Biochemical and Molecular Characterization of Minor Serogroups of Shiga Toxin-Producing *Escherichia coli* Isolated from Humans in Osaka Prefecture

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**ABSTRACT.** We have investigated 37 minor serogroup Shiga toxin-producing *Escherichia coli* (STEC) strains other than O157, O26, and O111 isolated from human specimens in Osaka prefecture to determine their serological and biochemical characteristics, virulence-associated genes, and clinical signs in patients. The same serotype strains were genotyped by pulsed-field gel electrophoresis (PFGE). The strains were not agglutinated with any serum. Four different Shiga toxin (Stx) types (1, 2, 2c, and 2f) were distributed in these isolates. The intimin gene was present in 83.8% of the strains and subtyped into intimin subtypes (14 serogroups) in 2005 [30, 31]. Recently there was increased from 33 isolates (9 serogroups) in 1997 to 52 isolates (14 serogroups) in 2005. STEC O26 accounted for 22% and O111 for 4.6% of the total STEC isolates in 2005.

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Shiga toxin-producing *Escherichia coli* (STEC) is known as an important cause of gastrointestinal disease in developed countries [5, 36, 42]. The public health impact of STEC infections is high because of their ability to cause secondary infections and systemic complications, such as hemolytic uremic syndrome (HUS) [3, 15, 23]. In Japan, STEC infection is classified as a category III notifiable infectious disease under the National Epidemiological Surveillance of Infectious Diseases (NESID) in compliance with the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections (Infectious Diseases Control Law), and reporting by physicians is mandatory. More than 3,000 new symptomatic and asymptomatic cases of STEC infection are reported every year, although reports have decreased slightly when compared with the reports from 2004 [30]. In 2005, 1,600 STEC isolates were reported from prefectural and municipal public health institutes (PHIs) to the Infectious Diseases Surveillance Center (IDSC) of the National Institute of Infectious Diseases (NIID). The ratio of O157 isolates was decreased from 75.4% in 1997 to 68.4% in 2005. O26 accounted for 22% and O111 for 4.6% of the total STEC isolates in 2005. In addition, serogroups other than O157, O26, and O111 increased from 33 isolates (9 serogroups) in 1997 to 52 isolates (14 serogroups) in 2005 [30, 31]. Recently there was an outbreak of STEC O121 infections among school children in Chiba Prefecture [1].

Many effective and selective media are widely used in routine laboratory examination for the isolation of STEC O157 that have sorbitol-nonfermenting and β-glucuronidase-negative characteristics. Rhamnose and sorbose are also utilized as indicators for the isolation of STEC O26 and STEC O111, respectively [16, 41]. The biochemical characteristics of various other STEC serogroups have not yet been reported. In Osaka Prefecture, some cases of HUS caused by non-O157 STEC have been reported since 1997. The development of rapid and effective methods for the isolation of ‘minor serogroup’ (serogroup other than O157, O26, and O111) STEC are desirable to prevent secondary infections and analyze the infectious route. Methods for the detection of Stx, the cardinal virulence factors, directly from human stool or colonies on selective agar plate have been described [18, 24, 34], but these have been failures due to low sensitivity and false positives [8]. Isolation of STEC is required not only for reporting to NESID but also for epidemiological investigation.

Symptoms range from mild diarrhea to hemorrhagic colitis, and the infection may be complicated with HUS. STEC has been isolated from healthy individuals as well. It was suggested that the clinical outcome of STEC infection is associated with the Stx type and intimin, an outer membrane protein responsible for the intimate adherence between the bacteria and the intestinal epithelial cell membrane [7, 10, 13]. The finding that enterohemorrhagic *E. coli* (EHEC) hemolysin, called enterohemolysin, might act as a virulence factor in STEC O157, O26, and O111 was reported [6]. We
therefore investigated minor serogroup STEC that had been collected from human cases from 1996 to 2006 in Osaka Prefecture to characterize the isolates for their serotypes, virulence factors, and their correlation with disease. Furthermore, we applied pulsed-field gel electrophoresis (PFGE) to analyze the similarity of isolates in the same serotype. The aims of this study were also to describe the characteristics as an indicator of effective detection of minor serogroup STEC and to evaluate the tools for epidemiological analysis.

MATERIALS AND METHODS

**Strains:** STEC identified to serogroups except O157, O26, and O111 were used in this study. These strains were isolated from 24 patients and 13 healthy carriers between January 1996 and December 2006. Their clinical features are summarized in Table 1.

