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Relationship of Genetic Type of Shiga Toxin to Manifestation of Bloody Diarrhea due to Enterohemorrhagic *Escherichia coli* Serogroup O157 Isolates in Osaka City, Japan

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One hundred sixty-nine strains of enterohemorrhagic *Escherichia coli* serogroup O157 were examined for the correlation between the genotype of their Shiga toxin genes (*stx*) and manifestation of bloody diarrhea (BD). It was shown that the strains carrying only *stx2vha* were probably less virulent and caused BD less frequently.

Enterohemorrhagic *Escherichia coli* (EHEC) of serogroup O157 was recognized as the causative agent following two outbreaks of hemorrhagic colitis (HC) in 1982 (12). The organisms are characterized by their ability to elaborate Shiga toxin (Stx) (2, 8). The association of EHEC with HC and hemolytic-uremic syndrome (HUS) implies that Stx is a major virulence factor in these diseases (4, 11, 15, 17). However, the role of Stx in the pathogenesis of HC remains to be elucidated. Tzipori et al. showed that Stx-negative variant strains still caused diarrhea (19). Attempts to link Stx with clinical manifestations of HC have been performed with epidemiological, clinical, and animal model investigations (6, 7, 10). However, these studies were performed prior to the discovery of Stx2 by Scotland et al. (14). Hence, it is not clear from reading the original sources whether single or multiple forms of Stx were being produced.

To assess the relationship between the manifestation of bloody diarrhea (BD) and Stx, EHEC O157 strains were examined by typing of the Stx genes (*stx*) and Stx production. The properties of the strains were correlated with the clinical symptoms of the persons from whom the strains were isolated.

A total of 357 EHEC O157 strains originally isolated from 1996 to 1998 in Osaka City, Japan, was used. They were assigned to groups on the basis of phage typing and DNA typing with pulsed-field gel electrophoresis as reported (Y. Nishikawa, A. Hase, J. Ogasawara, T. Cheasty, G. A. Willshaw, B. Rowe, and A. Yasukawa, presented at the Third Int. Symp. Workshop Verocytotoxin-Producing *Escherichia coli* Infections, Baltimore, Md., 1997). To provide independent clones for further analysis, 169 strains were chosen as the representatives of 162 types. The representative strain was the earliest isolate among strains belonging to each type. Thirteen strains that represented six types but were isolated in different years were treated as putative different clones. Finally, 169 strains of 162 types were examined to assess the relationship between Stx genotypes and manifestation of BD.

Stx2 genotypes were analyzed by the predicted restriction enzyme fragment length polymorphisms of PCR products (18). Stx2e and Stx2ev genes were examined using primer pairs reported by Cao et al. and Johnson et al., respectively (1, 3). The strains were examined for Stx production within two months after isolation using the RPLA test (VTEC-RPLA lots 31608, 29809, and 27808; Denka Seiken Co. Ltd., Tokyo, Japan) (5); it was confirmed that the sensitivities of the kits used differed by no more than a factor of 2 throughout the study.

The 169 strains were assigned to seven groups according to their type of *stx* (Table 1). There were no O157 strains that were positive for *stx2vhb*, *stx2e*, or *stx2ev* in this study. This is concordant with the study of Tyler et al., in which *stx2vhb* was found only among non-O157 Stx-producing *E. coli* strains (18). Symptoms of BD were reported for 50% (48 of 96) of patients from whom isolates possessing both *stx1* and *stx2* were isolated; BD was defined as diarrhea that contained blood that could be observed macroscopically. BD was reported for 55% (6 of 11), 39% (9 of 23), 18% (2 of 11), and 8% (2 of 25) of patients infected by isolates possessing only *stx2*, both *stx2* and *stx2vha*, both *stx1* and *stx2vha*, and only *stx2vha*, respectively (Table 1). After the strains were assigned to three groups on the basis of Stx2 genotypes, these results indicated that patients with strains that possessed *stx2* or both *stx2* and *stx2vha* often presented with BD. On the other hand, the patients from whom

