Characterization of Enterohemorrhagic Escherichia coli O111 and O157 Strains Isolated from Outbreak Patients in Japan

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In April and May 2011, there was a serious food-poisoning outbreak in Japan caused by enterohemorrhagic Escherichia coli (EHEC) strains O111:H8 and O157:H7 from raw beef dishes at branches of a barbecue restaurant. This outbreak involved 181 infected patients, including 34 hemolytic-uremic syndrome (HUS) cases (19%). Among the 34 HUS patients, 21 developed acute encephalopathy (AE) and 5 died. Patient stool specimens yielded E. coli O111 and O157 strains. We also detected both EHEC O111 stx1 and stx2-negative E. coli O111 strains in a stock of meat block from the restaurant. Pulsed-field gel electrophoresis (PFGE) and multilocus variable-number tandem-repeat analysis (MLVA) showed that the stx-negative E. coli O111 isolates were closely related to EHEC O111 stx1 isolates. Although the EHEC O157 strains had diverse stx gene profiles (stx1, stx2, and stx1

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nterohemorrhagic Escherichia coli (EHEC) strains cause a variety of human illnesses, such as uncomplicated diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome (HUS), and related acute encephalopathy (1). Shiga toxin 1 (Stx1) and Stx2 are EHEC virulence factors that cause endothelial cell damage with consecutive systemic thrombotic microangiopathy, resulting in hemorrhagic colitis and subsequent renal failure and involvement of other organs (1–4).

EHEC infection is a category III notifiable infectious disease in Japan, according to the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections (Infectious Diseases Control Law). All EHEC cases must be reported by the physician who made the diagnosis. Prefectural and municipal public health institutes (PHIs) conduct EHEC isolation, serotyping, and verotoxin (VT) typing and report their results to the Infectious Disease Surveillance Center (IDSC) of the National Institute of Infectious Diseases (NIID), Japan. Approximately 4,000 cases are reported annually. O157 is the most common EHEC serogroup in gastrointestinal tract infections, accounting for 60 to 70% of the reported EHEC infections. Among non-O157 EHEC serogroups, O26 is the second most common serogroup, accounting for 20 to 25% of the EHEC cases, followed by serogroups O111, O121, and O103.

Hemolytic-uremic syndrome (HUS) is an illness characterized by acute kidney injury, thrombocytopenia, and microangiopathic hemolytic anemia. Approximately 100 HUS cases associated with EHEC infections are reported annually in Japan, corresponding to 3 to 4% of the symptomatic EHEC infections. EHEC O157 is the most prevalent EHEC serogroup causing HUS, accounting for approximately 90% of the HUS cases identified among EHEC isolates. However, a wide variety of EHEC non-O157 serogroups might also cause HUS.

The EHEC O111 serogroup is the etiological agent of approximately 4% of the EHEC cases in Japan (5). During 2006 to 2010, there were 83 EHEC outbreaks in Japan, in which 10 or more EHEC-positive cases were reported. Six of these outbreaks were caused by EHEC O111 strains (6–10): three by EHEC O111

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Taniguchi, Y. Tada, N. Okabe, and E. coli Outbreak Investigation Team, unpublished data). A total of 941 individuals ate at the restaurant chain from 19 April to 4 May 2011, and 181 presented as outbreak-related cases, including 34 HUS cases. Only 55 of the 181 infections were confirmed by laboratory isolation to be from the EHEC O111 and/or O157 serogroups. Of the 34 HUS cases, 21 developed acute encephalopathy and 5 died. Here, we report studies characterizing the E. coli O111 and O157 strains isolated from the cases in this outbreak.

MATERIALS AND METHODS

Case definition. The case definition for the 181 patients in this outbreak was a person who developed illness >10 h after eating at one of the restaurants in the implicated restaurant chain in April 2011, and who presented with at least one of the following symptoms: (i) bloody stools, (ii) more than two gastrointestinal symptoms, such as diarrhea, nausea, or vomiting, abdominal pain, and tenesmus, (iii) one of the gastrointestinal symptoms in addition to at least two additional symptoms, such as fever (temperature of >37.5°C), malaise, or headache, or (iv) a stool culture positive for E. coli O111, EHEC O111, or EHEC O157.

