thrombotic thrombocytopenic purpura, and death (1). The Health and Disease Prevention Division, Shizuoka Prefectural Government of Japan reported the first outbreak of STEC O157 infection in Shizuoka Prefecture in 1987. A gradual increase in the incidence of STEC infections was observed in Shizuoka Prefecture between 2003 and 2007, which was unlike that observed in the first 15 years of surveillance (1987–2002). Among the infections with different strains of diarrheagenic *E. coli*, infection with the STEC strain has shown the highest mortality in Japan, which may be because of the severity of the clinical presentation in STEC infections (5). A high prevalence of pathogenic STEC in beef and beef cattle has been reported in Japan and other parts of the world (6,7). Therefore, there is a great risk of transfer of these STEC infections to humans. Masuda et al. reported the antimicrobial resistance of STEC strains isolated from humans between 1987 and 2002 (8).

The aim of the present study was to determine the frequency of occurrence of antimicrobial resistance in the STEC strains isolated from humans in Shizuoka Prefecture after 2002. We isolated 138 STEC strains between 2003 and 2007 and determined their serotypes, Stx-types, and antimicrobial resistance patterns.

MATERIALS AND METHODS

Isolates: A total of 228 STEC strains were isolated between 2003 and 2007 from fecal samples obtained in 99 cases of sporadic infections, 2 group outbreaks, and 37 familial outbreaks in Shizuoka Prefecture. To avoid overrepresentation of clonal strains, a single representative isolate was chosen from each group of isolates from the outbreak or household-contact groups.

Serotyping: For serotyping the *E. coli* isolates, we performed by slide and tube agglutination tests with anti-*E. coli* O and H sera (Denka Seiken Co., Tokyo, Japan), respectively, according to the manufacturer's

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instructions.

Stx-typing: The types of *stx* gene carried by each isolate were characterized by polymerase chain reaction (PCR) assay with the O-157 (Verocytotoxin Genes) One-Shot PCR Typing Kit (Takara, Ohtsu, Japan). The production of Stx type 1 (Stx1) and Stx type 2 (Stx2) in the isolates was determined by performing the reversed passive latex agglutination (RPLA) test (Denka Seiken) according to the manufacturer's instructions.

Characterization of β-lactamase genes: The bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{PSE\text{-}1}$, $bla_{CMY\text{-}1}$, $bla_{CMY\text{-}2}$, and bla_{FOX} genes and the promoter region of the *ampC* gene ($bla_{frdD\text{-}ampC}$) were amplified using the procedures previously described by Kojima et al. (9) and Shibata et al. (10). The amplified PCR products were sequenced using an Applied Biosystems 3730xl DNA Analyzer. The obtained sequences of the β-lactamase genes were compared with *bla* sequences previously described in the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations in the *ampC* promoter region were identified by comparing the sequence of this region with the sequence of the corresponding region in the *E. coli* K-12 strain LA5 (11). Mutations at the position −42 (C to T), −18 (G to A), −1 (C to T), and +58 (C to T) have been known to be potentially associated with AmpC hyperproduction (12).

Antimicrobial susceptibility testing: The antimicrobial resistance patterns of the STEC isolates were determined by the disk diffusion method using Mueller-Hinton agar according to the Clinical Laboratory Standards Institute (CLSI) procedure (13,14). The 16 disks of the following antibiotics (Becton Dickinson, Franklin Lakes, N.J., USA) were used: ampicillin (ABPC), chloramphenicol (CP), kanamycin (KM), streptomycin (SM), sulfamethoxazole/trimethoprim (ST), tetracycline (TC), nalidixic acid (NA), gentamicin (GM), fosfomycin (FOM), ciprofloxacin (CPFX), cefotaxime (CTX), cefuroxime (CXM), cefpodoxime (CPDX), ceftazidime (CAZ), aztreonam (AZT), and ceftriaxone (CTRX). In order to determine the minimum inhibitory concentration (MIC) for FOM and CPFX as the first-line antibiotics for STEC infection, the E-test (AB Biodisk, Solna, Sweden) was performed. *E. coli* ATCC 25922 was used as a quality control strain. The results of antimicrobial susceptibility testing were interpreted on the basis of the CLSI guidelines (13,14). Isolates that produced "intermediate" values were considered susceptible. We calculated the MIC_{50} and MIC_{90} values and the rates of resistance to FOM and CPFX. For isolates resistant to cephalosporins, the presence of extended-spectrum β-lactamases (ESBLs) was investigated by performing the CLSI-recommended confirmatory test, i.e., the standard disk diffusion test (13). BD Sensi-Discs (Becton Dickinson) was used in the disk diffusion testing. *Klebsiella pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) were used as the positive and negative controls, respectively, in the tests for ESBL production.