TABLE 1. Relationship among type of Stx genes, anti-Stx2 RPLA titer of EHEC O157 strains, and manifestation of BD

RPLA titer with anti-Stx2 latex	No. of BD patients with indicated genotype/total infected					
	stx1 and *stx2*	*stx2*	*stx2* and *stx2vha*	*stx1* and *stx2vha*	*stx2vha*	*stx1*, *stx2*, and *stx2vha*
0	0	0	0	0/1	0	0
1	0/1	0	0	1/6	0/3	0
10	0	0/1	0	0	0	0
20	0/1	0	0	0/1	0/5	0
40	1/4	0	0	0/2	0/3	0
80	3/5	1/3	0	0	0/2	1/1
160	7/13	2/2	3/5	0	2/5	0/1
320	11/22	1/2	0/2	0	0/3	0
640	8/15	1/1	0/6	1/1	0/4	0
1,280	14/29	1/2	5/7	0	0	0
2,560	4/6	0	1/3	0	0	0
Subtotal	48/96	6/11	9/23	2/11	2/25	1/2

[a] One strain possessed only *stx1*.

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TABLE 2. Relationship between type of Stx2 genes of EHEC O157 strains and manifestation of BD

Genotype of Stx2 genes	No. of BD patients/total infected (%)	Age (yr) (mean ± SE)	No. of infected persons	
			Male	Female
stx2	54/107 (50)[a]	24.9 ± 2.2	45	62
stx2 and *stx2vha*	10/25 (40)[b]	28.1 ± 4.5	8	17
stx2vha	5/36 (14)	24.1 ± 3.6	19	17

[a] Significantly different from the group of *stx2vha* at a 1% level (Fisher's exact test).
[b] Significantly different from the group of *stx2vha* at a 5% level (Fisher's exact test).

organisms having only *stx2vha* were isolated manifested BD with lower frequency (Table 2).

Significant differences were not observed in the ages and sexes of patient groups from which each *stx* type of organism was isolated (Table 2); however, the patients with BD were significantly younger than infected persons without BD (Table 3). It was not suggested that particular phage and/or DNA types were related to manifestation of BD (data not shown).

Why the strains that possessed only *stx2vha* caused BD less frequently remains to be elucidated. However, it may reflect functional difference between Stx2 and Stx2vha. Stx1 and Stx2 bind preferentially to Gb_3 [Galα(1→4)Galβ(1→4)Glc-ceramide] (20), while Stx2vha and Stx2e bind preferentially to Gb_4 [GalNAcβ(1→3)Galα(1→4)Galβ-(1→4)Glc-ceramide] (13). Takeda et al. observed that Vero cell cytotoxicity and mouse lethality of Stx2vh were somewhat lower than those of Stx2 (16). Tyler et al. (18) also reported that the culture supernatants of strains carrying only Stx2 variant genes gave low cytotoxin titers in HeLa cell assays. Differences in these biological activities can possibly explain why the strains that possessed only *stx2vha* caused BD less frequently.

TABLE 3. Relationship between the ages of infected persons and manifestation of BD

BD	Patient age (yr) (mean ± SE)	No. of infected persons	
		Male	Female
Positive	20.6 ± 2.6[a]	28	42
Negative	28.6 ± 2.2	44	55

[a] Significantly younger than infected persons without BD ($P < 0.01$, Mann-Whitney U test).

Alternatively, it was possible that the quantity of Stx produced was an important factor. The amount of Stx that was reactive to the anti-Stx2 latex particles depended on the Stx genotype. Strains possessing *stx2vha* tended to have lower titers than strains with *stx2*, regardless of the presence or absence of *stx1* (Fig. 1). It is possible that the anti-Stx2 latex was less reactive to Stx2vha. To address this, gene sequences in strains possessing only *stx2vha* were preliminarily examined. No difference was found in the sequence of *stx2vha* between two strains that produced high titers and two with low titers (data not shown), suggesting that Stx2vha molecules produced by these strains were identical. Therefore, the low titers expressed by the majority of strains possessing only *stx2vha* seemed to reflect a lower production of Stx2vha. If so, the discrepancy in manifestation of BD could be due to the Stx levels produced by each strain.

