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**Short Communication**

# Relationship between *stx* Genotype and Stx2 Expression Level in Shiga Toxin-Producing *Escherichia coli* O157 Strains

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**SUMMARY**: To determine the expression level of Shiga toxin (Stx) 2-related toxins (Stx2 and Stx2c) produced by each of 33 Stx-producing *Escherichia coli* (STEC) O157 strains, *stx2* and *stx2c* mRNAs (*stx2*-related mRNA) were measured using real-time PCR with primers that recognize sequences common to *stx2* and *stx2c*. The amount of Stx2 and Stx2c protein was measured using a reversed passive latex agglutination (RPLA) kit. Expression of *stx2*-related mRNA was significantly higher in STEC O157 strains carrying the *stx2* gene (i.e., *stx2*, *stx1/stx2*, or *stx2/stx2c*) than in most strains that carried the *stx2c* gene but not the *stx2* gene (i.e., *stx2c* or *stx1/stx2c*). RPLA might not measure the precise amount of each toxin variant; nevertheless, *stx2*-inclusive strains had 40-fold higher mean toxin titers than did strains that carried the *stx2c* gene but not the *stx2* gene, with the exception of 1 *stx2c* strain. Interestingly, 1 *stx2c* strain that was isolated from a patient with severe hemorrhagic diarrhea had the highest *stx2*-related mRNA expression and the highest toxin titer of all 33 STEC O157 strains. Taken together, these findings indicated that measurement of *stx2*-related mRNA expression could reflect differences in production levels of toxins among STEC strains.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are emerging pathogens of significant clinical and public health concern that can cause symptoms ranging from mild to severe, including diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome, in humans (1). A STEC strain can produce any one of several Stx proteins or some combination of these. These proteins include Stx1, Stx1c, Stx1d, Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g, and these toxins are encoded by the *stx1*, *stx1c*, *stx1d*, *stx2*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g* genes, respectively. In Japan, O157 is the dominant serogroup among STEC of human origin, and most O157 isolates from humans produce Stx1, Stx2, Stx2c, or some combination of these 3 Stx variants (2–5). In our previous study, we demonstrated that each of 268 STEC O157 strains of human (211 strains) or cattle (57 strains) origin had 1 of the following 6 genotypes: *stx1* alone (hereafter, *stx1*), *stx2* alone (hereafter, *stx2*), *stx2c* alone (hereafter, *stx2c*), both *stx1* and *stx2* (hereafter, *stx1/stx2*), both *stx1* and *stx2c* (hereafter, *stx1/stx2c*), or both *stx2* and *stx2c* (hereafter, *stx2/stx2c*) (3). Of these 6 genotypes, only 5 (*stx1* excluded) were detected among human isolates, and 5 genotypes (*stx2/stx2c* excluded) were detected among cattle isolates.

Siegler et al. and Boerlin et al. showed that enteric infection by Stx2-producing *E. coli* was more likely to cause severe human disease than infection by organisms that produce only Stx1 (6,7). Furthermore, most STEC O157 strains carrying the *stx2* gene (i.e., *stx1/stx2*, *stx2*, and *stx2/stx2c*) are more virulent than strains that do not carry the *stx2* gene (i.e., *stx1*, *stx1/stx2c*, and *stx2c*) (2–5); therefore, it is reasonable to speculate that the severity of STEC O157-related diseases in humans may be associated with the *stx* genotype of the STEC O157 strain and that the difference in virulence between Stx2 and Stx2c may be because of the toxin expression level or the affinity of the Stx variant for the glycolipid toxin receptor (globotriaosylceramide [Gb3]) that is present on target tissues in humans (8), or some other unnamed factors. It is not clear, however, which factors are most important in causing severe symptoms in humans. Here, we measured Stx2 and Stx2c proteins using a reversed passive latex agglutination (RPLA) kit (9) and *stx2* and *stx2c* mRNAs using real-time PCR (10).