RESULTS

Serotyping and Stx-typing: The serotyping results showed that the isolates belonged to 12 different O:H serotypes. Most of the isolates (73.2%) were of the *E. coli* O157:H7 serotype (Table 1), and 13.0% of the isolates were of *E. coli* O26:H11 serotype. In the *E. coli* O157 isolates, the most frequently observed Stx-type was Stx1 and Stx2 (67.3%), followed by Stx2 (30.8%; Table 1). In *E. coli* O26 isolates, the Stx-type of all the 21 isolates was Stx1 (Table 1).

Table 1. Serotypes and Stx-types of STEC isolates in Shizuoka Prefecture between 2003 and 2007

Serotype	No. of isolates			
	Toxin type			Total (%)
	Stx1	Stx2	Stx1/2	
O26:H11	18			18 (13.0)
O26:H-	2			2 (1.4)
O26:HUT	1			1 (0.7)
O103:H2	1			1 (0.7)
O103:H51	1			1 (0.7)
O111:H-	2		2	4 (2.9)
O121:H19		3		3 (2.2)
O157:H7	1	32	68	101 (73.2)
O157:H-	1		2	3 (2.2)
O165:H-		1		1 (0.7)
OUT:H-	1	1		2 (1.4)
OUT:HUT		1		1 (0.7)
Total	28	38	72	138

H-, nonmotile; HUT, H untypeable; OUT, O untypeable; Stx1/2, Stx1 and Stx2.

Resistance phenotypes: Of the 138 STEC isolates, 31 (22.5%) showed resistance to 1 or more antimicrobial agents (Table 2). Of the 104 *E. coli* O157 isolates, 16 (15.4%) were resistant to 1 to 5 antimicrobial agents. Of the 21 *E. coli* O26 isolates, 11 (52.4%) were resistant to 1 to 7 antimicrobial agents. Of the 4 *E. coli* O111 isolates, 2 (50.0%) were resistant to 1 to 4 antimicrobial agents. Of the 9 remaining isolates of other serotypes, 2 (22.2%) were resistant to 5 to 6 antimicrobial agents. Of all the *E. coli* O157 isolates, 1 showed resistance to 5 antimicrobial agents: ABPC, KM, SM, ST, and TC. Among the *E. coli* O26 isolates, 1 showed resistance to 7 antimicrobial agents: ABPC, CP, KM, SM, ST, TC, and NA. The most frequently observed combination was resistance to SM and TC, which was detected in 10 isolates, out of which 7 were *E. coli* O157 isolates and 3 were *E. coli* O26 isolates (Table 2). We did not detect resistance to multiple clinically relevant drugs (CPFX, FOM, and KM) in any of the strains. The STEC O26:H- isolate appeared to hyper-express a broad-spectrum β-lactamase, as it showed resistance to ABPC, CXM, and CPDX (Table 2). However, the disk confirmatory test indicated that this cephalosporin-resistant isolate was not an ESBL producer.

All the STEC isolates were susceptible to the following 6 antimicrobial agents: FOM, CPFX, CTX, CAZ, AZT, and CTRX (Table 3). Among the 138 isolates that were tested, 24 (17.4%) were resistant to TC, 23 (16.7%) to SM, 12 (8.7%) to ABPC, 7 (5.1%) to CP, 7 (5.1%) to KM, 4 (2.9%) to ST, 3 (2.2%) to NA, 1 (0.7%) to GM, 1 (0.7%) to CXM, and 1 (0.7%) to

Table 2. Distribution of multiresistance in STEC isolates in Shizuoka Prefecture (2003–2007)