Strains. A total of 104 EHEC O111, EHEC O157, and stx-negative E. coli O111 isolates were collected from three public health centers in Toyama Prefecture, Japan, and sent to the Toyama Institute of Health (THI). EHEC strains were also obtained from public health institutes in Fukui Prefecture, Ishikawa Prefecture, Kanazawa City, and Yokohama City, where related cases were detected. EHEC O111 strain H11128 (19) and EHEC O157 strain Sakai (20), used as reference strains, were obtained from the National Institute of Infectious Diseases (Tokyo, Japan) and the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan), respectively.

Isolation of EHEC and serotyping. Specimens were collected from seven public health and medical laboratories (listed in Acknowledgments). Stool specimens (n = 188) from patients, samples from leftover food from the implicated restaurants (n = 20), and smear samples from the implicated restaurant kitchens (n = 14) were collected and sent to the THI for analysis. For EHEC isolation, the specimens were analyzed by enrichment culture, immunomagnetic separation (IMS) (21) with O111 or O157 lipopolysaccharide (LPS) antibodies, and acid treatment before plating on selective agar medium, as described below (22). The enriched samples were spread on cefixime-tellurite (CT)-MacConkey sorbitol agar, CT-MacConkey sorbose agar, CHROMagar O157 TAM, CT-CHROMagar O157 TAM, or CHROMagar Shiga toxigenic E. coli (STEC). After the plates were incubated overnight at 35°C, the isolated colonies were transferred to Trypticase soy agar (Becton, Dickinson and Company), and these plates were incubated at 35°C overnight. The colonies (>4) isolated from the TSA plates were tested by PCR for the presence of the stx gene and serotyped using anti- E. coli O111 and O157 antisera (Denka Seiken Co., Ltd., Tokyo, Japan). Determination of the flagellar antigen type of the EHEC O111 outbreak strain was carried out by fliC typing with PCR-restriction fragment length polymorphism (23). The PCR amplicons were digested with Hhal (Nippon Gene Co., Ltd., Toyama, Japan) and separated by 2% agarose gel electrophoresis.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed using the PulseNet protocol (24). Genomic DNA in agarose plugs was digested overnight with 30 U XbaI (Nippon Gene Co., Ltd., Toyama, Japan) at 37°C. Electrophoresis of the XbaI-treated plugs was performed with the CHEF Mapper system (Bio-Rad Laboratories) using pulsed-field certified agarose (Bio-Rad Laboratories) with 0.5× Tris-borate-EDTA (TBE) running buffer. The electrophoretic conditions were as follows: 6 V/cm for 19 h, pulse time ranging from 2.2 to 54.2 s, and 0.5× TBE buffer at 12°C. *Salmonella enterica* subsp. enterica serovar Braenderup genomic DNA in an agarose plug was also digested overnight with 30 U XbaI and used as a molecular size marker. After electrophoresis, the gels were stained with ethidium bromide (final concentration, 50 ng/ml), destained by washing with distilled water, and photographed with ChemiDoc XRS (Bio-Rad Laboratories).

Multilocus variable-number tandem-repeat analysis. Multilocus variable-number tandem-repeat analysis (MLVA) was carried out as described in previous reports (25, 26). The PCR products labeled at the 5′ termini of the target loci for MLVA were separated using an ABI 3130xl Genetic Analyzer (Applied Biosystems). The repeat copy number for a null allele (i.e., when no PCR product was obtained) was designated −2.

