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Original Article

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 $lpfA_{O113}$ 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 *stx2b* (7), and those from bovine products carried *stx2g* (8). Stx2e can cause edema in neonatal piglets (9). Stx2f is mostly found in SETC isolated from bird (10), but recently it has 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 A_2 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.

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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 weve 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 $lpfA_{O113}$) 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 32 *stx2a*, 11 *stx2d*, 8 *stx2c*, 3 *stx2b*, and 2 *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 *stx2c*, 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:H− 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 Phe67 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 A_2 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. KSQSLYTTGE at position 288–297 in the A_2 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 Glu297 (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*		$lpfA_{O113}$	1
O1 : H45		*2a*		*estA1*				*iha*	*espP*			1
O6 : H34		*2a*	*2d*				*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O8 : H2		*2d*			*astA*			*iha*	*espP*		$lpfA_{O113}$	1
O8 : H19		*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O8 : H49	*1a*	*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
		2a	*2d*		*astA*			*iha*			$lpfA_{O113}$	1
O8 : H–		*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O18 : H7	*1a*	*2a*		*estA1*	*astA*						$lpfA_{O113}$	1
		2a									$lpfA_{O113}$	1
O22 : H21		*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O22 : H–		*2b*	*2d*					*iha*			$lpfA_{O113}$	1
O38 : H–	*1a*	*2a*	*2d*				*saa*	*iha*	*espP*		$lpfA_{O113}$	2
O79 : H7		*2a*		*estA1*					*espP*			1
O109 : H16		*2a*	*2g*	*estA1*	*astA*						$lpfA_{O113}$	2
		2a		*estA1*	*astA*						$lpfA_{O113}$	1
O109 : H–	*1a*			*estA1*	*astA*						$lpfA_{O113}$	2
		2a		*estA1*	*astA*	*eae*			*espP*			1
		2a			*astA*	*eae*			*espP*			2
O113 : H21		*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O113 : H–		*2a*	*2d*	*cdtV*			*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O118 : H12		*2a*			*astA*				*espP*			1
O150 : H–		*2a*									$lpfA_{O113}$	1
O157 : H7		*2c*				*eae*		*iha*	*espP*	*toxB*		2
		2c				*eae*			*espP*	*toxB*		1
O174 : H21		*2c*						*iha*			$lpfA_{O113}$	2
O178 : H19	*1a*	*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	3
		2a	*2d*					*iha*			$lpfA_{O113}$	2
O183 : H18	*1a*			*cdtIII*	*cnf2*		*saa*	*iha*	*espP*		$lpfA_{O113}$	1
O187 : H29		*2a*	*2b*	*2c*				*iha*				1
OUT : H11		*2a*					*saa*	*iha*	*espP*		$lpfA_{O113}$	1
OUT : H19		*2a*	*2c*				*saa*	*iha*	*espP*		$lpfA_{O113}$	1
OUT : H29		*2a*	*2b*	*2c*				*iha*				1
OUT : H–		*2a*						*iha*	*espP*		$lpfA_{O113}$	1
		2d						*iha*	*espP*		$lpfA_{O113}$	2

Table 2. Amino acid sequences of the C-terminal end of the A_2 subunit and the part of the B subunit of Stx2 subtypes

Subtype of Stx2	Serotype	Origin	A_2 subunit 288									297	11	B subunit						18
2a	O157 : H7	human	K	S	Q	F	L	Y	T	T	G	K	S	K	Y	N	E	D	D	T
2a	O91 : H21	human	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–[1)]
2a	O109 : H16	cattle	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2a	O178 : H19	cattle	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2a	O8 : H19	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	–	–	–
2a	O8 : H–	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	–	–	–
2b	O22 : H–	cattle	R	A	H	S	–	N	–	S	–	E	–	–	–	–	–	N	–	–
2c	O157 : H7	human	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–
2c	O157 : H–	human	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–
2c	O157 : H7	cattle	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–
2c	O174 : H21	cattle	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–
2d	O6 : H34	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O8 : H2	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O8 : H49	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O22 : H–	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O38 : H–	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O113 : H–	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2d	O178 : H19	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	–	N	–	–
2g	O109 : H16	cattle	–	–	–	S	–	–	–	–	–	E	–	–	–	–	G	D	N	–

[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). *astA* was detected in 12 strains (27%), and *estA1* and *astA* were detected together in 7 strains (O18:H7 and O109:H16/H−). *cdtIII* and *cnf2* coexisted in 2 strains (O1:H7 and O183:H18), and *cdtV* 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-O157 STEC strains (16). *saa*, which is the major adherence 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 O157. 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). *stx2a* of O8:H19 and O8:H− showed the same amino acid sequences as *stx2d* in the A_2 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 $lpfA_{O113}$ are 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:H8, 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−, O178:H19, and OUT:H−) from cattle; all of them were *eae*-negative, and 3 (1 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*, and *espP*; 2 O109:H− carried *stx1a*, *estA1*, *astA*, 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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