Identification and Epidemiological Description of Enterohemorrhagic *Escherichia coli* O157 Strains Producing Low Amounts of Shiga Toxin 2 in Aichi Prefecture, Japan

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**SUMMARY:** Out of 68 *Escherichia coli* O157 field isolates tested in vitro for Shiga toxin (Stx2) 2 production, 12 (17.6%) produced no or a limited amount of Stx2 (Stx 2 non- or low-producing strain; TNLP) even though all 68 possessed the *stx*2 gene. The remaining 56 were Stx2 high-producing strains. The 12 TNLPS carried the *q21* gene allele, which encodes a transcription antiterminator Q protein and is highly homologous to that of *Φ21* phage. They also carried nucleotide substitutions and insertions in the promoter region of the *stx*2 gene compared with that of O157 EDL933, producing a considerable amount of Stx2. In contrast, the Stx2 high-producing strains carried the *q933* gene allele, which was first reported on an *stx*2 (933W), but not the *q21* gene allele, and did not have mutations in the promoter region of the *stx*2 gene. These 2 genetic characteristics, i.e., replacement of the *q* gene and mutation in the promoter region of the *stx*2 gene, seemed to determine the amount of Stx2 produced by each strain. The TNLPS were more frequently isolated from healthy carriers than from patients (*P* < 0.05), suggesting that TNLPS are less virulent than those with normal Stx2 production.

**INTRODUCTION**

Since a large outbreak of food-borne enteritis caused by enterohemorrhagic *Escherichia coli* (EHEC) O157 occurred in 1996 in Sakai City (1), O157 has become one of the most serious public health concerns in Japan. Shiga toxin (Stx) is a major virulence factor in O157 and is closely related to its clinical manifestations. Recently, Ishiguro et al. (2) reported that a significant portion of their clinical O157 isolates (33/106, 31.1%) produced no- or a low-level of Stx2; these isolates are designated here as TNLPs (S *lates*) (3). Among these isolates (933W, 31.1%) produced no- or a limited amount of Stx2; these isolates are designated here as TNLPs (S *lates*) (33/106, 31.1%) produced no- or a low-level of Stx2; these isolates are designated here as TNLPs (S *lates*) (3). Out of 68 *Escherichia coli* O157 field isolates tested in vitro for Shiga toxin (Stx2) 2 production, 12 (17.6%) produced no or a limited amount of Stx2 (Stx 2 non- or low-producing strain; TNLP) even though all 68 possessed the *stx*2 gene. The remaining 56 were Stx2 high-producing strains. The 12 TNLPS carried the *q21* gene allele, which encodes a transcription antiterminator Q protein and is highly homologous to that of *Φ21* phage. They also carried nucleotide substitutions and insertions in the promoter region of the *stx*2 gene compared with that of O157 EDL933, producing a considerable amount of Stx2. In contrast, the Stx2 high-producing strains carried the *q933* gene allele, which was first reported on an *stx*2 (933W), but not the *q21* gene allele, and did not have mutations in the promoter region of the *stx*2 gene. These 2 genetic characteristics, i.e., replacement of the *q* gene and mutation in the promoter region of the *stx*2 gene, seemed to determine the amount of Stx2 produced by each strain. The TNLPS were more frequently isolated from healthy carriers than from patients (*P* < 0.05), suggesting that TNLPS are less virulent than those with normal Stx2 production.

**MATERIALS AND METHODS**

**Bacterial strains and detection of *stx1* and *stx2* genes:** The 68 O157 strains analyzed were isolated from 55 patients and 13 healthy carriers in Aichi Prefecture between 1998 and 2006 (Table 1A, B). For sequencing- and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analyses, representative strains were chosen considering the year of isolation and toxin type. Stx genes, i.e., *stx1* and *stx2*, were detected by PCR using Kobayashi’s primer sets (4). *stx1* gene (5’-agtaatgtgguggaa-3’ and 5’-gacctctcagtgcg-3’), *stx2* gene (5’-tctggatcttctccg-3’ and 5’-tccttgtgtattac-3’) and/or a commercially available primer set (TAKARA BIO Inc., Otsu, Japan).
Reversed passive latex agglutination (RPLA) assay of Stx1 and Stx2: Each culture supernatant of all 68 O157:H7 strains was assayed for both the presence and the amount of Stx1 and Stx2. Sample preparation and a RPLA test were performed as described by Koitabashi et al. (3). The test strain was grown in CA-YE medium with shaking (120 rpm) at 37°C overnight. The bacterial culture was centrifuged (800 × g, 10 min), and the supernatant was obtained and used as the culture supernatant sample. Stxs in the culture supernatant were detected by a commercially available RPLA kit (VTec-RPLA; Denka-Seiken Co., Ltd., Niigata, Japan) according to the manufacturer’s specifications. This kit consisted of latex particles coated with rabbit anti-Stx (Stx1 or Stx2) immunoglobulin G, latex particles coated with immunoglobulin G from a non-immunized rabbit (a control), latex particles coated with purified Stxs (controls), and dilution buffer. The agglutination test was carried out in a 96-well U-bottom microtitre plate for 20 h at room temperature. For a quantitative assay, culture supernatant was twofold serially diluted from 2^1 to 2^11. The multiplier of the highest dilution of the sample that gave a positive reaction was defined as the amount of Stx. Finally, the amount of Stx for the sample was expressed as the mean of the results obtained by assays performed in triplicate.