Detection of virulence-related genes. Test strains were selected from 10, nine, five, two, and two representative isolates of the EHEC O111:H8 stx<sub>2</sub>, stx-negative E. coli O111, EHEC O157 stx<sub>2</sub>, EHEC O157 stx<sub>1</sub>, and EHEC O157 stx<sub>1</sub> isolates, respectively. The E. coli strains were resuspended in 200 μl of 5% (wt/vol) Chelex 100 resin (27) and heated for 10 min at 100°C. After centrifugation, the supernatants were quantified by using a NanoDrop ND-1000 (Thermo Fisher Scientific). The DNA preparations were diluted to a final concentration of 10 ng/μl and used as a template for PCR. The PCR primers used to detect stx genes were commercially purchased EVC-1 and EVC-2 to simultaneously detect common stx genes, EVT-1 and EVT-2 to detect stx<sub>1</sub>, and EVS-1 and EVS-2 to detect stx<sub>2</sub> (TaKaRa Bio, Inc.). Other virulence-related genes were analyzed by a multiplex PCR-based protocol (28). The target genes were stx<sub>1</sub>, stx<sub>2</sub>, eae, cvd342, aggR, invE, elt (labile toxin [LT] gene), esth (saithoin [StH] gene), esp (sulfotransferase [StP] gene), bfp, EAF, and astK. The Stx-encoding genes, stx<sub>1</sub> and stx<sub>2</sub>, of the EHEC isolates in this outbreak were subtyped using PCR (29).

To detect the norV gene (30), a primer pair was designed from the EHEC O111 genome norV sequence (GenBank accession no. AP010960): norV-337F (5′-CAT ACC TCA CCG AGT G-3′) and norV-914R (5′-GAG CGG AAG ACA TTG GTG AGG-3′). To detect the espG gene (31), the primer pair was espG-F (5′-CCA TTT GAT AAT AAT TCT CAT GGT G-3′) and espG-R (5′-GCC TTA GTA ATC GTC GGT CGA TAA TC-3′).

Titration of Stx in EHEC cultures. To detect Stx1 toxin in the culture medium of the EHEC isolates, the strains were grown in Casamino Acid-yeast extract (CA-YE) medium (Denka Seiko Co., Ltd., Tokyo, Japan) overnight with shaking at 35°C. Each culture was centrifuged (900 × g for 15 min), and the supernatant was used for the Stx1 and Stx2 assays. Each toxin was detected in a 2-fold dilution series of the supernatant by a verotoxin E. coli reversed passive latex agglutination assay (VTEC-RPLA) (Denka Seiko Co., Ltd., Tokyo, Japan), according to the manufacturer’s specifications. The toxin titers were expressed as the maximum dilution with a positive reaction.

RESULTS

Isolation of EHEC from patients. During this outbreak, we identified 34 patients with HUS cases and, in the early phase of the outbreak, stx-negative E. coli O111-positive cases were identified. Therefore, we reexamined the stool specimens that had given culture-negative results. After enrichment using anti-LPS (O111 or O157) antibody-conjugated magnetic beads, multiple colonies (usually 4 or more; at most, 800 colonies) were checked for stx genes. A total of 55 patients were found to be positive for EHEC O111 with fliC<sub>111</sub> and/or O157 with fliC<sub>157</sub>. These laboratory-confirmed cases were divided into three groups: only EHEC O111 isolated (group 1), both EHEC O111 and O157 isolated (group 2), and only EHEC O157 isolated (group 3). The groups contained 25, 12, and 18 cases, respectively (Table 1). Of the 37 EHEC O111-positive cases (groups 1 and 2), 17 developed HUS, including 13 cases of acute encephalopathy. However, of the 30 EHEC O157-positive cases (groups 2 and 3), only 9 cases developed HUS, including 6 cases of acute encephalopathy. Therefore, the HUS rate in patients infected with group 1 EHEC was 28%, with group 2 EHEC was 67%, and with group 3 EHEC was 6%.

We did not isolate any EHEC strains from 126 of the 181 cases...
TABLE 1 Numbers of patients in the groups defined by isolation of EHEC in stool specimens

<table>
<thead>
<tr>
<th>Clinical presentationa</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUS + AE, death</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>HUS + AE</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>HUS</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Non-HUSh</td>
<td>16</td>
<td>4</td>
<td>17</td>
<td>110</td>
<td>147</td>
</tr>
</tbody>
</table>

Total no. of patients 25 12 18 126 181

a HUS, hemolytic uremic syndrome; AE, acute encephalopathy.

