Epidemiological Study of a Food-Borne Outbreak of Enterotoxigenic Escherichia coli O25:NM by Pulsed-Field Gel Electrophoresis and Randomly Amplified Polymorphic DNA Analysis

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This study investigated the applicability of molecular epidemiological techniques to the identification of the causal agent of an outbreak of diarrhea caused by ingestion of food contaminated with enterotoxigenic Escherichia coli (ETEC). The outbreak occurred at four elementary schools in July 1996 and affected more than 800 people. Illness was most strongly associated with eating tuna paste (relative risk, 1.79; 95% confidence interval = 1.16 to 2.79; P = 0.0001). To evaluate the epidemiological characteristics of the pathogen, the DNAs from numerous isolated ETEC strains were subjected to randomly amplified polymorphic DNA analysis, pulsed-field gel electrophoresis of nuclease S1-treated plasmid DNA, and analysis of genomic DNA restriction fragment length polymorphisms. All ETEC isolates were of the O25:NM (nonmotile) serotype, which carries a heat-stable enterotoxin Ib gene. Genotypic analysis demonstrated that the strains isolated from the patients at all four schools were identical. The isolates of ETEC O25:NM obtained from the tuna paste that had been served for lunch at these schools were genetically indistinguishable from those isolated from the patients. Results suggest that this outbreak was food borne. The molecular biology-based epidemiological techniques used in this study were useful in characterizing the causal agent in this food-borne epidemic.

MATERIALS AND METHODS

Description of the diarrhea outbreak. The outbreak of food-borne ETEC O25:NM infections described here involved four elementary schools (designated schools A, B, C, and D) with a total of 2,019 students and 118 staff members. Among these, 737 students and 64 school staff exhibited symptoms of ETEC infection. The symptoms included abdominal cramps (84% of the patients), diarrhea (78%), fever (59%), and vomiting (10%). Most patients sought medical attention between 16 and 18 July 1996; the peak occurrence of symptoms among the patients was on 17 July 1996. Three patients were hospitalized due to dehydration, but all patients recovered without complications. Stool specimens from 641 symptomatic patients and 791 asymptomatic patients, as well as 296 environmental specimens including 34 school lunch meals stored in refrigerators, were examined. Routine cultures of the stool specimens were negative for Salmonella, Shigella, Campylobacter, and Yersinia. Cultures of stool specimens obtained from asymptomatic and asymptomatic patients were positive for ETEC strains (serotype O25:NM) that produced heat-stable toxin (ST).

Epidemiological studies and statistical methods. A retrospective cohort study was carried out on 24 July 1996 to identify food items that may have been associated with an increased risk of illness in schools A, B, C, and D. Relative risks with 95% confidence interval (CI) values are reported as measures of association. Also, the χ2 test was used to evaluate the data. A P value of <0.05 was accepted as statistically significant.

Bacterial strains. The E. coli isolates used in the molecular biology-based epidemiological study (strains 1 to 14) were obtained from symptomatic and asymptomatic people, including students, teachers, and licensed cooks, and from foods that included tuna paste served at lunch in the schools. The control strains of E. coli O25:NM (strains 15 and 16), isolated from two patients with sporadic diarrhea in July 1996 whose infections were unrelated to the school outbreak, were obtained from the Division of Pathology and Bacteriology of the Kanagawa Prefectural Institute of Health. Strain 16 was isolated from a patient who had previously traveled to the Maldives, whereas strain 15 was isolated from a patient who did not have a history of travel before the onset of diarrhea. For the isolation of E. coli from stool specimens, samples were plated onto a desoxycholate hydrogen sulfide lactose agar plate and a Salmonella-Shigella agar plate (BBL prepared plate medium; Becton Dickinson Microbiology Systems, Sparks, Md.). A total of five screened colonies were picked from each plate. After the identification of each strain as E. coli by standard procedures, all E. coli isolates were reidentified with a Microscan instrument (DADE International, Tokyo, Japan) with Neg Combo 31 panels. Each isolate was serologically screened for both E. coli O- and H-antigen type by the slide agglutination technique with an antisera kit (Escherichia coli Antiserum Setken; Denka-Setken, Tokyo, Japan).

