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Characterization of third-generation cephalosporin-resistant Shiga toxin-producing strains of *Escherichia coli* O157:H7 in Japan

Ryuji Kawahara,[a]# Kazuko Seto,[a] Masumi Taguchi,[a] Chie Nakajima,[b] Yuko Kumeda,[a] Yasuhiko Suzuki[b]

[a]Division of Bacteriology, Osaka Prefectural Institute of Public Health, Osaka, Japan

[b]Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

Running Head: Cephalosporin-resistant STEC O157:H7 in Japan

Address correspondence to Ryuji Kawahara, kawahara@iph.pref.osaka.jp.

Abstract

We isolated Shiga toxin-producing *Escherichia coli* O157:H7 strains resistant to third-generation cephems. The resistant strains harbored *bla* $_{CMY-2}$, one of the plasmid-mediated AmpC ß-lactamases. Genotyping of isolates revealed the possible spread of this problematic bacterium. Results suggested the importance of the investigation and surveillance of enterobacteria with plasmids harboring *bla*$_{CMY-2}$.

Text

Shiga toxin-producing *Escherichia coli* (STEC) is one of the most important recently emerged pathogens. It causes fatal infections, such as hemolytic-uremic syndrome (HUS) and hemorrhagic colitis. Among STECs, O157:H7 is the most important serotype (1, 2).

ß-lactam resistance among Enterobacteriaceae has emerged worldwide. Broad-spectrum ß-lactamases, such as extended-spectrum ß-lactamases (ESBLs) and plasmid-mediated AmpC ß-lactamases, are found in many species of Enterobacteriaceae, including *E. coli*, *Klebsiella pneumonia*, *Salmonella enterica*, and *Shigella* spp (3, 4).

STEC infections caused by cephalosporin-resistant isolates have been reported in both Europe and Japan. For example, a large outbreak of STEC O104:H4 harboring $bla_{\text{CTX-M-15}}$ occurred in Germany in 2011 (5). In addition, STEC O26:H11 with plasmid–encoded $bla_{\text{CTX-M-18}}$ was isolated from a human infection in Japan (6). However, only a few reports have described the detection of cephalosporin resistance among STEC O157 strains. In the United States, STEC O157 with bla_{CMY} has been detected among human isolates (7), and cephalosporin-resistant STEC O157 strains were isolated from bovine feces in Japan, although the resistance genes were not identified (8).

We collected 2167 STEC O157:H7 and O157:HNM strains associated with infections in Osaka Prefecture, Japan between 1996 and 2011, and examined their drug susceptibility using the disk diffusion method (9). Among these, seven isolates exhibited ß-lactam resistance, including third-generation cephalosporins, but were susceptible for meropenem. These were isolated from five independent diarrhea patients and two asymptomatic family members of a patient between 2006 to 2007 (Table). The common source of infection in each case and epidemiological link between the five symptomatic patients was not detected. All strains were identified as O157:H7 with antisera and their production of both Stx1 and Stx2 was confirmed using a VTEC-RPLA assay (VTEC-RPLA; Denka Seiken, Tokyo, Japan). The results of biochemical characterization indicated the typical phenotype of STEC O157; sorbitol-negative and ß-glucuronidase-negative.

The susceptibility of the isolates to ampicillin (AMP), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), imipenem (IPM), gentamicin (GEN), amikacin (AMK), minocycline (MIN), nalidixic acid (NAL), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), and fosfomycin (FOF) was determined by the broth micro-dilution method using Dry Plate Eiken (Eiken Chemical Co., Ltd., Tokyo, Japan) and the results were interpreted as described by the Clinical and Laboratory Standards Institute(9).

ESBL and AmpC beta-lactamase production were determined by the ESBL confirmatory test(9) and the double-disk synergy test with 3-aminophenylboronic acid (Boronic acid test, [10]), respectively. As shown in Table, the MICs of ß-lactams, including penicillins, cephalosporins, and ATM, were high, with the exception of IPM. In addition, these isolates were susceptible with meropenem and gave negative results in the ESBL confirmatory test, but positive in the boronic acid test. These results suggested that they were producers of AmpC ß-lactamase, but neither ESBL nor carbapenemase. On the other hand, they were susceptible to aminoglycosides, FOF, MIN, NAL, CIP, and SXT (data not shown).