TABLE 2 Profiles of serotypes and toxins of EHEC strains isolated from outbreak patients

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Serogroup O111</th>
<th>Serogroup O157</th>
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<tbody>
<tr>
<td></td>
<td>stx1</td>
<td>stx2</td>
<td>stx1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>+</td>
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<td>−</td>
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</tr>
<tr>
<td>102</td>
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</tr>
<tr>
<td>24</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Total no. of positive isolates 37 52c 25 10 4

a Groups 1 to 4 are defined in the Table 1 footnotes.
b Symbols indicate that the strain was isolated (+) or not isolated (−).
c Of these 52 isolates, 3 were not available and were therefore excluded from further analysis.

An interesting feature of this outbreak was that stx-negative E. coli O111 strains were isolated from patient stool specimens. In addition, EHEC O157 strains with several types of toxins were isolated. These results are summarized in Table 2, stx-negative E.

c O111 organisms were isolated from 28 of the 55 EHEC-positive cases (groups 1, 2, and 3) and from 24 of the 126 EHEC-negative cases (group 4). Further analysis was carried out on 37 EHEC O111 stx1, 49 stx-negative E. coli O111, 25 EHEC O157 stx1, 10 EHEC O157 stx2, and 4 EHEC O157 stx1 stx2 strains, in addition to an EHEC O111 isolate and a stx-negative E. coli O111 isolate from a beef sample from the same lot as the suspected contaminated food, the raw beef dish yu kohoe.

Molecular typing of E. coli isolates. If an EHEC strain loses its Stx prophage during infection, the resulting strain is an stx-negative eae-positive strain. Therefore, PFGE molecular typing was carried out to investigate the genetic relationships between the stx-positive and -negative EHEC O111 strains in this study. The PFGE patterns are shown in Fig. 1. All EHEC O111 stx1 strains, including an isolate from a beef sample, had the same XbaI digestion pattern (Fig. 1A, lane 1), except one strain that had a similar pattern but with a three-band difference (Fig. 1A, lane 3). Of the 50 stx-negative EHEC O111 strains, 48 (96%), including an isolate from a beef sample, had the pattern shown in Fig. 1A, lane 2. There was only a two-band difference between the patterns of almost all stx-positive and -negative EHEC O111 strains (cf. Fig. 1A, lanes 1 and 2). A comparison of these PFGE patterns identified an approximately 550-kb band in stx-positive EHEC O111 strains that was not present in the patterns of stx-negative EHEC O111 strains, as well as an approximately 490-kb band in stx-negative EHEC O111 strains that was not present in the patterns of stx-positive EHEC O111 strains. The size difference of these bands, 60 kb, corresponds to the genome size of Stx-converting phages (33).
To confirm the genetic relationship of the EHEC O111 isolates from the outbreak, MLVA was carried out. Of the 88 E. coli O111 isolates, including the isolate from a beef sample, 80 (including both stx-positive and -negative isolates) had an identical MLVA profile. The remaining eight EHEC O111 isolates had similar MLVA profiles, but each had a repeat number variation at one locus among the 18 loci. These data strongly suggest that the E. coli O111 strains isolated during this outbreak were genetically closely related. The isolates from beef had a PFGE type (Fig. 1A, lane 1) and MLVA type (MLVA-O111) that appeared to be identical to those of the most prevalent stx-negative E. coli O111 isolates from clinical specimens, supporting the hypothesis that yukhoe beef was the vehicle for this outbreak.

The EHEC O157 isolates in this study were also analyzed by PFGE and MLVA to investigate the genetic relatedness of the three different toxin types present in these strains. There were three groups of EHEC O157 isolates based on a PFGE analysis of their XbaI restriction digest patterns, with one- to three-band differences (Fig. 1B). Of the 39 O157 isolates, 30 showed an identical PFGE pattern (Fig. 1B, lane 1). This pattern was found in all the EHEC O111 strains isolated during this outbreak were genetically closely related. The isolates from beef had a PFGE type (Fig. 1A, lane 1) and MLVA type (MLVA-O111) that appeared to be identical to those of the most prevalent stx-positive and -negative E. coli O111 isolates from clinical specimens, supporting the hypothesis that yukhoe beef was the vehicle for this outbreak.