It appeared that the strains carrying *stx2* tended to cause BD more than strains possessing only *stx2vha*. Classical HUS develops typically a few days after the onset of an acute diarrheal prodromal illness, which is often bloody. In cases of patients infected with EHEC O157, it may be that HUS is also caused by organisms having *stx2* or both *stx2* and *stx2vha* rather than bacteria with only *stx2vha*, although BD is not an essential symptom for HUS. In fact, all six strains isolated from HUS

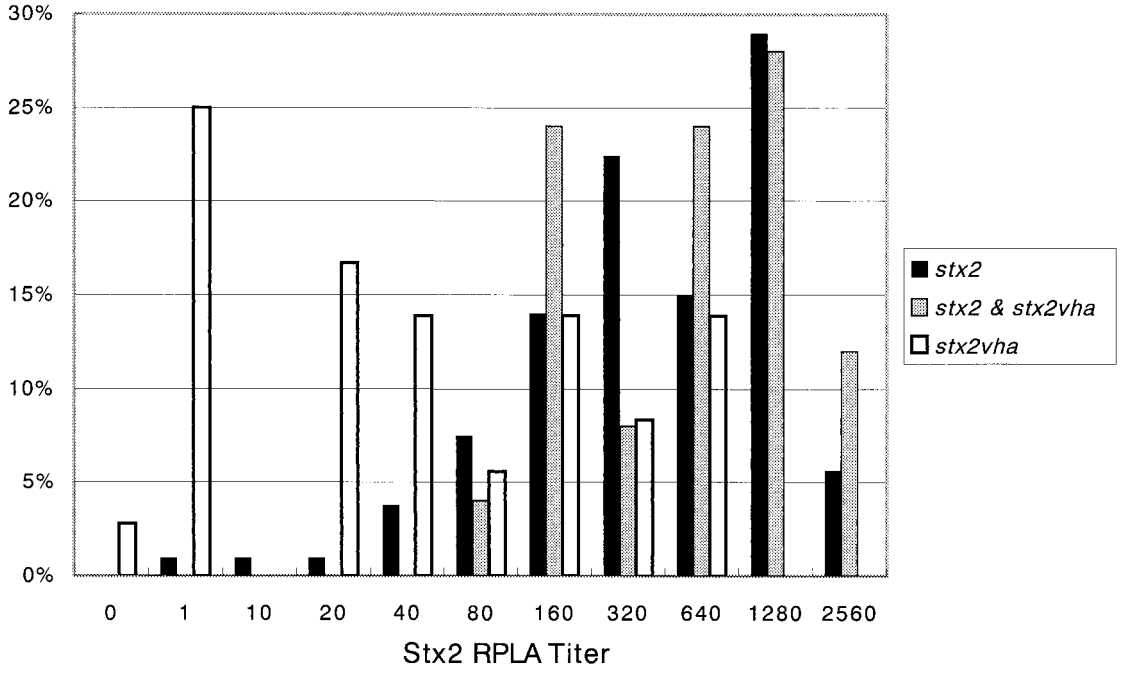

FIG. 1. Relationship between Stx2 RPLA titer and the genotype of EHEC O157 strains. One hundred sixty-eight strains of EHEC O157 were assigned to three groups on the basis of their types of Stx2 genes. Proportions of strains that showed each RPLA titer in the genogroup are indicated.

patients who first contracted BD in this study possessed both *stx1* and *stx2*. Ostroff et al. suggested that EHEC O157 strains that produced Stx2 alone were more likely to be associated with the development of HUS than strains that produced Stx1 alone or Stx1 and Stx2 (9). In the present investigation, however, the possession of *stx1* did not have any negative influence on manifestation of BD. BD was found more among patients infected by strains with both *stx1* and *stx2vha* (18%) than among those infected by strains with *stx2vha* alone (8%) (Table 1). Of 18 strains that did not possess *stx1* and gave a low RPLA titer of Stx2 (<80), only one was from a patient with BD. On the other hand, 6 of 22 strains that gave an Stx2 titer of less than 80 but had *stx1* were from patients with BD. These differences, however, were not statistically significant, and the role of Stx1 in BD could not be evaluated since there was only a single strain that produced only Stx1 in this study.