Identification of q gene and sequencing of the promoter region in the stx genes: Identification of the q gene was performed by PCR using the corresponding primer sets (5,6). Briefly, 5 μL of DNA obtained from boiled stationary-phase bacteria was added to a 50-μL PCR master mix containing a final concentration of 1.5 (q933) or 2.5 (q21) mmol MgCl2, 200 μmol/L each deoxynucleoside triphosphate, 1U Taq polymerase, 0.6 μg/μL of 595 primer (6) (5'-ccgagaaa accgtaaacag-3') and 0.6 μg/μL of either primer q933 (5'- cggagggattgggaaggg-3') or q21 (5'-gaaatctcaaatgcctgtgct-3'). PCR consisted of an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 52°C (q933) or 55°C (q21) for 1 min, and 72°C for 2 min; and a final 10-min extension step at 72°C. All PCR products were separated by gel electrophoresis (100 V) in 2% agarose gels, stained with ethidium bromide, and visualized by UV illumination. For the analysis of the stx3 promoter region, a part of the PCR product was used for sequencing with a 595 primer and a slt2s-2 primer (5'-gttattatcctacgctcggccctt-3').

Pulsed-field gel electrophoresis (PFGE): PFGE was done according to a standard method (7). Briefly, bacterial cells grown overnight at 37°C on LB agar plates were suspended in 200 μL of distilled water, and the samples were mixed with an equal amount of 1% SeaKem Gold agarose (Lonza, Basel, Switzerland) in order to induce plug formation. The bacterial cells were lysed within the plugs by incubation with a lysis solution (0.5 M EDTA [pH 8], 1% sarcosine, and 1 mg of protease K per mL [Roche Diagnostics, Tokyo, Japan]) overnight at 50°C. The plugs were then washed twice with 500 μL of TE buffer (10 mM Tris and 1 mM EDTA [pH 8]) containing 4 mM Pefabloc SC at 50°C, and then 1 mL of TE buffer, and finally 200 μL of appropriate buffer for restriction enzyme digestion. Appropriately cut slices of plugs were incubated overnight with XbaI (Roche Diagnostics) at 37°C. The plugs were then loaded onto 1% SeaKem Gold agarose gels. PFGE was performed with the CHEF DR III (Bio-Rad Laboratories, Tokyo, Japan) using the following run parameters: a switch time of 2.2 to 54.2 s and a run time of 21 h. Dendrograms were created with the Fingerprinting II (Bio-Rad Laboratories) by using the Dice coefficient, the unweighted pair group method with arithmetic means (UPGMA), and a position tolerance of 1.0%.

PCR-RFLP and detection of EHEC virulence plasmid and eae gene: Genotyping of stx3 gene was performed by a PCR-RFLP method (8). PCR products of 285- and 346-bp were amplified using two primer sets: VT2-c&-d and VT2-e&-f, respectively. To confirm the identification of stx3 genotype, the product obtained with VT2-c&-d was digested with HaeIII and RsaI (SibEnzyme, Novosibirsk, Russia). The ampiclon obtained with VT2-c&-d was digested with PvuII (SibEnzyme). The resultant fragments were separated by agarose gel electrophoresis in 2% Agarose X (Wako Pure Chemical Industries Ltd., Osaka, Japan) and visualized by staining with ethidium bromide. EHEC virulence plasmid and eae gene were examined by PCR using the special primer sets MFS1-F&-R and AE-9&-10, respectively (9,10).

RESULTS

Detection of TNLP in the 68 O157 strains in Aichi: Of 68 strains, 43 turned out to possess both stx1 and stx2 genes, as well as a successful PCR for q933 and q21. 44 strains were noted to have q933 alone and one strain had q21 alone.

Table IA. Results of PCR-, sequencing- and PFGE-based analyses of 68 O157 strains

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>Source</th>
<th>stx gene</th>
<th>q gene</th>
<th>Type of mutation</th>
<th>PFGE Group</th>
<th>stx2 genotype</th>
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</thead>
<tbody>
<tr>
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<td>patient</td>
<td>stx1 &amp; 2s</td>
<td>q21</td>
<td>Thai-12</td>
<td>I(2)</td>
<td>stx1,2s</td>
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<tr>
<td>1998-2006</td>
<td>patient</td>
<td>healthy carrier</td>
<td>q933</td>
<td>933W</td>
<td>II(2)</td>
<td>stx2</td>
</tr>
<tr>
<td>2001</td>
<td>healthy carrier</td>
<td>stx3</td>
<td>ND</td>
<td>933W &amp; Thai-12</td>
<td>I(2)</td>
<td>stx1,2s</td>
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Table IB. Chronological distribution of TNLP and Stx2 high-producing strains