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Shiga toxin production and other virulence markers. This outbreak produced an extraordinary number of cases of HUS and encephalopathy. In this outbreak, proinflammatory cytokines were induced in most patients with acute encephalopathy and severe HUS (34,35). In addition to E. coli LPS, Stx1 and Stx2 can induce expression and synthesis of cytokines in Caco-2 cells, a human colon epithelial cell line (36). Therefore, we attempted to estimate the amount of Stx1 and Stx2 present in the culture media of EHEC strains isolated from outbreak patients. The 8 isolates of EHEC O111 stx2 and 3 isolates of EHEC O157 stx1 stx2 produced Stx2 titers of 1:64 and 1:32, respectively, which were lower than those produced by EHEC O111 strain 11128 and EHEC O157 strain Sakai (1:128 and 1:256, respectively). The Stx1 titer of the EHEC O157 stx1 stx2 strains was also lower than that of the EHEC O157 strain Sakai (1:32 versus 1:128).
strains (data not shown), indicating that an Stx2 prophage had been lost during subculture. Collectively, these data indicate that some Stx2 prophages in the EHEC O111 strains isolated in this outbreak were unstable during in vitro cultivation, and they suggest that loss of the Stx2 prophage may have occurred in the infected patients.

**DISCUSSION**

Several reports have identified EHEC-associated HUS cases that no longer shed EHEC but do shed stx-negative EHEC organisms that presumably lost stx during infection (18, 37–39). Stx is the most important EHEC virulence factor, causing the specific clinical features of EHEC infection (1–4). Therefore, stx-negative E. coli organisms may be produced by excision of the Stx-converting phage during infection. In agreement with this suggestion, more stx-negative EHEC strains have been isolated at follow-up testing of EHEC-infected patients (38). In HUS patients who shed only stx-negative EHEC, the etiology of HUS can be missed using current bacteriological methods based on detecting only the stx gene or Stx. This can hamper epidemiological investigations and lead to inappropriate clinical management, especially with a cluster of sporadic cases.

The etiology of the EHEC O111/O157 outbreak studied here was confirmed in only 18 of the 34 HUS cases by isolating EHEC O111 (9 cases), EHEC O157 (1 case), and both EHEC O111 and O157 (8 cases). The remaining 16 HUS cases were EHEC negative, even after extensive efforts to isolate EHEC, with up to 800 E. coli colonies examined after O111 enrichment using immunomagnetic beads. Since serologically positive results for the E. coli O111 antigen were found from all but one of the EHEC-negative HUS cases (32), EHEC O111 may be the primary cause of the severe HUS complications. An stx-negative E. coli O111 strain was isolated from most EHEC-positive HUS cases (13 of 18 HUS cases) and from a few EHEC-negative HUS cases (3 of 16 HUS cases). We also isolated EHEC O111 stx2 and stx-negative E. coli O111 organisms from a beef sample from the same lot as the suspected infection vehicle. Therefore, conversion from stx-positive to stx-negative E. coli O111 may occur in contaminated food, during infection, and/or bacteriological testing. The subculture data presented here show that the EHEC O111 stx2 isolates in this study can lose the Stx2 prophage during in vitro subculture (Fig. 3). This may have biased our bacteriological analyses, resulting in an underestimation of the scale of the outbreak. Since the loss of the stx gene was not consistent in all EHEC O111 isolates in this study, with some isolates remaining stx2 positive during subculture and other isolates losing stx2, the molecular mechanism and factors affecting the loss of the stx2 phage require further study.

Bielaszewka et al. (40) reported the isolation of stx-negative EHEC organisms from approximately 5% of the HUS patients. In that report, the majority of the stx-negative/eae-positive isolates belonged to serogroups O26, O103, O145, and O157:H7/NM, but no stx-negative/eae-positive E. coli O111 isolates were reported. The absence of stx-negative/eae-positive E. coli O111 isolates in the HUS patients may be explained by the fact that a majority of the EHEC O111 isolates possessed stx2 solely or in combination with stx2 (13), and the Stx1 prophage of EHEC O111 is thought to be defective, resulting in immobilization and stability in the EHEC chromosome (41). In contrast to a previous investigation (39), there were 16 HUS cases (47.1% [16 in 34 HUS cases]) in this outbreak from patients whose stools contained stx-negative E. coli.

