Shiga Toxin Subtypes and Virulence Genes in *Escherichia coli* Isolated from Cattle

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**SUMMARY:** Subtypes of *stx1* and *stx2* in 45 Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from cattle were investigated by PCR. Only subtype *stx1a* was detected among all the *stx1*-positive strains. The major *stx2* subtype was *stx2a* followed by *stx2d, stx2c, stx2b*, and *stx2g* in decreasing order of frequency. *stx2c* was found in strains of serotypes O157 and O174. *stx2d* was found in 11 strains. These strains were confirmed by DNA sequencing to carry both the activatable tail and the END motif; all were eae-negative, and 3 contained *stx2d* as the only *stx*. *stx2g* was found in 2 strains in association with *stx2a, estA1*, and *astA*. In addition, 7 hybrid strains of shigatoxigenic and enterotoxigenic *E. coli* (STEC/ETEC) were found to harbor one or both of *stx1a* and *stx2a* (*stx1a/stx2a*) and *estA1*. Among 27 serotypes of STEC strains isolated from cattle, O157:H7 and O109:H– strains were *eae*-positive. Other putative adhesin genes, such as *saa, iha, espP*, and *lpfA* were detected in more than 12 serotypes.

**INTRODUCTION**

*Escherichia coli* are usually harmless inhabitants of the intestinal tract of various animals and humans; however, some strains carry characteristic diarrhea-causing genes in humans. Shiga toxin-producing *E. coli* (STEC) is the most potent strain causing not only gastroenteritis but also hemolytic-uremic syndrome (HUS) and encephalitis.

Shiga toxin (Stx) produced by *E. coli* is classified into 2 types (*Stx1* and *Stx2*) based on their antigenicity, and each type has many genetic subtypes. Scheutz et al. established a protocol for the subtyping of both *stx1* and *stx2* by PCR and standardized the nomenclature of 3 *Stx1* and 7 *Stx2* subtypes (1). Strains producing subtype *Stx2a, Stx2c*, or *Stx2d* are often associated with development of hemorrhagic colitis and HUS (2). In contrast, *Stx2b* (3), *Stx2e* (4), *Stx2f* (5), and *Stx2g* (6) are scarcely associated with serious human disease. STEC strains isolated from red deer, sheep, and goat products were reported to carry *Stx2a* (7), and those from bovine products carried *Stx2g* (8). *Stx2a* can cause edema in neonatal piglets (9). *Stx2f* is mostly found in SETC isolated from bird (10), but it has recently been detected in human clinical isolates (11). *Stx2d* is distinct because it is activated by elastase, which largely increases cytotoxicity by the cleavage of 2 amino acids from the C terminal end of the A2 subunit (12). Each subtype displays dramatic differences in potency (2), and determining the clinical symptoms and distribution of Stx subtypes in host organisms according to each subtype is an important goal.

Characterization of STEC isolates from cattle is important to understand the source of new strains in human patients. The aim of this study was to evaluate the virulence potential of each STEC strain. We applied the PCR method proposed by Scheutz et al. (1) to subtype of *stx1* and *stx2* found in STEC strains from cattle and also investigated relationships of these subtypes with serotypes and other virulence genes.

**MATERIALS AND METHODS**

**Bacterial strains and template DNA preparation:** *E. coli* strains carrying virulence genes were isolated from the intestinal ingredients of cattle, as previously described (13). Forty-five STEC strains isolated from 32 Japanese Black cattle (20 oxen and 12 cows) bred in Hyogo Prefecture were used in this study. They were brought into the slaughterhouse between November 2012 and August 2013; 31 cattle were fed for 28–34 months and 1 cow for 147 months. Human STEC strains (17 strains of O157, 5 of O26, and 1 each of O91, O103, O111, O121, and O145) were collected from January 2012 to December 2013. These strains were isolated from stool samples obtained from patients with enterohemorrhagic *E. coli* infections notified to the Health and Welfare Office of Hyogo Prefecture, Japan.

Written informed consent for investigation of these pathogens and publication of results was obtained from all patients or their parents. Control strains for the *stx*-subtyping PCR were kindly provided by the National Institute of Infectious Diseases (NIID).