No. of antibiotics	Resistance pattern	No. of isolates									
		O157						O26	O111	Others	Total
		2003	2004	2005	2006	2007	Subtotal				
7	ABPC, CP, KM, SM, ST, TC, NA							1			1
6	ABPC, CP, KM, SM, TC, NA									1	1
5	ABPC, CP, KM, SM, TC									1	1
	ABPC, KM, SM, ST, TC				1		1				1
4	ABPC, CP, KM, TC							1			1
	CP, KM, SM, TC							1			1
	ABPC, KM, SM, TC								1		1
3	ABPC, SM, TC					1	1				1
	ABPC, SM, ST					2	2				2
	ABPC, CXM, CPDX							1			1
	CP, SM, TC							1			1
2	ABPC, SM	1	1				2				2
	SM, TC	3	2	1		1	7	3			10
	CP, TC							1			1
	TC, GM							1			1
1	TC			2	1		3				3
	SM							1			1
	NA								1		1
0		5	15	30	20	18	88	10	2	7	107
Total		9	18	33	22	22	104	21	4	9	138

ABPC, ampicillin; CP, chloramphenicol; KM, kanamycin; SM, streptomycin; ST, sulfamethoxazole/trimethoprim; TC, tetracycline; NA, nalidixic acid; CXM, cefuroxime; CPDX, cefpodoxime; GM, gentamicin.

CPDX (Table 3). The distribution of MIC values of FOM and CPFX against *E. coli* O157 and *E. coli* O26 isolates is shown in Table 4. The MIC values of FOM against *E. coli* O157 ranged from 0.25 to 16 μg/ml; the MIC_{50} value was 1 μg/ml and MIC_{90} value was 4 μg/ml. The MIC_{50}/MIC_{90} of FOM was much higher against *E. coli* O26 isolates, at 8/32 μg/ml. The MIC_{50} value of CPFX was 0.016 μg/ml against both *E. coli* O157 and *E. coli* O26 isolates, and the MIC_{90} value of CPFX was 0.032 μg/ml against both *E. coli* O157 and *E. coli* O26 isolates, indicating that there was no difference between the MICs against *E. coli* O157 and *E. coli* O26 isolates.

We observed that 1 *E. coli* O26:H11 isolate, 1 *E. coli* O111:H- isolate, and 1 *E. coli* OUT:H- isolate were resistant to NA. The MIC values of CPFX against these 3 isolates ranged from 0.25 to 0.5 μg/ml, which shows their low susceptibility to CPFX.

Characterization of β-lactamase genes: In 1 cephalosporin-resistant *E. coli* O26:H- isolate, we amplified the $bla_{\text{frdD-ampC}}$ gene and sequenced the transcriptional regulatory region of *ampC* to identify mutations in the promoter region. In this isolate, mutations at position -42 (C to T), -18 (G to A), -1 (C to T), and $+58$ (C to T) with respect to the transcriptional start site ($+1$) of the *ampC* gene were detected. Though we did not perform enzyme expression experiments, mutations at these points could be associated with AmpC hyperproduction (12), which explains the resistance phenotype of this isolate.

DISCUSSION

In 2006, the Infectious Agents Surveillance Report (IASR) reported that the proportion of STEC infection caused by the STEC O157:H7 strain was gradually decreasing; the proportion of STEC infections caused by the O157:H7, STEC O26, and STEC O111 were 52%, 24%, and 3.3%, respectively (15). Masuda et al. reported that the majority of STEC isolates in Shizuoka Prefecture between 1987 and 2002 were of O serogroups O157 (69.8%) and O26 (19.3%) (8). In contrast, we observed a higher proportion of isolates of O serogroup O157 between 2003 and 2007.