**Serotype identification:** Serotyping of O antigen (lipopolysaccharide) and H antigen (flagellar) of motile strains was performed according to the agglutination test [32] using Escherichia coli antisera set 1 and set 2 (Denka Seiken, Tokyo, Japan). The strains untypeable with commercially available serum were identified at the National Institute of Infectious Disease. The H types of nonmotile strains were investigated for the flagellin genes (fliC) by PCR followed by HhaI digestion of fliC PCR products and evaluation of restriction fragment length polymorphism (RFLP) patterns, as previously described [28].

**Biochemical characterization:** The strains were examined for biochemical properties using conventional methods [11]. The carbohydrate-fermenting ability was determined with pepton water containing Andrade’s indicator (1%) and one of the following 14 carbohydrates (1%) (Wako Pure Chemicals, Osaka, Japan): adonitol, arabinose, dulcitol, glucose, inositol, lactose, maltose, mannitol, rhamnose, salicin, sorbitol, sucrose, trehalose, or xylose, after 3 days incubation at 37°C. The activity of β-glucuronidase was observed by inoculating on CLIG medium (Kyokuto Pharmaceutical, Tokyo, Japan).

**Typing of Shiga toxins and stx genes:** Production of Stx1 and Stx2 was tested using a reverse passive latex agglutination test (VTEC-RPLA; Denka Seiken), according to the manufacturer’s instructions. The detection and subtyping of stx genes was performed by HincII digestion of a 900 bp DNA product, which was obtained by PCR with primers Lin5' and Lin3' [2].

**Detection and subtyping of intimin gene (eae):** The eae genes were detected by PCR using primers eaek1 and EA2 [26]. The subtyping of eae genes into intimin α, β, γ, ε, and ζ was performed by PCR with primer SK1 in combination with primers LP2 to LP6B [44].

**Hemolytic phenotype and detection of EHEC hemolysin gene (ehxA):** The hemolytic activity was assayed on enterohemolysin agar plates (Kanto Chemical, Tokyo, Japan) containing washed sheep blood and 10 mM calcium chloride, and sheep blood agar plates (Kanto Chemical). Enterohemolysin is only observed on the enterohemolysin agar plate, Table 1. Source and number of isolates and clinical features of individuals infected with minor serogroup STEC

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number of Persons</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>O28:H20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O63:H6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>O65:NM</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>O91:H14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O103:H2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O103:H11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O119:H4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O119[H25]</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O121:H19</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O126:H8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O165[HUT]</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O177[HUT]</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Out:H2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Out:H14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Out:H25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Out:HUT</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a) Family cases.

b) Sporadic cases.

c) AD; abdominal pain, D; nonbloody diarrhea, BD; bloody diarrhea, HUS; hemolytic uremic syndrome, None; asymptomatic.

d) Nonmotile and negative in the fliC-specific PCR.

e) An H type in brackets indicates the presence of non-motile (NM) strains, which were analyzed for their fliC type by PCR-RFLP.

f) HUT means untypeable with PCR-RFLP of fliC.

g) OUT means untypeable with antisera specific for O1 to O181.
and α-hemolysin reveals hemolysis on both agar plates [6]. Detection of ehxA was performed with primers hlyAF and hlyAR [35].

**Growth on CT-SMAC and MIC of potassium tellurite:** The growth of the strains was evaluated by comparing the colonies on MacConkey sorbitol agar (SMAC; Nissui, Tokyo, Japan) with and without CT supplements (cefotaxime:final 0.05 mg/liter and potassium tellurite: 2.5 mg/liter, ASKA Diagnostics, Tokyo, Japan). The bacterial solutions were adjusted to the 0.5 MacFarland standard (ca. 10⁸ CFU/ml) with BBL Trypticase Soy Broth (Becton, Dickinson and Company, Sparks, MD, U.S.A.). Ten microliter of the solution was applied to SMAC and CT-SMAC, and incubated for 20 hr at 37°C. The MIC of potassium tellurite (Dynal A. S., Oslo, Norway) was measured by the agar dilution method [22] using SMAC as a substitute for Mueller Hinton agar.

**Antimicrobial susceptibility testing:** The antimicrobial susceptibilities were determined by the disk diffusion method [29] with the following 12 antimicrobial agents (Becton, Dickinson and Company): ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, fosfomycin, gentamicin, kanamycin, nalidixic acid, ofloxacin, streptomycin, sulfamethoxazole-trimethoprim, and tetracycline.

**PFGE:** PFGE was performed according to the method of PulseNet Japan [43], using XbaI and BlnI (Roche Diagnostics, Mannheim, Germany). *Salmonella Braenderup H9812* PulseNet Standard Strain was kindly provided by the Centers for Disease Control and Prevention (CDC) [19]. FingerprintingII Version 3 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used for calculating the Dice similarity indices (tolerance 1.2%, unweighted pair group method using arithmetic averages) in the cluster analysis.