<table>
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<th>Year</th>
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<th>2001</th>
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<th>2005</th>
<th>2006</th>
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<tr>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>12</td>
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<tr>
<td>Stx2 high-producing strain</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>56</td>
</tr>
</tbody>
</table>

Only one strain per incident was included for this study.
but the remaining 25 were stx₁-negative and possessed the stx₂ gene as determined by PCR. The amount of Stx1 produced by the 43 stx₁ gene-positive strains ranged from 5.3 to 9.7 with an average of 7.1 (Fig. 1A). In contrast to Stx1, two clusters were seen in the amount of Stx2 produced by the 68 stx₂ gene-positive strains (Fig. 1B). Of the 68 strains, each of the 56 produced a significant amount, ranging from 6.7 to 11.0 with an average of 8.9, and hence were designated as Stx2 high-producing strains. The remaining 12 (12/68, 17.6%) were considered to be TNLPs. Seven out of the 12 TNLPs definitely produced Stx2 at a low level (3.0–5.3), the Stx2 production of 1 strain was unreplicable, and 4 were Stx2 non-producers.

Identification of the q gene and the mutation in the stx₂ promoter region in TNLP: Of 12 TNLPs, 11 (91.7%) carried the q21 gene (Table 1A) and 1 carried neither the q933 nor q21 gene. Sequence analyses of 10 representative strains having the q21 gene found that all of the strains had a Thai-12 type mutation in the promoter region of the stx₂ gene. Regarding the 56 Stx2 high-producing strains, 50 possessed the q933 gene, 4 carried both the q933 and q21 genes and 2 had only the q21 but not the q933 gene. Consequently, 54 (96.4%) of the 56 high-producing strains carried the q933 gene. The stx₂ gene promoter regions of 9 representative strains with q933 genes were determined, and all turned out to be of the 933W type. As for the strains carrying both the q933 and q21 genes, 933W type and Thai-12 type mutations were found in the stx₂ promoters located downstream of the q933 and q21 genes, respectively. The 2 high-producing strains with the q21 gene carried the Thai-12 type mutation. Stx2 production seemed to be regulated by both the q gene and the mutation in the stx₂ promoter. However, since exceptional strains such as Stx2 high-producers with a q21 gene were observed, additional unknown factors, for example, the infection of another phage having a sufficient amount of a functional Q protein (3), may be responsible for the regulation of Stx2 expression.

PFGE- and PCR-RFLP-based analysis of TNLP: PFGE- and PCR-RFLP-based analyses were performed to further characterize the 12 TNLPs. PFGE identified 3 genetically similar groups (Groups I-III) showing over 85% similarity (Fig. 2A, B). The band patterns among the 3 groups and the other 5 strains were genetically different from each other. The 2 Stx2 high-producing strains obtained in 2006 were also

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![Fig. 1.](image1) Stx1 and Stx2 productions by 68 O157 field isolates in Aichi Prefecture. Stx1, Shiga toxin 1; Stx2, Shiga toxin 2; TNLP, Stx 2 non-or low-producing strain.

![Fig. 2.](image2) (A) PFGE fingerprint pattern and (B) dendrogram of 12 TNLPs and 4 Stx2 high-producing strains. (A): Lambda DNA concatemers were placed in the left side end.
of Stx2 production may not always reflect toxin production, which would imply that Stx1 production may be critically important for the in vivo pathogenicity of TNLP.

Epidemiological analysis revealed the relationship between Stx2 production and the possible pathogenicity of O157 strains (Fig. 3). Of 55 strains from patients, 48 were Stx2 high-producing strains. The remaining 7 were TNLPs. On the other hand, of 13 strains from healthy carriers, 8 were Stx2 high-producing strains and 5 were TNLPs. Chi-square ($\chi^2$) test revealed that the TNLPs tended to be more often isolated from healthy carriers than were Stx2 high-producing strains ($P < 0.05$). Although the results of in vitro quantitative assay of Stx2 production may not always reflect toxin production in vivo, the TNLPs seemed to be less associated with pathogenicity. Of the 7 TNLPs from patients, 5 produced Stx1, but only 2 out of 5 TNLPs from asymptomatic carriers were Stx1-producers, which would imply that Stx1 production may be critically important for the in vivo pathogenicity of TNLP. Although the reason the TNLPs are attenuated remains to be elucidated, Nishikawa et al. (11) reported that the strains carrying only stx2vha caused bloody diarrhea less frequently, suggesting that the quantity of Stx2 produced and the stx2 genotype were important determinants of the severity of the disease.

TNLPs were originally reported to be isolated from non-clinical resources, e.g., beef and bovine feces in Thailand. Thailand is renowned for a scarcity of patients with EHEC infection and a higher incidence of anti-O157 seroprevalence compared to Japan. Based on the epidemiological analyses, they assumed that TNLPs more frequently caused asymptomatic infection than EHEC, which produces a high amount of Stx2 in vitro, and that the low incidence of severe O157 infection and higher seroprevalence against O157 in Thailand could be explained by the prevalence of TNLPs (3)

**REFERENCES**


**ACKNOWLEDGMENTS**

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