In conclusion, the present study indicates that the typing of Stx2 genes of EHEC O157 isolates provides useful information not only for epidemiological analysis but for prognosis, as strains carrying only *stx2vha* are possibly less virulent and cause BD less frequently.

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REFERENCES

1. **Cao, C., S. Yamasaki, Z. Lin, H. Kurazono, and Y. Takeda.** 1994. Specific detection of a Verotoxin 2 variant, VT2vp1, by a bead-enzyme-linked immunosorbent assay. Microbiol. Immunol. **38:**435–440.
2. **Johnson, W. M., H. Lior, and G. S. Bezanson.** 1983. Cytotoxic *Escherichia coli* O157:H7 associated with haemorrhagic colitis in Canada. Lancet **i:**76.
3. **Johnson, W. M., S. D. Tyler, G. Wang, and H. Lior.** 1991. Amplification by the polymerase chain reaction of a specific target sequence in the gene coding for *Escherichia coli* verotoxin (VTe variant). FEMS Microbiol. Lett. **84:**227–230.
4. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.
5. **Karmali, M. A., M. Petric, and M. Bielaszewska.** 1999. Evaluation of a microplate latex agglutination method (Verotox-F assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. J. Clin. Microbiol. **37:**396–399.
6. **Keenan, K. P., D. D. Sharpnack, H. Collins, S. B. Formal, and A. D. O'Brien.** 1986. Morphologic evaluation of the effects of Shiga toxin and *Escherichia coli* Shiga-like toxin on the rabbit intestine. Am. J. Pathol. **125:**69–80.
7. **Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154:**338–341.
8. **O'Brien, A. D., G. D. LaVeck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **146:**763–769.
9. **Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi.** 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994–998.
10. **Pai, C. H., J. K. Kelly, and G. L. Meyers.** 1986. Experimental infection of infant rabbits with verotoxin-producing *Escherichia coli*. Infect. Immun. **51:** 16–23.
11. **Riley, L. W.** 1987. The epidemiologic, clinical, and microbiologic features of hemorrhagic colitis. Annu. Rev. Microbiol. **41:**383–407.
12. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
13. **Samuel, J. E., L. P. Perera, S. Ward, A. D. O'Brien, V. Ginsburg, and H. C. Krivan.** 1990. Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect. Immun. **58:** 611–618.
14. **Scotland, S. M., H. R. Smith, and B. Rowe.** 1985. Two distinct toxins active on Vero cells from Escherichia coli O157. Lancet **ii:**885–886.
15. **Smith, H. R., and S. M. Scotland.** 1988. Vero cytotoxin-producing strains of *Escherichia coli*. J. Med. Microbiol. **26:**77–85.
16. **Takeda, Y., H. Kurazono, and S. Yamasaki.** 1993. Vero toxins (Shiga-like toxins) produced by enterohemorrhagic *Escherichia coli* (Verocytotoxin-producing *E. coli*). Microbiol. Immunol. **37:**591–599.
17. **Tarr, P. I.** 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. **20:**1–10.
18. **Tyler, S. D., W. M. Johnson, H. Lior, G. Wang, and K. R. Rozee.** 1991. Identification of verotoxin type 2 variant b subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. **29:**1339–1343.
19. **Tzipori, S., H. Karch, K. I. Wachsmuth, R. M. Robins-Browne, A. D. O'Brien, H. Lior, M. L. Cohen, J. Smithers, and M. M. Levine.** 1987. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. **55:**3117–3125.
20. **Waddell, T., S. Head, M. Petric, A. Cohen, and C. Lingwood.** 1988. Globotriosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2. Biochem. Biophys. Res. Commun. **152:**674–679.