**RESULTS**

**Serological diversity of STEC isolates:** Of a total of 1,705 STEC strains, 1,519 O157 strains (89.1%), 136 O26 strains (8.0%), and 13 O111 strains (0.8%) were isolated during the period from January 1996 to December 2006. A total of 37 minor serogroup STEC strains were isolated from 5 family cases and 23 sporadic cases, and classified into 16 different O:H serotypes. Twenty-two motile strains belonged to the following 11 serotypes: O28:H20, O63:H6, O91:H14, O103:H2, O103:H11, O119:H4, O121:H19, O126:H8, O1217MINOR SEROGROUP STEC ISOLATED FROM HUMAN

**Clinical features:** Twenty-four strains were isolated from patients, and the other strains were obtained from asymptomatic carriers (Table 1). HUS developed in 4 patients, and abdominal pain was the dominant symptoms in 20 patients. Bloody diarrhea and nonbloody diarrhea appeared in 14 and 10 patients, respectively. The serotypes isolated from HUS patients were O165:[HUT] (2 isolates), O177:[HUT], and OUT:[HUT]. The strains belonging to serotypes O91:H14, OUT:H2, and OUT:H14 were isolated from food-providing workers who showed no symptoms. The remaining asymptomatic carriers were family members of patients. O63:H6 (2 isolates) and O119:H4 were isolated from family members of STEC O157 patients. O119[H25] (3 isolates) was isolated from family members of STEC O26 patients.

**Biochemical characterization of isolates:** The strains belonging to same serotypes showed the same results except for some carbohydrate fermentation results. The serotype O165:[HUT], O177:[HUT], and OUT:[HUT] strains revealed the most atypical phenotype, negative reaction for lysis decarboxylase and gas production from glucose. The serotype O119:[H25] and OUT:H25 strains were lysine decarboxylase-negative, and the serotype O65:NM strains did not produce gas from glucose. Sorbitol was not fermented in 5 serotypes; O63:H6, O119[H25], O177[HUT], OUT:H14, and OUT:H25. Four serotypes (O103:H11, O165[HUT], O177[HUT], OUT:[HUT]) and two serotypes (O119:H4, O165:[HUT]) were rhamnose-negative and xylose-negative, respectively. Although it is well known that STEC O157 is β-glucuronidase negative, the minor serogroup STEC strains were positive except for O65:NM, which was positive on day 2 (Table 2).

**Characterization of virulence factors:** The production of Stx1 and Stx2 was examined with the VTEC-RPLA assay, and stx genotypes of the strains were determined by PCR-RFLP (Table 2). The serotype O65:NM strains produced both Stx1 and Stx2. Either Stx1 or Stx2 was detected in the other strains. The stx types of all strains were identical to the Stx types. The production of Stx2 and presence of stx2 were found in 16 strains of 6 serotypes (O28:H20, O63:H6, O121:H19, O165:[HUT], O177:[HUT], and OUT:[HUT]). O28:H20 (1 strain), O165:[HUT] (3 strains), and OUT:[HUT] (1 strain) had stx2c in addition to stx2. Stx2 was also detected in O63:H6 (4 strains) carrying stx2f.

The eae gene was detected in 31 strains (83.8%) belonging to 10 serotypes. In the eae-positive strains, four intimin types, namely α, β, ε, and ζ, were detected (Table 2). Intimin ε was most frequent and detected in serotypes O103:H2, O121:H19, O165:[HUT], O177:[HUT], and OUT:[HUT]. Intimin α was found in O63:H6, β in O65:NM and O103:H11, and ζ in O119[H25] and OUT:H25. The ehxA gene was detected in 31 strains including all except for 3 serotypes (O63:H6, O119:H4, and O126:H8), but eight strains, O165:[HUT], O177:[HUT], OUT:H14, and OUT:[HUT] showed no enterohemolytic activity. The 6 ehxA negative strains were negative for enterohemolytic activity (Table 2). There were no strains demonstrating α-hemolysis.
Growth on CT-SMAC and MIC of potassium tellurite: In 11 strains of 7 serotypes (O28:H20, O91:H14, O126:H8, O165:HUT, O177:HUT, OUT:H2, and OUT:HUT), there were few colonies on CT-SMAC that was used as a selective agar plate for STEC O157. MICs of potassium tellurite for these strains were shown to be below 1.25 µg/ml (Table 3).