Genes belonging to the CIT (*bla* $_{CMY-2}$-related genes) group were detected among the s even STEC O157:H7 strains by multiplex PCR screening for plasmid-mediated AmpC ß-lact amase genes, as described previously (11). The entire coding regions of CIT-like ß-lactamase genes harbored by the isolates were amplified with primers CMY21-120F (5'-GGCCCGGA CACCTTTTTG-3') and CMY21-1324R (5'-CCTGGGCCTCATCGTCAG-3') using standard PCR conditions and sequenced with an ABI 3130 Genetic Analyzer (Life Technologies, Carl sbad, CA). The DNA sequences were identical to the bla_{CMY-2} gene, a plasmid-mediated Amp C ß-lactamase, in the GenBank database (accession no. X91840), as determined using the BL AST program { http://blast.ncbi.nlm.nih.gov/Blast.cgi, , (11). In addition, bla_{TEM-1} penicillina

58 se genes were detected and identified in the three isolates from the same family members by
59 PCR using primers TEM-19F (5'-AAAGGGCCTCGTGATACGC-3') and TEM-1077R (5'-A
60 GTTACCAATGCTTAATCAGTGAGGC-3') and sequencing.
61 We performed IS-typing to compare the strains harboring *bla*$_{CMY-2}$ with other
62 cephalosporin-susceptible STEC O157 human isolates. For IS-typing, a multiplex PCR-based
63 typing method for STEC O157 (12) was performed using the IS-Printing System (Toyobo
64 Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The results for the 7
65 STEC O157:H7 strains and 506 randomly selected STEC O157 strains from cephalosporin-
66 susceptible STEC O157 human isolates in Osaka between 1996 and 2011 were converted to
67 binary profiles and visualized using the minimum spanning tree algorithm with Bionumerics
68 software (version 6.5; Applied Maths, Kortrijk, Belgium). The results of minimum spanning
69 tree analysis indicated that the seven strains harboring *bla* $_{CMY-2}$ formed a large cluster (Fig. 1).
70 The IS type of three isolates from the family members was identical. We also confirmed
71 these strains showed same patterns by pulse field gel electrophoresis with both *Xba*I and *Bln*I
72 restriction enzyme (data not shown). In contrast, the other four isolates showed different
73 patterns with double- or triple-locus variations. In addition, strains harboring the same IS type
74 as 19H131, 19H252, and 19H311 (Fig. 1, arrows 2, 6, and 7), but which did not encode the

75 *bla*$_{CMY-2}$ gene, were detected among STEC O157 strains isolated in Osaka Prefecture, Japan

76 between 1996 and 2011.

77 Plasmids of the isolates were extracted using a NucleoBond Xtra Midi kit (Clontech,

78 Heidelberg, Germany) according to the manufacturer's instructions and visualized using

79 agarose gel electrophoresis (Fig. 2A). Plasmids isolated from *Salmonella* Enteritidis L156

80 and *E. coli* NR1 were used as DNA size markers. Each strain contained several plasmids with

81 approximately 90–110 kbp plasmids in common. Then, mating experiments with a nalidixic

82 acid-resistant *E. coli* C600 as the recipient was performed to select ß-lactam-resistant

83 conjugants on sorbitol MacConkey agar supplemented with cefotaxime (0.5 μg/mL) and

84 nalidixic acid (25 □g/mL) (13). All conjugants with each isolate acquired resistance to ß-

85 lactams except for IPM, and *bla* $_{CMY-2}$ but not *bla*$_{TEM-1}$ (Table). The plasmids detected were

86 approximately 110 kbp in C600/18H093 and 95 kbp in others (Fig. 2B).

87 To identify the plasmids harboring the *bla* $_{CMY-2}$ gene among these transconjugants, the

88 southern hybridization assay was carried out with a probe of the internal *bla* $_{CMY-2}$ fragment.