DNA was extracted by alkaline- and heat-treatment, and the extracted DNA was stored at −20°C until use.
Primers and PCR amplification conditions used to subtype Stxs: Primers presented by Scheutz et al. (1) were used for subtyping and sequencing. Multiplex PCR was performed to detect the following groups of genes: (i) stx1a, stx1c, and stx1d; (ii) stx2a, stx2b, and stx2f; and (iii) stx2e and stx2g. Single PCR using one primer pair was performed to detect stx2c and stx2d, and genes were confirmed by multiplex method.

DNA templates (2.4 μL) were mixed with 2 × GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), added to 25 μL of reaction mixture, along with 0.3 μM of each primer, and amplified under the following conditions: an initial denaturation at 95°C (10 min); 35 cycles of 94°C (50 s), 64°C (40 s), and 72°C (1 min); and a final extension at 72°C (3 min) with a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). For the resolution of stx2c and stx2d, we also used AmpliTaq Gold 360 Master Mix (Applied Biosystems). Subtypes of suspicious amplicons were confirmed by determining their nucleotide sequences.

Partial sequencing of stx1 and stx2: PCR-amplified DNA was directly sequenced using an ABI3500 genetic analyzer and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the previously mentioned primers (1). The thermal cycling conditions were the same as those used for subtyping, except for an annealing temperature of 56°C. The nucleotide sequences were aligned with those of the reference sequences and translated into amino acids, using SeaView software (14).

Detection of virulence genes by PCR: Based on the protocol presented by the NIID (15), multiplex PCR was performed to detect stx1, stx2, estA1, estA2, eltA, invE, eae, aggR, afaD, and astA. Previously mentioned PCR primers (13) were used to detect and subtype cdt and cnf. Putative adhesin genes (saa, iha, toxB, espP, and lpfA0118) were detected by PCR using primers described by Monaghan et al. (16) and the conditions described by Tatarczak et al. (17).

Serotyping of E. coli isolates: Genetic O serogrouping by PCR targeting O-serogroup-specific sequences in mostly the wzx, wzy, wzt, and wzm genes (18) was performed for STEC strains that could not be serotyped with commercially available antisera (Denka Seiken, Tokyo, Japan). In addition, O- and H-serotypes were confirmed using E. coli antisera (Statens Serum Institute, Copenhagen, Denmark; Veritas, Mountain View, CA, USA) at the NIID.

RESULTS

Stx subtypes and serotypes of STEC strains from cattle: Among the 45 isolates from cattle, 11 carried stx1, 41 carried stx2, and 7 carried both of these. stx1 was determined to be stx1a subtype, and stx2 was subtyped into stx2a, stx2b, and stx2g (Table 1). Multiple subtypes of stx2 were present in 13 strains: 7 carried both stx2a and stx2d, 2 carried both stx2a and stx2g, 2 carried stx2a, stx2b, and stx2c, 1 carried both stx2a and stx2e, and 1 carried both stx2b and stx2d. Coexistence of stx1a, stx2a, and stx2d was observed in 2 strains, and that of stx1a and stx2a in 5 strains.

Twenty-seven serotypes were identified in the isolates from cattle; the major serotypes were O109:H1 and O178:H19 (5 each), followed by O109:H16, and O157:H7 (3 each). O157:H7 has been predominant STEC serotype isolated from humans and cattle, and it carries stx2c and eae at a high rate (19). O8:H19, O113:H21, O157:H7, O174:H21 (20–23), and O178:H19 (23,24) have been reported to be associated with human STEC-causing HUS, and have also been isolated from cattle (16,24,25). In addition, O1:H7 was associated with human STEC-causing HUS (20), and O8:H2, O8:H−, O113:H−, O118:H12, and O150:H− were associated with human STEC-causing diarrhea or other gastrointestinal alterations (20–22). O8:H49 (23), O109:H16 (8), and O109:H− have been found in animals (26).

Sequencing analysis of stx1 and stx2 genes: Twenty stx1 genes derived from 15 human isolates (7 O157, 5 O26, and 1 each of O103, O111, and O145) and 5 cattle isolates (1 each of O1, O8, O38, O109, and O178) were sequenced, and 864 nucleotides and 287 translated amino acids (from Ser34 of the A subunit to Pro3 of the B subunit) were compared. All of the nucleotide sequences analyzed belonged to the stx1a subtype, and 3 stx1a from cattle corresponded with Shigella spp. Other stx1a sequences included 1 nucleotide substitution without amino acid exchange.