Antimicrobial resistance: Antimicrobial resistance patterns are shown in Table 4. Fifteen (40.5%) of the strains showed resistance to one or more antibiotics. The 5 strains belonging to serotype O65:NM that were isolated from 2 individual families showed identical resistance patterns. On the other hand, in one family case, the strain O103:H2 isolated from a patient showed multiple resistance, but the other strains from the family members were susceptible. There were no strains resistant to fosfomycin or ciprofloxacin (Table 4).

PFGE: A dendrogram of the XbaI digest pattern is shown in Fig. 1 for discrimination of the strains within the same serotype isolated from independent cases. The strains isolated from family cases were closely related (96% to 100% similarity) within the respective family. In the cluster of O65:NM the similarity of the strains isolated from Family B and Family C was 97%. In sporadic cases, the two O121:H19 strains isolated in 2003 showed the identical PFGE profile, and the two O63:H6 strains isolated in 2003 and 2004 showed 97% similarity as high as the strains of Family D. In the cluster of O165:HUT, five strains were heterogeneous but three strains isolated in 2006 were related (93% similarity). The strains with the identical patterns by XbaI digestion revealed also high similarity by BlnI digestion (data not shown).
Table 4. Antimicrobial resistance pattern of minor serogroup STEC isolates

<table>
<thead>
<tr>
<th>Resistance pattern&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of strains</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM, TC, CP</td>
<td>1</td>
<td>O103:H2</td>
</tr>
<tr>
<td>SM, TC, KM</td>
<td>1</td>
<td>O119:H4</td>
</tr>
<tr>
<td>ABPC, SM</td>
<td>1</td>
<td>O103:H11</td>
</tr>
<tr>
<td>SM, KM</td>
<td>1</td>
<td>O103:H2</td>
</tr>
<tr>
<td>SM, TC</td>
<td>6</td>
<td>O65:NM(5)&lt;sup&gt;b&lt;/sup&gt;, O165:[HUT]&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TC, CP</td>
<td>1</td>
<td>O165:[HUT]</td>
</tr>
<tr>
<td>CP</td>
<td>2</td>
<td>O121:H19, OUT:&lt;sup&gt;d&lt;/sup&gt;:[HUT]</td>
</tr>
<tr>
<td>SM</td>
<td>1</td>
<td>O165:[HUT]</td>
</tr>
<tr>
<td>TC</td>
<td>1</td>
<td>O126:H8</td>
</tr>
<tr>
<td>Total</td>
<td>15 (40.5%)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> SM; streptomycin, TC; tetracycline, CP; chloramphenicol, KM; kanamycin, ABPC; ampicillin.
<sup>b</sup> Number in parentheses are number of isolates.
<sup>c</sup> HUT in blackets means untypeable with PCR-RFLP of fliC of non-motile strains.
<sup>d</sup> OUT means untypeable with antisera specific for O1 to O181.

DISCUSSION

Reports of STEC isolation from PHIs to IDSC have totalled 12,477 since 2000. Although O157 was the predominant serogroup, over 100 strains belonging to serogroups O103 or O121 that were untypeable with commercially available antisera until August 2005 were isolated [20]. In Osaka Prefecture, STEC O103 and STEC O121 were sometimes isolated, but the STEC identified as rare serogroups such as O65, O165, and O177, were found among clinical isolates. O177 is a new serogroup that was designated in 2004 [38]. The earliest clinical isolate of serotype O177:NM was stx<sup>2</sup>, eae, and ehxA positive and was provided from CDC in USA in 1998. The first STEC O177 isolate in Osaka Prefecture was isolated from a girl (4-years-old) born in Osaka City in the same month, but these two isolates showed different PFGE patterns [40]. Since the 5 isolates of STEC O65:NM used in this study were only reported to IDSC, they were isolated from five individuals of two families eating together at a yaki-niku restaurant. STEC O65:NM strains were isolated from swine feces in North America [9, 12]. The strains from humans in Osaka Prefecture were widely different from swine isolates, because the human isolates had stx<sup>1</sup>, stx<sup>2</sup>, and eae (intimin β) although most of swine isolates were stx<sup>2e</sup> positive [12], and eae negative [25]. STEC O165:[HUT] was isolated from two children in 1998 and 2001 who presented complicated HUS and three children in 2006 who presented diarrhea or bloody diarrhea. It became easier to detect E. coli O165 because seven antisera, including O165 antisera, came onto the market in August in 2005. STEC belonging to serotypes O165:NM, O165:H19, and O165:H25 were isolated in Europe and Australia [13, 37]. The PCR-RFLP patterns of fliC of STEC O165 isolated in Osaka Prefecture was identical to the patterns of neither H19 nor H25, whereas they were the same with those of O177 and OUT isolated from HUS patients. It is of interest whether any strain of nonmotile STEC reported to IDSC shows the same pattern. Although ten isolations of STEC O63:H6 were reported from 2000 to 2006 in Japan, there are no reports of isolation of O63 from other countries [37]. The four O63:H6 strains in Osaka Prefecture produced Stx<sup>2</sup>, and had stx<sup>2f</sup> and intimin α genes. STEC O128 strains harboring stx<sup>2f</sup> were isolated from pigeons and infant patients [14, 21, 39]. In addition, intimin α was found frequently in entero-pathogenic E. coli (EPEC) [33] and it was detected in only two STEC O177:H7 isolates in Germany [5]. In this study, we isolated and characterized the unique STEC O63:H6 harboring the stx<sup>2f</sup> and intimin α genes.