Enterotoxigenic Escherichia coli (ETEC) strains have been etiologically associated with diarrheal illnesses that affect individuals of all age groups and at diverse locations around the world. In underdeveloped countries, the organisms frequently cause diarrhea in infants and in visitors from industrialized countries. The etiology of this cholera-like illness has been recognized for about 20 years. In Japan, ETEC infections were first recognized in 1979, and since then, ETEC has been identified among patients with diarrhea who had not traveled abroad (9, 24). In 1996, a large outbreak of ETEC infections described here involved four elementary schools (designated schools A, B, C, and D) with a total of 2,019 students and 118 staff members. Among these, 737 students and 64 school staff exhibited symptoms of ETEC infection. The symptoms included abdominal cramps (84% of the patients), diarrhea (78%), fever (59%), and vomiting (10%). Most patients sought medical attention between 16 and 18 July 1996; the peak occurrence of symptoms among the patients was on 17 July 1996. Three patients were hospitalized due to dehydration, but all patients recovered without complications. Stool specimens from 641 symptomatic patients and 791 asymptomatic patients, as well as 296 environmental specimens including 34 school lunch meals stored in refrigerators, were examined. Routine cultures of the stool specimens were negative for Salmonella, Shigella, Campylobacter, and Yersinia. Cultures of stool specimens obtained from asymptomatic and asymptomatic patients were positive for ETEC strains (serotype O25:NM) that produced heat-stable toxin (ST).

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Antisera Seiken; Denka-Seiken, Tokyo, Japan).
Detection of heat-stable enterotoxin protein and DNA by ELISA and PCR.

Heat-stable enterotoxin from each E. coli isolate was detected with the Colistin enzyme-linked immunosorbent assay (ELISA) kit (Denka-Seiken), which can detect both STla and STlb heat-stable enterotoxins (22). For PCR analysis, genomic DNA from each E. coli strain was prepared by using the SepaGene kit (Sanko Pharmaceutical Co., Tokyo, Japan). PCR was performed with the EC Nucleotides Mix (Nippon Shoji, Ltd., Tokyo, Japan), which includes four sets of primers for the detection of the ST gene (171-bp PCR product) and the heat-labile toxin gene (132-bp PCR product) of enterotoxigenic E. coli, the verotoxin 1 and 2 gene (228 bp) of enterohemorrhagic E. coli, and the invE gene (382 bp) of enteroinvasive E. coli (10, 12). The following were the primer sequences for these genes: for ST, 5'-TTTATTCTTTTTCACTCGTCTTT-3' (forward) and 5'-ATACAACACATCATCAG-3' (reverse); for the heat-labile toxin, 5'-AGCAGTGTCTCAGATTCTGG-3' (forward) and 5'-GTGCTCAGATTCTGGTGCCTC-3' (reverse); for verotoxin, 5'-TTTACGATAGACTTCTCGAC-3' (forward) and 5'-CAGATATAAAATTTATTCCGC-3' (reverse); and for the invE gene, 5'-ATATCTTATATTCACTGG-3' (forward) and 5'-GATGGCGAGAATTATATCCCG-3' (reverse). PCR products were resolved by electrophoresis in a 2.5% agarose gel and were detected by ethidium bromide staining. Amplified products were confirmed by sequencing (4.5% agarose gel) and were detected by ethidium bromide staining.

RESULTS

Epidemiological study. The association between clinically defined illness and the consumption of specific foods on 15 July 1996 in three schools (schools A, B, and C) is presented in Table 1. Results of interviews with all people in the three schools, which included students, school staff, and licensed cooks, suggested that the most significant association with illness was the ingestion of tuna paste, which was served for lunch in these schools on 15 July 1996 (relative risk, 1.79; 95% CI = 1.16 to 2.79; P = 0.0001). The tuna paste contained flakes of tuna, shredded carrots, onions, and cucumbers, as well as mayonnaise and mustard, but only the carrots, onions, and cucumbers were ingredients commonly delivered to these three schools by the same wholesaler. This wholesaler delivered carrots, onions, and cucumbers to the four schools, which included students, school staff, and licensed cooks, and was associated with illness in all three schools.

Table 1. Association of clinically defined illness and consumption of specific foods on 15 July 1996 in three schools