The STEC O157 strains ($n$ = 33) used in this study were isolated from 25 humans and 8 cattle. All samples were collected in Miyazaki Prefecture, Japan; the human fecal samples were collected from 2000 to 2010, and the cattle fecal samples were collected from 2008 to 2009. The 25 isolates of human origin comprised 12 representative isolates, 1 from each of 12 outbreaks (1 group outbreak and 11 family outbreaks), and 13 isolates from 13 sporadic cases. All 5 genotypes (*stx2, stx2c, stx1/stx2, stx1/stx2c*, and *stx2/stx2c*) detected among the 211 strains of human origin in our previous study were represented in this group of 25 human strains; obviously, none of these 25 strains had the *stx1*

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genotype. There were 2 serotypes of O157:H7 (24 strains) and O157:H- (1 strain) among the 25 isolates of human origin. The strains of cattle origin were isolated from 8 healthy cattle in slaughterhouses. Only 4 (*stx2, stx2c, stx1/stx2*, and *stx1/stx2c*) of the 5 genotypes detected among the 57 strains of cattle origin in our previous study were represented in this group of 8 cattle strains; none of these 8 strains had the *stx1* or the *stx2/stx2c* genotype. The 2 isolates in each of the 4 genotypic groups were derived from cattle on different farms, and the genetic patterns, which were determined using pulsed-field gel electrophoresis (PFGE), of all 8 cattle isolates were different from each other. All strains isolated from cattle had the O157:H7 serotype.

The bacterial strains were grown in CAYE broth (Denka Seiken Co., Tokyo, Japan) at 37°C and shaken for 2, 4, 5, 6, 7, 8, 9, or 24 h; each culture had a beginning concentration of between $9 \times 10^4$ and $1.1 \times 10^5$ cfu/ml. For each culture, the amount of *stx2* and *stx2c* mRNA, the amount of Stx2 and Stx2c, and the number of bacteria were determined.

To measure mRNA, individual total RNA samples were extracted and purified from 0.5 ml of each bacterial culture using RNAprotect Bacteria Reagent (Qiagen, Tokyo, Japan). DNA was removed from total RNA samples using DNase I (RNase-free; TaKaRa Bio Inc., Shiga, Japan) digestion, and each total RNA sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif., USA). Aliquots of the resulting cDNA samples were used as templates for real-time PCR. Real-time PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) according to the Taqman PCR method described by Nielsen and Andersen (10) to quantitatively measure mRNA of *stx2* and *stx2c* (absolute quantification). This real-time PCR assay can specifically detect 3 *stx2* variants (*stx2, stx2c, stx2d*) because the primers and probes used recognize sequences common to the *stx2*, *stx2c*, and *stx2d* genes, but not the *stx2e* gene. In this study, the real-time PCR assay method was used to measure the mRNA expressed from the *stx2* or *stx2c* gene, or from the *stx2* and *stx2c* genes together in each of the STEC O157 strains; hereafter, *stx2* mRNA, *stx2c* mRNA, and *stx2*-related mRNA (both together).

To accurately assess the differences in *stx2*-related mRNA expression among STEC O157 strains, we made relative quantifications, which normalize expression values of the target mRNA using the expression level of a housekeeping mRNA (an internal standard) in the RNA samples (11). The *tufA* gene encodes the EF-Tu elongation factor that functions in protein synthesis; EF-Tu is an abundant cellular housekeeping protein in *E. coli*, and *tufA* mRNA was used as the internal standard. Primers and a probe for real-time PCR amplification of *tufA* were prepared according to the method reported by Sablet et al. with the following changes: the forward primer was mTufAqF (CATGGTTGATGAC GAAGAGCTG), the reverse primer was mTufAqR (CACGCTCTGGTTCCGGAAT) (11), and a new probe, TufAqP (FAM-CGATCGTTCGTGGTTCTGC TCTGAA-TAMURA), was designed. Real-time PCR amplification of *stx2*-related mRNA and *tufA* mRNA was performed in parallel in separate wells of the same multi-well plate. The amount of *stx2*-related mRNA and *tufA* mRNA in each sample was determined using the method described by Sablet et al. with slight modification (11). In this method, PCR amplifications of genomic DNA from a *stx2* genotype O157 culture were used to generate standard curves.