89 The PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) was use

90 d as recommended by the manufacturer with primers CMY21-399F (5'-TTGAGCTAGGATC

91 GGTTAGTAAGACG-3') and CMY21-1039R (5'-CATCTCCCAGCCTAATCCCTG-3'). Th

92 e results demonstrated that the *bla* $_{CMY-2}$ gene existed on the 110 and 95 kbp plasmids in C600
93 /18H093 and other transconjugants, respectively (Fig. 2C).
94 Plasmid genotypes were examined by the detection of integrons, replicon typing and
95 IncI1 plasmid multilocus sequence typing (IncI1 pMLST) as previously described (14-16).
96 The PCR assay showed that all the conjugants contained a plasmid with the IncI1 replicon
97 (Table). The presence of the class 1 integron gene was confirmed in only C600/18H093.
98 IncI1 pMLST was used to examine the similarity among the *bla* $_{CMY-2}$-harboring plasmids and
99 to compare with pMLST profiles in the global database (http://pubmlst.org/plasmid/). As
100 shown in Table, all plasmids, except for that found in C600/18H093, showed identical
101 pMLST profiles and were identified as ST55. The pMLST profile of C600/18H093, which
102 only differed from the other transconjugants with respect to repI1, did not match any STs in
103 the global database.
104 Here, we identified seven STEC O157:H7 isolates from Japan that produce *bla*$_{CMY-2}$ ß-
105 lactamase, a plasmid-mediated AmpC ß-lactamase. Due to the lack of epidemiological links
106 among the patients and the various IS-typing profiles that were observed among the STEC
107 O157:H7 isolates, with the exception of the closely related strains isolated from the three

family members, the infections were considered to be caused by distinct STEC strains in five independent incidences.

Generally, it's not recommended to use antibiotics for treatment of STEC infections(1). However, it's still necessary to determine drug susceptibility of STEC strains. Because therapeutic or prophylactic administration of antibiotics is required in a severe situation, for instance, the German outbreak by STEC O104:H4 producing ESBL, 2011(17).

The genotype of the plasmids carrying the *bla*$_{\text{CMY-2}}$ gene indicated that all plasmids contained an IncI1 replicon, and six of the strains isolated in 2007 carried a plasmid with the ST55 sequence type. In Taiwan, several isolates of *S. enterica* Choleraesuis, Typhimurium, Agona, and Enteritidis with ceftriaxone resistance were isolated from patients between 2007 to 2010, and included eight strains that carried *bla*$_{\text{CMY-2}}$-harboring IncI1 plasmids (18). In addition, a *S.* Typhimurium strain isolated in 2010 was found to harbor an IncI1 plasmid of ST55. Notably, our present results suggest that the emergence of O157:H7 strains resistant to ß-lactams, including third-generation cephalosporins, may have been caused by the spread of an IncI1 plasmid of ST55 among animals and/or humans.

The data obtained in the present study and those reported for isolates from Taiwan suggest that IncI1 plasmids have high transmissibility across species barriers. Although the

sources are presently unknown, these ß-lactam-resistant isolates may have emerged by horizontal transfer of similar plasmids containing *bla*$_{CMY-2}$ among enterobacteria and therefore may be a threat for the control of not only O157:H7, but also other pathogenic Enterobacteriaceae. For this reason, further investigation and surveillance for enterobacteria with plasmids harboring the *bla*$_{CMY-2}$ gene are strongly recommended to clarify the transmission dynamics of this plasmid and design countermeasures for preventing its further spread.

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FIGURE LEGENDS

FIG 1 Minimum spanning tree analysis of IS profiles. Circle size indicates the number of isolates. The number of locus mismatches between the IS type profiles was used as distance. The O157:H7 strains harboring $bla_{\text{CMY-2}}$ indicated as1 (18H093), 2 (19H131), 3 (19H180), 4 (19H187), 5 (19H188), 6 (19H252), and 7 (19H311).

FIG 2 Profiling and hybridization of plasmids extracted from the CMY-2-producing *E. coli* O157:H7 (A) and transconjugant strains (B, C). (A) Lanes: M1, *Salmonella* Enteritidis L156; M2, *E. coli* NR1; 1, 18H093; 2, 19H131; 3, 19H180; 4, 19H187; 5, 19H188; 6, 19H252; 7, 19H311; and 8, *E. coli* C600. (B) Lanes: 1, C600/18H093; 2, C600/19H131; 3, C600/19H180; 4, C600/19H187; 5, C600/19H188; 6, C600/19H252; 7, C600/19H311; and 8, *E. coli* C600. The plasmids in the agarose gel were hybridized with the $bla_{\text{CMY-2}}$ probe after being transferred to a nylon membrane (C).