<table>
<thead>
<tr>
<th>Food</th>
<th>No. of subjects</th>
<th>Exposed Affected</th>
<th>Exposed Unexposed</th>
<th>Unexposed Affected</th>
<th>Unexposed Unexposed</th>
<th>Attack rate (%) for the following subjects:</th>
<th>Relative risk</th>
<th>95% CI</th>
<th>P value by ( \chi^2 ) test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread</td>
<td>646</td>
<td>23</td>
<td>985</td>
<td>58</td>
<td>39.7</td>
<td>28.4</td>
<td>1.40</td>
<td>0.85–2.29</td>
<td>0.0427</td>
</tr>
<tr>
<td>Milk</td>
<td>642</td>
<td>27</td>
<td>989</td>
<td>52</td>
<td>39.4</td>
<td>34.2</td>
<td>1.15</td>
<td>0.72–1.85</td>
<td>0.3564</td>
</tr>
<tr>
<td>Tuna paste</td>
<td>640</td>
<td>26</td>
<td>948</td>
<td>93</td>
<td>40.4</td>
<td>22.5</td>
<td>1.79</td>
<td>1.16–2.79</td>
<td>0.0001</td>
</tr>
<tr>
<td>Soup</td>
<td>640</td>
<td>26</td>
<td>953</td>
<td>51</td>
<td>40.2</td>
<td>24.8</td>
<td>1.62</td>
<td>1.05–2.50</td>
<td>0.0010</td>
</tr>
<tr>
<td>Ice cream</td>
<td>252</td>
<td>5</td>
<td>636</td>
<td>31</td>
<td>28.4</td>
<td>13.9</td>
<td>2.04</td>
<td>0.79–5.31</td>
<td>0.0572</td>
</tr>
</tbody>
</table>

Chromosomal REP family analysis by PFGE. Genomic DNA for PFGE analysis was prepared by using the GenePath plug Kit 2 (Bio-Rad) by following the manufacturer's protocol, with some modifications (16). Dispersible 100-μl scale plug molds were used to prepare agarose plugs for each sample. Overnight cultures of the ETEC isolates grown in brain heart infusion broth were resuspended at a concentration of 5 × 10⁶ cells/ml in PBS. Fifty microliters of each sample was used for plug preparation. After serial treatment with proteinase K, PMSF, and washing buffer (50 mM Tris-HCl [pH 7.5]), one-eighth of each plug was digested with 10 U of AvrI, SphI, SpeI, or XbaI. The gels were processed with the CHEF-DR II PFGE apparatus (Bio-Rad) with the following electrophoresis conditions: 7 h at 170 V with an initial time of 5 s and a final time of 20 s, followed by 14 h at 170 V with an initial time of 5 s and a final time of 80 s. Electrophoresis was performed with 0.5 × TBE buffer at 11°C. A 48.5-kb lambda DNA ladder (FMc BiosProducts) was used as a molecular size marker. After electrophoresis, the gels were stained with ethidium bromide and were photographed under UV light at 302 nm.

Isolation of ETEC O25:NM, phenotyping, and toxin detection. Examination of stool, food, and environmental samples resulted in the isolation of E. coli strains of serotype O25:NM producing ST, i.e., ETEC O25:NM. The samples had been obtained from students, teachers, licensed cooks, and tuna paste. ETEC O25:NM strains were isolated from a total of 393 people, including 270 of 641 symptomatic patients tested (42%) and 123 of 791 asymptomatic patients tested (16%). ETEC O25:NM was detected from tuna paste from schools A, B, and C. All other foods tested were negative for ETEC O25:NM. A total of 14 strains from all four schools were selected at random for further genotypic analysis. Strains 1 to 4 were obtained from school A, strains 5 to 8 were obtained from school B, strains 9 to 11 were obtained from school C, and strains 12 to 14 were obtained from school D. Strains 1, 5,
9, 12, and 13 were isolated from symptomatic students; strains 2, 6, and 10 were obtained from asymptomatic students; strains 3, 7, and 14 were from licensed cooks; and strains 4, 8, and 11 were from tuna paste. Mixed-primer PCR of strain 1 detected an ST gene of the STIb subtype which was identical to the toxin gene of two control ETEC O25:NM strains that had been isolated from unrelated patients with sporadic infections (Fig. 1). The toxin genes of the other ETEC O25:NM isolates analyzed were identical to that of strain 1 (data not shown).