In the sequencing analysis of stx2 genes, 20 human isolates (17 O157, and 1 each of O91, O111, and O121) and 26 cattle isolates distributed on 17 serotypes were used, and 579 nucleotides and 192 amino acids (from Val196 of the A subunit to Pro3 of the B subunit) were compared. To confirm the subtypes determined by PCR, cattle isolates carrying stx2c or stx2d were preferentially sequenced, and 1 isolate with stx2a and stx2g, and 8 with stx2a were added. As previously reported (1), stx2a, stx2c, and stx2d were most closely related; that is, 547 nucleotides were corresponded with 29 stx2a, 16 stx2c, and 11 stx2d variants, and missense mutations resulting in amino acid substitutions were only observed at 11 nucleotide positions. Based on the nucleotide sequences, we found that 1 O174:H21 strain carried a variant of stx2c with a large insertion sequence (approximately 2 kb) at the intergenic spacer region between the A subunit and the B subunit.

Sequences of the last 10 amino acids in the C-terminal end of the A2 subunit as well as the 8 amino acids at position 11–18 in the B subunit are shown in Table 2; these represent each subtype identified in this study. KSSQSLYTTGE at position 288–297 in the A2 subunit has been referred to as ‘‘the activatable tail’’, and the combination of the activatable tail and END at position 15–17 in the B subunit seem to be responsible for the activatable property of the toxin (1).

All 11 Stx2d detected in samples from cattle carried the activatable tails and END motifs. Characteristic substitutions in the activatable tail are Ser291 and Gln297 (12), and these were found in variants of Stx2a and Stx2g without the END motif. Moreover, the END motif was found in Stx2b and Stx2c without the activatable tail.

Prevalence of other virulence-related genes in STEC strains from cattle: Virulence-related genes detected in cattle isolates are shown in Table 1. Among the 45 strains, eae was detected in 6 strains (13%); and all
### Table 1. Prevalence of virulence related genes on 45 STEC strains isolated from cattle

| Serotype | Subtype of stx1 and stx2 | Virulence gene | No. of strains |
|----------|--------------------------|----------------|---------------|
| O1 : H7  | 1a                       | cdtIII, cnf2, saa, iha, espP | lpfA<sub>O113</sub> 1 |
| O1 : H45 | 2a                       | estA1          | iha, espP     | lpfA<sub>O113</sub> 1 |
| O6 : H34 | 2a, 2d                   | astA           | iha, espP     | lpfA<sub>O113</sub> 1 |
| O8 : H19 | 2a                       | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| O8 : H49 | 2a, 2d                   | astA           | iha           | lpfA<sub>O113</sub> 1 |
| O8 : H-  | 2a                       | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| O18 : H7 | 2a                       | estA1          | astA          | lpfA<sub>O113</sub> 1 |
| O22 : H21 | 2a                     | iha             | espP          | lpfA<sub>O113</sub> 1 |
| O22 : H-  | 2b, 2d                  | iha             |               | lpfA<sub>O113</sub> 1 |
| O38 : H-  | 2a, 2d                  | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| O79 : H7 | 2a                       | astA1          | espP          | lpfA<sub>O113</sub> 1 |
| O109 : H16 | 2a, 2g                 | estA1, astA     |               | lpfA<sub>O113</sub> 2 |
| O109 : H-  | 2a                      | estA1, astA     |               | lpfA<sub>O113</sub> 1 |
| O113 : H21 | 2a                   | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| O113 : H-  | 2a, 2d                 | cdtV, saa, iha, espP | lpfA<sub>O113</sub> 1 |
| O118 : H12 | 2a                  | astA           | espP          | lpfA<sub>O113</sub> 1 |
| O150 : H-  | 2a                      | iha, espP      |               | lpfA<sub>O113</sub> 1 |
| O157 : H7 | 2c                      | eae, iha, espP, toxB |               |
| O174 : H21 | 2c                   | iha             | toxB          | lpfA<sub>O113</sub> 2 |
| O178 : H19 | 2a, 2d              | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| O183 : H18 | 2a, 2d           | cdtIII, cnf2, saa, iha, espP | lpfA<sub>O113</sub> 1 |
| O187 : H29 | 2a, 2b, 2c          | iha             |               | lpfA<sub>O113</sub> 1 |
| OUT : H11 | 2a                      | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| OUT : H19 | 2a, 2c                  | saa, iha, espP |               | lpfA<sub>O113</sub> 1 |
| OUT : H29 | 2a, 2b, 2c            | iha             |               | lpfA<sub>O113</sub> 1 |