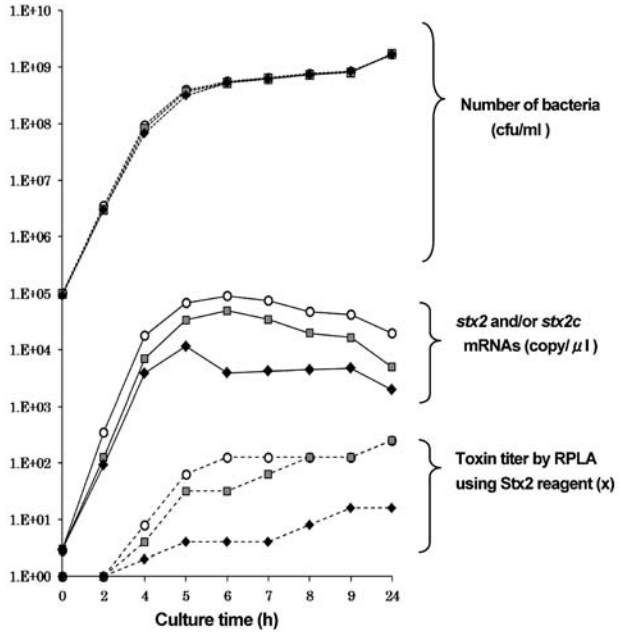


Fig. 1. The kinetics of *stx2* and *stx2c* mRNA expression and Stx2 and Stx2c toxin production in STEC cultures. The dotted line graphs show the number of bacteria. The line graphs show the amounts of *stx2*- and/or *stx2c*-mRNA measured by real-time PCR. The dashed line graphs show the amounts of Stx2 and/or Stx2c as measured by RPLA. The genotype of each of the three bacterial strains was *stx1/stx2* (○), *stx2* (■) or *stx2c* (◆).

Toxin production was assessed using RPLA; culture supernatants treated with polymyxin B were used as samples. RPLA was performed using a commercial kit (VTEC-RPLA; Denka Seiken) according to the manufacturer's instructions (9). The Stx2–Stx2c titer of each culture was expressed as the reciprocal of the highest dilution factor (×) that caused agglutination using the latex reagent. The number of bacteria in each culture was calculated from the optical density at 630 nm ($OD_{630}$) of each culture using a standard curve.

The kinetics of *stx2*-related mRNA expression (absolute amount) and titers of Stx2 and Stx2c in 3 strains—*stx2*, *stx1/stx2*, and *stx2c* (Nos. 3, 14, and 25 in Fig. 2, respectively)—were compared to assess differences among genotypes in toxin production (Fig. 1). During the first 24 h after inoculation, increases in the number of bacteria were similar between cultures, regardless of the *stx* genotype. In contrast, mRNA expression varied among the strains. In *stx2* and *stx1/stx2*, mRNA expression reached a peak value 6 h after inoculation, but in *stx2c*, expression peaked 5 h after inoculation. The peak value in *stx2* and *stx1/stx2* were 4-fold and 8-fold greater, respectively, than that in the *stx2c* strain.

The Stx2 and Stx2c titer in each strain began to increase 4 h after bacterial inoculation and reached a peak 9 or more hours after inoculation. The peak titers in the *stx2* and *stx1/stx2* strains were 256×, but that in the