RAPD analysis, plasmid profiles, and chromosomal DNA RFLP analysis. RAPD analysis of DNA from 14 isolates from the outbreak and the 2 control strains produced six to seven bands by agarose gel electrophoresis (Fig. 2). All 14 food-borne outbreak-associated strains showed identical banding patterns. The banding pattern of strain 15 is indistinguishable from that of food-borne outbreak-associated strains 1 to 14. Slight differences in the banding pattern of strain 16 are apparent from those of strains 1 to 15. Compared with the RAPD patterns of strains 1 to 15, strain 16 lacked the 1,050-bp band and presented an additional 650-bp band. The isolates were then compared by establishing plasmid profiles by using nuclease S1 digestion and PFGE (Fig. 3). All 14 isolates exhibited identical profiles, with each strain having five large plasmids (100, 82, 67, 43, and 20 kb). The plasmid profiles of the sporadic ETEC O25:NM strains, in contrast, differed somewhat from this pattern. In particular, both sporadic strains lacked the 100-kb plasmid. In addition, strain 16 carried an additional 170-kb plasmid. The 14 ETEC O25:NM isolates also were compared by RFLP analysis after digestion of the chromosomal DNA with SpeI (Fig. 4A), XbaI (Fig. 4B), and ApaI and SfiI (data not shown). All isolates exhibited identical RFLP patterns. A comparison of one of the isolates (strain 1) with the
During the past 20 years, between 700 and 9,000 cases of food poisoning due to *E. coli* have annually been reported in Japan (9). In addition, occasional large outbreaks of ETEC infections have occurred since 1967. ETEC has also been identified as the causative agent of illness in about 20% of patients who developed diarrhea after traveling to foreign countries (24). The ETEC serotypes most commonly associated with large outbreaks in Japan are O6:NM and O6:H16, which produce both ST and heat-labile toxin, as well as O27:H7, O27:H20, and O159:H20, which produce only ST. ETEC serotypes O8, O25, O148, and O167 have also been reported in the literature (4, 9, 23, 24). To our knowledge, this is the first report of a large food-borne outbreak caused by ETEC O25:NM in Japan. In the United States, in contrast, 13 outbreaks of gastroenteritis caused by ETEC have been reported to the Centers for Disease Control and Prevention since 1975, 9 of which were food borne. Reported sources of the infections included water contaminated with human sewage (19), infected food handlers (21), dairy products such as semisoft cheeses (13), and salads containing raw vegetables (15). In 1996, large outbreaks of verotoxin-producing *E. coli* (enterohemorrhagic *E. coli*; EHEC) O157:H7 infection occurred among students in several Japanese schools and day-care centers. The outbreaks affected 9,451 patients and caused 12 deaths and were thought to be caused by contaminated

\[ \text{FIG. 5. Comparison of the chromosomal restriction patterns of three ETEC O25:NM strains by PFGE. Strain 1 (lanes 1, 4, 7, and 10) was isolated from a symptomatic student. Strain 15 (lanes 2, 5, 8, and 11) and strain 16 (lanes 3, 6, 9, and 12) were isolated from two patients with sporadic cases of ETEC O25:NM infection. The isolates were digested with } \text{SfiI (lanes 1 to 3), } \text{SstI (lanes 4 to 6), } \text{SpeI (lanes 7 to 9), and } \text{XhoI (lanes 10 to 12). Lanes M, molecular size marker (48.5-kb lambda DNA ladder).} \]

\[ \text{DISCUSSION} \]

This study demonstrates that practical molecular biology-based epidemiological approaches can be used to identify the source and route of infection of food-borne ETEC poisoning in the community. PFGE analysis is considered the most reliable and practical tool for molecular biology-based epidemiological analyses of bacterial infections (1–3, 6, 8, 11, 14, 18, 20). As demonstrated here, additional RAPD analysis (5, 6) and the determination of plasmid profiles by nuclease S1 treatment and PFGE (3) can increase the accuracy of these analyses. PCR-based RAPD analysis is increasingly being used for molecular biology-based epidemiological applications, such as subtyping of *E. coli* isolates. Although RAPD analysis can provide RFLP data very rapidly, the resolution and reproducibility of these data are somewhat limited. Nevertheless, RAPD analysis can provide valuable preliminary molecular biology-based epidemiological information during bacterial outbreaks while more time-consuming PFGE analyses are performed. The analysis of plasmid profiles by conventional electrophoresis has resolution problems including the instability of the plasmids. However, nuclease S1 treatment and PFGE have improved the resolution in analyzing large plasmids compared with the resolution of conventional procedures. The major limitation of PFGE analysis includes the requirements for technical skill and an expensive apparatus and the long duration until the completion of the analysis (4 to 7 days). Moreover, the analysis of PFGE-RFLP data may be difficult. For example, after isolating the bacterial strain responsible for an epidemic, this strain must be compared to strains involved in other outbreaks. Because PFGE conditions vary, however, it may be difficult to compare PFGE data from different studies. It is therefore necessary to establish databases that contain reliable RFLP information on each bacterial strain obtained by molecular biology-based methods involving several restriction enzymes. Such information will allow international collaboration for identifying the causative agents involved in large outbreaks until rapid, automated partial or full genomic sequencing techniques replace the current RFLP technologies. In the ETEC O25:NM outbreak described here, we used several molecular biology-based epidemiological techniques. PFGE is one of the most informative tools for characterizing strains, but it is not a perfect technique. To avoid misunderstanding the molecular biology-based epidemiological results, we recommend analysis of epidemic strains not only by PFGE but also by other supportive techniques in the case of a similar outbreak.

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REFERENCES