### Table 2. Amino acid sequences of the C-terminal end of the A2 subunit and the part of the B subunit of Stx2 subtypes

| Subtype of Stx2 | Serotype | Origin | A<sub>2</sub> subunit | B subunit |
|-----------------|----------|--------|-----------------------|-----------|
| 2a              | O157 : H7 | human | K S Q F L Y T G K S Y N E D D T |           |
| 2a              | O91 : H21 | human |                        |           |
| 2a              | O109 : H16 | cattle |                        |           |
| 2a              | O178 : H19 | cattle |                        |           |
| 2a              | O8 : H19  | cattle |                        |           |
| 2a              | O8 : H-   | cattle |                        |           |
| 2b              | O22 : H-  | cattle |                        |           |
| 2c              | O157 : H7 | human |                        |           |
| 2c              | O157 : H- | human |                        |           |
| 2c              | O157 : H21 | cattle |                        |           |
| 2d              | O6 : H34  | cattle |                        |           |
| 2d              | O8 : H2   | cattle |                        |           |
| 2d              | O8 : H49  | cattle |                        |           |
| 2d              | O22 : H-  | cattle |                        |           |
| 2d              | O38 : H-  | cattle |                        |           |
| 2d              | O113 : H- | cattle |                        |           |
| 2d              | O178 : H19 | cattle |                        |           |
| 2g              | O109 : H16 | cattle |                        |           |

1): Horizontal line indicates no change of amino acid.
these strains were serotypes O109:H− or O157:H7. estA1 was detected in 9 strains (20%) that were hybrids of shigatoxigenic and enterotoxigenic E. coli (STEC/ETEC). estA1 was detected in 12 strains (27%), and estA1 and astA were detected together in 7 strains (O18:H7 and O109:H16/H−). cdhIII and cnf1 coexisted in 2 strains (O1:H7 and O183:H18), and cdh was detected in O113:H−.

Forty-five cattle isolates were tested for 5 putative adhesion genes (saa, iha espP, toxB, and lpfA_{O113}) that have been detected previously in non-O115 STEC strains (16). saa, which is the major adhesion factor gene in eae-negative STEC (16), was detected in 16 strains (36%). As previously reported (16), all saa-positive strains were eae-negative. toxB was detected only in O115. The frequency of detection of iha, espP, and lpfA_{O113} was relatively high; iha was detected in 31 (69%), espP in 29 (64%), and lpfA_{O113} in 34 (76%) isolates.

**DISCUSSION**

In this study, the primary subtypes of Shiga toxin detected in cattle isolates were stx1a, stx2a, stx2c, and stx2d. Regardless of the source or serotype of E. coli, the sequence of stx1a, stx2a, and stx2c were relatively well conserved, and there was no more than 1 amino acid substitution in each subtype, with the exception of 2 variants classified as stx2a (O8:H19 and O8:H−; Table 2), stx2d of O8:H19 and O8:H− showed the same amino acid sequences as stx2d in the A2 subunit (the activatable tail), but did not have the END motif (Table 2). These sequences were similar to Stx2a-O104-G5506 (EF441619) described by Scheutz et al. (1). For stx2d, 2 or less amino acid substitutions were observed within the variation shown by Scheutz et al. (1).