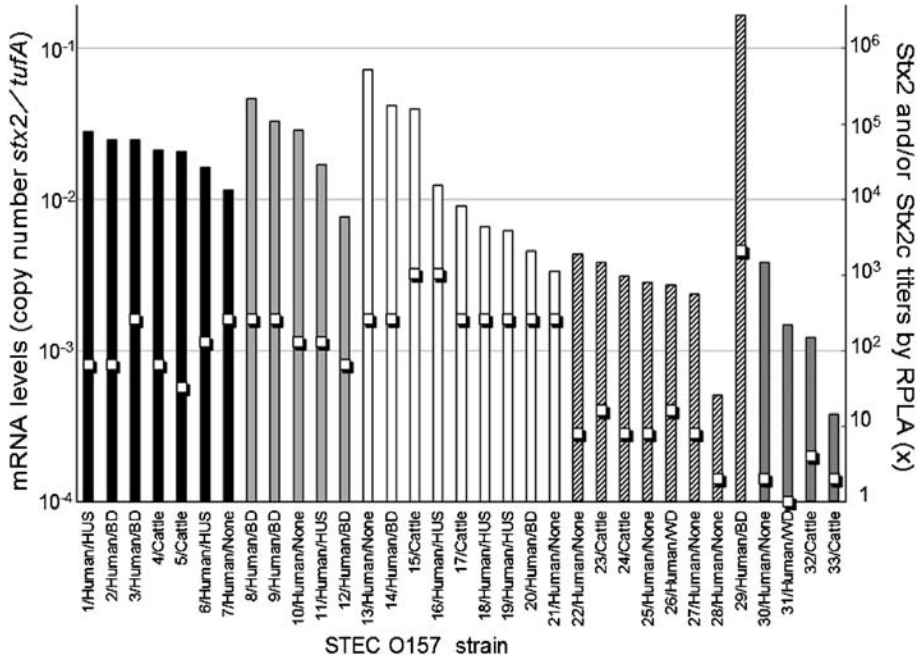


Fig. 2. Expression of *stx2* and *stx2c* mRNA and production of Stx2 and/or Stx2c in 33 STEC isolates. Bar graphs show the relative amounts of *stx2* and/or *stx2c* mRNA to *tufA* mRNA as measured by using real-time PCR in bacterial cultures after 6-h incubation of the *stx2* (■), *stx2/stx2c* (▒), *stx1/stx2* (□), *stx2c* (▨), and *stx1/stx2c* (▒) strains. The white square (□) indicates the Stx2 and/or Stx2 titers measured by RPLA in bacterial cultures after 24-h incubation. HUS, hemolytic uremic syndrome; BD, bloody diarrhea; WD, watery diarrhea; None, asymptomatic.

*stx2c* strain was 16×. The peak value of the toxin titer for the *stx2* and *stx1/stx2* strains was 16-fold greater than that for the *stx2c* strain.

Total *stx2*-related mRNA and Stx2 and Stx2c was measured in 33 O157 strains, which represented 5 different genotypes. The strains were cultured for the appropriate number of hours, which was determined by examination of the 3 representative strains described above. Expression of *stx2*-related mRNA was measured 6 h after inoculation, and toxin production was measured 24 h after inoculation.

The expression of *stx2*-related mRNA in each of the 33 isolates was measured by relative quantitative real-time PCR (Fig. 2). The isolates with genotypes that included the *stx2* gene (i.e., *stx2*, *stx1/stx2*, and *stx2/stx2c* strains) had significantly higher mRNA expression levels than isolates with genotypes that included the *stx2c* gene but not the *stx2* gene (i.e., *stx2c* strains [except No. 29 in Fig. 2] and *stx1/stx2c* strains) (*t* test, $P = 6.08 \times 10^{-8}$). The mean mRNA level of the strains carrying the *stx2* gene was about 10-fold higher than that of the strains that carried the *stx2c* gene but not the *stx2* gene. Only 1 *stx2c* strain (No. 29 in Fig. 2), which had a remarkably high level of *stx2c* mRNA, did not conform to this pattern. For each genotype, no difference in mRNA expression was evident between isolates of cattle origin and those of human origin.

For each of the 33 isolates, the amount of Stx2 and/or Stx2c produced in culture was measured by RPLA (Fig. 2). Isolates with *stx2*-inclusive genotypes (i.e., *stx2*, *stx1/stx2*, and *stx2/stx2c*) had high Stx2 and/or Stx2c titers that ranged from 32× to 1,024×. In contrast, each isolate, except 1, with a genotype that included the *stx2c* gene but not the *stx2* gene (i.e., *stx2c* and *stx1/stx2c* strains), had low titers that ranged from 1× to 32×. The 1 exception was the *stx2c* isolate with remarkably high *stx2c* mRNA expression level. It had a toxin titer of 2,048×. If the *stx2c* strain (No. 29 in Fig. 2) with remarkably high mRNA expression level and toxin titer was excluded from the analysis, the mean toxin titer of the *stx2*-inclusive strains was about 40-fold higher than that of the strains that did not include *stx2*.

RPLA is based on antigen-antibody reactions. Karmali et al. showed that the anti-Stx2 latex reagent could react with both Stx2 and Stx2c, but that it was 30-fold less sensitive to purified Stx2c than purified Stx2 (9); therefore, RPLA might not be able to measure the precise amount of each toxin variant (i.e., the 40-fold difference in titers observed between *stx2*-inclusive strains and *stx2c*-inclusive non-*stx2* strains in this study may not have represented the actual quantitative difference in toxin production).