![FIG 3 Stability of stx2 genes of the five EHEC O111 stx2 strains in successive subcultures. Ten O111 colonies (1 to 10) isolated from a stool specimen were picked, and each colony was spread on a TSA plate (A). A portion of each overnight subculture was transferred to a new TSA plate, and then this procedure was repeated to produce two subcultures. Following overnight incubation, the subcultures were tested using colony-sweep PCR for stx2 (A, B, and C), and the five stx2-positive colonies (1, 2, 4, 5, and 6) from the primary isolates were selected for the second round of subculture (C). The stx2 and 16S rRNA gene amplicons were 404 bp and 544 bp, respectively. Sakai, EHEC O111 stx-positive; Saikai strain as a positive control; NC, template DNA-free reaction as a negative control.](http://jcm.asm.org/Downloaded from http://jcm.asm.org)
O111 strains. It is probably because EHEC O111 in this outbreak possessed the Stx2 prophage only, which was unstable and progressively lost from the genome. An HUS outbreak in Italy in 1992 may have involved a similar loss of Stx phages during EHEC O111 infection (15). That HUS outbreak had nine HUS cases, including 6 cases that were diagnosed by detecting a serum antibody to E. coli O111 LPS. Stx-producing E. coli was isolated from a stool specimen in only one case. Unfortunately, there were no data on the isolation of stx-negative strains.

The prevalence of HUS (19% [34 of 181 EHEC-infected patients]) was unexpectedly high in the 2011 outbreak, even if the suspected cases are included in the calculation. The most probable explanation for this relatively high HUS prevalence is that the concentration of EHEC in the EHEC-contaminated food was high. However, the EHEC O111 and EHEC O157 concentrations in the vehicles for the outbreak studied here are not known. We were unable to examine the raw beef dish, yukhoe, which was the vehicle for EHEC O111, and thus far, the vehicle for EHEC O157 has not been confirmed. The cytokine profiles of the patients with serious complications in this outbreak indicate that massive induction of proinflammatory cytokines may have contributed to the development of serious complications (34, 35). The high level of cytokine induction may have been due to LPS release in the intestinal tract, although it remains unclear how LPS-dependent induction might have occurred in this outbreak. In addition, although Stx1 and Stx2 may induce cytokines, including tumor necrosis factor alpha, the amount of Stx2 production in the EHEC O111 strains, as well as Stx1 and Stx 2 production in the EHEC O157 strains, in this outbreak was low or similar to that in EHEC O111 strain 11128 and EHEC O157 Sakai by in vitro testing. Unfortunately, we did not determine the reasons for this high HUS prevalence using the in vitro bacteriological testing alone. Another possibility is that the instability of the Stx2 phages in the EHEC O111 strains may have played a role in the high HUS prevalence in this outbreak. Mellmann et al. (42) suggested that stx-negative EHEC strains might be the recipients of Stx2-converting phages from isogenic stx-positive strains. In fact, we detected many Stx2 phage plaques in the culture lysates of some EHEC O111 isolates following incubation with mitomycin C, as well as in some of the bloody stool specimens (data not shown). Almost all of these plaques were shown to be stx positive by the PCR assay, and these phages were found to be functional Stx2-converting phages. This would suggest a highly dynamic system that converts in both directions by the loss or gain of Stx2 phages. This cycling lifestyle might be enhanced by a mixture of stx-positive EHEC organisms and its isogenic stx-negative strain in the contaminated vehicle or during infection. Further studies of integrated prophages in both the EHEC O111 and EHEC O157 strains isolated during this outbreak need to be carried out, because the dynamic conversion of these strains might influence the outcome of disease.

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We declare that we have no conflicts of interest.

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