We also found that the antimicrobial resistance rate of the STEC strains isolated between 2003 and 2007 was lower than that of the strains isolated between 1987 and 2002 reported by Masuda et al. (8). Although the term of our survey was 5 years, the number of antimicrobial resistance patterns observed in this study were 6 more than those observed by Masuda et al. between 1987 and 2002 (8). This indicates that the STEC isolates have evolved to show a variety of antimicrobial resistance patterns. The resistance rate of the STEC isolates to NA (2.2%) in our study is higher by 1.6% than the corresponding value reported by Masuda et al. (8). All the 3 STEC isolates resistant to NA showed a low susceptibility to CPFX (MIC, 0.25–0.5 μg/ml). The reports on the increasing MIC values of fluoroquinolones against *Shigella* strains raise concerns about the possibility of the treatment failures (16). In fact, a decreased suscepti-

Table 3. Antimicrobial resistance properties of STEC isolates

Serotype	Toxin type	No. of isolates	No. of resistant isolates (%)	No. of STEC isolates (%) resistant to															
				ABPC	CP	KM	SM	ST	TC	NA	GM	FOM	CPFX	CTX	CXM	CPDX	CAZ	AZT	CTRX
O26:H11	Stx1	18	9 (50.0)	2 (11.1)	5 (27.8)	3 (16.7)	6 (33.3)	1 (5.6)	8 (44.4)	1 (5.6)	1 (5.6)								
O26:H-	Stx1	2	1 (50.0)	1 (50.0)											1 (50.0)	1 (50.0)			
O26:HUT	Stx1	1	1 (100)				1 (100)		1 (100)										
O103:H2	Stx1	1	0																
O103:H51	Stx1	1	0																
O111:H-	Stx1	2	1 (50.0)							1 (50.0)									
	Stx1/2	2	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)		1 (50.0)										
O121:H19	Stx2	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)		1 (33.3)										
O157:H7	Stx1	1	0																
	Stx2	32	7 (21.9)				5 (15.6)		7 (21.9)										
	Stx1/2	68	8 (11.8)	5 (7.4)			7 (10.3)	2 (2.9)	4 (5.9)										
O157:H-	Stx1	1	0																
	Stx1/2	2	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)										
O165:H-	Stx2	1	0																
OUT:H-	Stx1	1	0																
	Stx2	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)		1 (100)	1 (100)									
OUT:HUT	Stx2	1	0																

ABPC, ampicillin; CP, chloramphenicol; KM, kanamycin; SM, streptomycin; ST, sulfamethoxazole/trimethoprim; TC, tetracycline; NA, nalidixic acid; GM, gentamicin; FOM, fosfomycin; CPFX, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; CPDX, cefpodoxime; CAZ, ceftazidime; AZT, aztreonam; CTRX, ceftriaxone; Stx1/2, Stx1 and Stx2.

Table 4. MIC distribution of STEC O26 and O157 isolates

Antimicrobial agent	O serogroup	Range of MIC (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
FOM	O26	0.5–64	8	32
	O157	0.25–16	1	4
CPFX	O26	0.008–0.25	0.016	0.032
	O157	0.004–0.032	0.016	0.032

FOM, fosfomycin; CPFX, ciprofloxacin.

bility to fluoroquinolones has been associated with decreased clinical responses of *Salmonella* infections to fluoroquinolones (17). A similar problem can occur with STEC infections as well.

The *E. coli* O26 isolates clearly showed a decreased susceptibility against FOM. Decreased susceptibility to FOM has been associated with decreased clinical responses to STEC O26 infections. The essential antimicrobial agents for the treatment of STEC infection must be used prudently in veterinary medicine, particularly for antimicrobial therapy of cattle, given that cattle are the main reservoir of STEC strains. It is necessary to control the multiplication of these strains with low susceptibility to clinically relevant drugs such as fluoroquinolones, FOM, and aminoglycosides. Resistance phenotypes and sequence analysis results were consistent with observation of AmpC cephalosporinase hyperproduction in the *E. coli* O26:H- isolate. ESBL-producing *E. coli* O26 strains have been detected in humans previously (18,19). To our knowledge, this is the first report on the mutations in the promoter region of chromosomal AmpC β-lactamase in STEC O26 strains isolated from humans. This type of resistance does not disseminate via horizontal gene transfer mechanisms. In addition, cephalosporins such as CXM and CPDX are not efficient for treating STEC infections by such strains. However, the increasing antimicrobial resistance is a public health concern. In future studies, we intend to perform antimicrobial susceptibility tests on STEC strains, taking into account the usage of antimicrobial agents.

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Conflict of interest None to declare.

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