Since serotypes of O8:H19, O113:H21, O174:H21, and O178:H19 have been frequently isolated from cattle and found to be eae-negative, the prevalence of putative adhesion genes has been extensively investigated (16,23,24). Our results from O8:H19 and O113:H21 strains carrying stx2a, saa, iha, espP, and lpfA_{O113} is consistent with those of previous reports (23). The combination of stx2c, iha, and lpfA_{O113} has been reported in O174:H21 strains (23). O178:H19 strains have been divided into 2 groups based on their genetic profiles; one carries stx1a, stx2a, saa, iha, espP, and lpfA_{O113}, and the other carries stx2a, stx2d, iha, and lpfA_{O113}. The former combination was observed by Miko et al., and a strain with stx2a, stx2d, saa, iha, espP, and lpfA_{O113} was also reported (24).

Among the eae-negative STEC, Stx2d_{activatable} is believed to cause severe diseases, including bloody diarrhea and HUS, especially when the strain carries stx2d_{activatable} as the only stx, and is serotypes O8:H2, O22:H6, O91:H21, O113:H21, or O174:H21 (21). Coexistence of additional toxins, such as Stx1a and Stx2c, which are more cytotoxic for Vero cells than Stx2d alone, have been shown to mask the activation phenotype of Stx2d (1,12). We found stx2d with an activatable tail and END motif in 11 strains (1 each of O6:H34, O8:H2, O8:H49, O22:H−, and O113:H−, and 2 each of O38:H−, O113:H19, and OUT:H−) from cattle; all of them were eae-negative, and 3 (O8:H2 and 2 OUT:H−) carried stx2d as the only stx. Those 3 strains also carried adherence factors iha, espP, and lpfA_{O113} (Table 1).

STEC/ETEC hybrid strains carrying both stx and est have been isolated from humans and animals (6, 27–30). Of those, O101:H− isolated from an infant with HUS carried stx2a, estA1, eae, and espP; O2:H27 isolated from a child with diarrhea, an asymptomatic adult, and bovine feces carried stx2a, estA1, and astA (27,28); and O2:H2 isolated from cattle carried stx1a, estA1, and astA (27,29). In addition, STEC/ETEC strains with stx2d (28), stx2e (30), and stx2g (6,28) have also been reported.

We isolated 9 STEC/ETEC hybrid strains from cattle, and 6 of these strains were serotype O109 from 6 different cattle; 1 O109:H− carried stx2a, estA1, astA, eae, espP, and lpfA_{O113}; 2 O109:H− carried stx1a, estA1, astA, espP, and lpfA_{O113}; and 3 O109:H16 carried stx2a, stx2g, estA1, astA, and lpfA_{O113}. Although Bonardi et al. isolated an O109:H− strain carrying stx1 and stx2 without eae from a cow (26), the O109:H− strains detected in our study possessed either stx1a or stx2a, and only stx1a-positive strains lacked eae. Since the attaching-effacing effect of eae as well as mucosal damage and colonization of espP, are associated with high cytotoxicity, human infections by STEC/ETEC strains with eae and espP detected in our study may show high pathogenicity similar to the O101:H− strain isolated from an HUS patient in Finland (27). Horizontal transfer of plasmid-associated genes is believed to produce genetically diverse STEC/ETEC strains, and infection with these hybrid strains may cause more severe disease in patients (27).

We found 2 STEC/ETEC strains (O109:H16) carrying stx2g in association with stx2a, estA1, astA, and lpfA_{O113}. An STEC strain (O109:H16) carrying stx2g was previously isolated from semi-hard cows’ milk cheese, indicating the ease with which humans can be exposed to these STEC types through the food chain (8). Prager et al. revealed that 0.6% of human STEC strains possess stx2g. In that study, the main serotypes, including animal strains, were O15:H16 and O175:H28, and they carried estA1, astA, and lpfA_{O113} as the pathogenicity-related genes associated with stx2g (6).

In our institute, stx2d has not been obtained from human isolates, but a STEC/ETEC hybrid strain (O168:HUT) carrying stx2g, estA1, and astA was isolated from a patient with diarrhea in 2015 (unpublished data). Humans are confronted with a significant risk of STEC infection by consuming food contaminated with newly introduced E. coli strains that have acquired virulence genes in cattle. Since many combinations of toxin genes and other virulence factors are predicted to affect the overall toxicity of strains, further research on STEC isolates from cattle are needed to clarify their risk to humans.

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**Conflict of interest** None to declare.
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