The Vero cell assay is a well-known and standard method used to quantify Stx proteins. In our previous experiments, among strains of the 5 different *stx* genotypes (*stx2, stx2c, stx1/stx2, stx1/stx2c*, and *stx2/stx2c*), most *stx2c* strains showed lower cytotoxicity on Vero cells than strains with any of the other 4 genotypes (data not shown); however, Lindgren et al. showed that purified Stx2vhb (approximately 5 pg/$CD_{50}$), which has B subunit sequences that are identical to those of Stx2c, is approximately 100-fold less reactive than purified Stx2 (approximately 500 pg/$CD_{50}$) on Vero cells. This difference is a result of the lower affinity of Stx2vhb for the glycolipid receptor Gb3 that is caused by the 1-amino-acid difference shared by the B subunits of Stx2vhb and Stx2 (12); therefore, because of the limitations of the Vero and RPLA assays, it is difficult to as-

sess the precise differences in toxin production among strains expressing these Stx variants using these assays.

Here, *stx2* and *stx2c* mRNAs were quantified to assess the quantitative difference in toxin production among 33 O157 strains, each of which had 1 of 5 different *stx* genotypes. Based on our analysis of the 33 O157 isolates, expression of *stx2*-related mRNA in *stx2*-inclusive isolates (i.e., *stx2, stx1/stx2*, and *stx2/stx2c* strains) was significantly higher on average than that in isolates with a genotype that included the *stx2c* gene but not the *stx2* gene (i.e., *stx2c* and *stx1/stx2c*). This finding strongly indicated that O157 strains with a genotype that included the *stx2* gene produced more *stx2*-related toxin than did those with a genotype that included the *stx2c* gene but not the *stx2* gene. Furthermore, this difference in mRNA expression must affect pathogenicity in humans; however, it is not currently known whether the levels of toxin production observed in vitro correspond to the amount of toxin produced in vivo.

Our results were consistent with those of Zhang et al. (13) who divided *E. coli* O157:H7 strains into 3 major genetic lineages—lineage (L) I (which includes strains with the *stx2* gene), LI/II (which includes strains with the *stx2* gene or *stx2/stx2c* genes), and LII (which includes strains with the *stx2c* gene)—and showed that LI and LI/II strains produced significantly higher levels of *stx2* and/or *stx2c* mRNA than LII strains. They also showed that LI and LI/II strains are more frequently associated with human diseases than LII strains. Their classification (lineage) seems to differ from our classification (*stx* genotype); however, both systems are highly dependent on the *stx2* genotype. Our results were thought to be comparable with their results; therefore, the differences in the amount of toxin produced might be 1 of the factors that cause differences in virulence between Stx2 and Stx2c. There may, however, be other causal factors such as a difference in affinity of Stx for the toxin receptor Gb3 that is present on target tissues in humans.

Another interesting finding was the high levels of toxin production in 1 particular *stx2c* STEC O157 strain. In general, the *stx2c* O157 strains expressed less *stx2*-related mRNA than did the *stx2* strains; however, 1 *stx2c* strain, isolated from a patient with severe hemorrhagic diarrhea, had the highest *stx2*-related mRNA expression and the highest toxin titer of all 33 strains (Fig. 2). Only 1 such highly virulent *stx2c* strain was isolated so far in our study; therefore, the probability of detecting such a strain is very low. Nevertheless, the existence of this strain indicated that even *stx2c* strains can cause serious symptoms.

In conclusion, we measured *stx2* and *stx2c* mRNA levels in 33 STEC isolates using real-time PCR and found that isolates with *stx2*-inclusive genotypes (i.e., *stx2, stx1/stx2*, and *stx2/stx2c* strains) had significantly higher mean values of mRNA expression than isolates with genotypes that included the *stx2c* gene but not the *stx2* gene (i.e., *stx2c* and *stx1/stx2c* strains). Only 1 exceptional isolate had the *stx2c* genotype and a remarkably high mRNA expression level.

Taken together, these findings indicate that measurement of *stx2*-related mRNA expression could reflect the differences in toxin production levels among STEC strains that expressed different toxin variants; therefore, measurement of *stx2* and *stx2c* mRNA could be used to assess the actual production of Stx2 and Stx2c, and this production is thought to be important in determining the severity of illness.

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**Conflict of interest** None to declare.

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