It is probable that rhamnose, sorbitol, or xylose can be used as a discriminative marker, because 80% of E. coli fermented these carbohydrates [11]. The serotypes O63:H6, O119:[H25], OUT:H14, OUT:H25, and O103:H11 can be detected on CT-SMAC or CT-RMAC used as selective medium for STEC O157 or STEC O26, because they were sorbitol- or rhamnose-negative and resistant to potassium tellurite. We attempted to prepare medium without potassium tellurite to detect STEC O165:[HUT], O177:[HUT], and OUT:[HUT] that did not ferment sorbitol and/or rhamnose, because these serotypes were susceptible to the compound. Since it is impossible to detect all minor serogroup STEC by using one media, we should pick up sorbitol-fermenting colonies on CT-SMAC, and use the medium without CT supplements, for example desoxycholate-hydrogen sulfide-lactose (DHL). When public health agencies investigate follow-up cultures, MacConkey agar base (Becton, Dickinson and Company) and DHL agar base (Nihon Pharmaceutical, Osaka, Japan) supplemented with a discriminative carbohydrate are useful. It is widely known that enteroinvasive E. coli reveal characteristics similar to Shigella. Since some of the minor serogroup STEC produced a negative reaction for lysine decarboxylase and gas production from glucose, we performed Stx examination of the atypical E. coli.

It was reported previously that enterohemolysin agar plates were useful as screening media for detection of non-O157 STEC [4]. In this study the serogroups O103 and O121 that were reported to IDSC frequently showed enterohemolytic activity, but 14 strains of 7 serotypes including isolates from HUS patients were negative. In addition, ehxA was not associated with disease severity [10], so that it is suggested that enterohemolysin agar plates are not suitable to detect minor serogroup STEC.

The National Veterinary Assay Laboratory reported that antimicrobial resistance was more frequent in serogroups O26 and O145 than serogroup O157 among bovine STEC strains [25]. Similar results were found in our study, where the resistance rate to antibiotics of minor serogroup STEC (40.5%) was higher than STEC O157 (10.8%) isolated in Osaka Prefecture in 2006. Although there were no strains...
resistant to the drugs for therapy of STEC infection, an outbreak due to multiple-drug resistant O26:H11 and the isolation of O26:H11 producing extended-spectrum β-lactamase have been reported in Japan [17, 27], so that the careful monitoring of antimicrobial resistance of minor serogroup STEC should be continued.

PFGE analysis revealed that all of the O65:NM strains isolated from Family B and Family C were closely related, which confirmed epidemiologically and genotypically that they had been exposed to the same infectious source. In sporadic cases, the PFGE profiles of some strains suggested a genomic relationship, but there was no epidemiological information to support these relationships. Since high diversity was shown in O63:H6, O103:H2, and O165:[HUT], the PFGE profiles of these strains should be compared with the strains of the domestic PFGE network.

An epidemiological study for 6 years in Denmark indicated that risk factors for HUS were the combined presence of stx2 and eae rather than serogroup O157 [10]. The present study shows that the strains isolated from HUS patients were harboring both stx2 and eae. Ten of 13 strains isolated from asymptomatic carriers were Stx1-positive and Stx2-negative, whereas 10 of 14 strains isolated from patients with bloody diarrhea produced Stx2, supporting the previous reports that Stx2 was associated with severity of disease [7, 10].
The current methods for the detection of STEC have been developed to isolate serogroups O157 and O26. We should consider that some of minor serogroup STEC strains present atypical phenotypes and form no colonies on CT-SMAC. In addition, only 50 serogroups are typeable with commercially available antiserum, while there are over 130 serogroups reported as STEC [37]. Our results indicate that it is important to examine Stx production of all isolates on selective agar plates with and without CT supplement for the detection of various serogroups of STEC.

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