Short Communication

Isolation of Shiga Toxin 2f-Producing Escherichia coli (O115:HNM) from an Adult Symptomatic Patient in Fukuoka Prefecture, Japan

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SUMMARY: Shiga toxin 2f-producing Escherichia coli (O115:HNM) with eae was isolated from a symptomatic patient in Fukuoka Prefecture, Japan. The patient was a 23-year-old male and his symptoms were diarrhea, abdominal pain, headaches and a fever (37.7°C). He had eaten raw chicken meat, raw chicken eggs, cooked chicken meat and raw vegetables about 13 h prior to the onset of the symptoms. The patient’s specimen was examined, and no diarrheagenic agents were detected except for Shiga toxin 2f-producing E. coli (STEC2f) with eae. This is the first report of the serotype O115:HNM possessing stx2f. We discuss the necessity of routinely using stx2f-detecting PCR primers for detection of this enteric pathogen.

Shiga toxin-producing Escherichia coli (STEC) possesses Shiga toxins (Stxs), consisting of Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (1), along with other virulent factors. The Stx1 group consists of three variants (Stx1, Stx1c, Stx1d) whereas the Stx2 group comprises a number of variants including Stx2, 2e, 2dact, 2-O118 (former Stx2d-Unt), 2f and 2g (2-4). The Stx2c or Stx2dact (mucus-activatable toxin) variants are often associated with the life-threatening hemolytic-uremic syndrome (HUS) in children, whereas other toxin variants, e.g., Stx2-O118 or Stx2e (restricted to virulence in pigs), are found to be associated mainly with uncomplicated diarrhea (3,4). Typical STEC strains possess additional virulent factors such as large plasmids (e.g., FII-like plasmid), or pathogenicity islands (e.g., the locus of enterocyte effacement, characterized by the eae gene) other than Stx (5). The eae gene was detected more frequently in strains isolated from HUS patients than in those associated with cases of diarrhea involving STEC (6). However, Shiga toxin 2f-producing E. coli (STEC2f) were identified among eae-harboring E. coli from feral pigeons in Europe (7).

It is important to record cases involving STEC2f to further our understanding of STEC2f infection because there have been contradictory observations about the occurrence of STEC2f in human beings. On the one hand, STEC2f is very rarely associated with human infections; it has been thought that STEC2f might be a pigeon-adapted Stx variant with a limited impact on human diseases (8). On the other, an increasing number of STEC2f isolates from humans have been observed among clinical isolates (3). In some previous cases involving SETC2f, the pathogen was retrospectively identified as SETC2f (3,9), because common stx2 PCR never detected the stx2f (7,10) and common reverse passive latex agglutination test (RPLA) responded only weakly to Stx2f (7). Thus, few episodes of infections including incubation periods or suspected vehicles have been recorded in cases with the pathogen.

Both the patient and his associate showed diarrhea after having a dinner together. The patient was a 23-year-old male from Fukuoka Prefecture, Japan. His symptoms (October 20, 2008) were diarrhea, abdominal pain and headaches with a fever (37.7°C). The patient used the lavatory due to diarrhea seven times on day one, four times on day two and one time on day three. He had eaten raw chicken meat, raw chicken egg, grilled chicken skewers, deep fried chicken meat and raw vegetables (tossed salad) with his associate (a 23-year-old female) about 13 h prior to the onset of the symptoms. His associate also showed diarrhea about 30 times with a low-grade fever (37°C) on day one of onset. However, none of her specimens was examined in our laboratory. She ate the same meal except for the deep fried chicken about 12 h prior to the onset of the symptoms. On the day before the onset day, the patient and his associate had eaten together for the first time in a week.

The patient’s sample on day two after the onset was examined to detect diarrheagenic agents using standard culture procedures and PCR methods. E. coli, Salmonella, Shigella, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Campylobacter jejuni, C. coli, Aeromonas hydrophila, A. sobria, Plesiomonas shigelloides, Vibrio parahaemolyticus, V. mimicus, V. cholerae, adenovirus, Aichi virus, astrovirus, enterovirus, norovirus, parechovirus and sapovirus were tested on the sample. PCR for the colony sweep method from Salmonella-Shigella agar (SS) and deoxycholate-hydrogen sulfide-lactose agar (DHL) were used with the primers for eae described by Kobayashi et al. (11), and with commercial primers (Stx-related gene; Takara Bio Inc., Otsu, Japan). Then, isolates were examined for biochemical, serological and genetic characters using culture methods, commercial kits, antisera (Denka Seiken Co., Tokyo, Japan) and PCR methods. PCR methods were used

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to determine pathogenic genes for isolates with primers for eae, bfpA, aggR and astA described by Kobayashi et al. (11), primers for enterohemorrhagic E. coli (EHEC)-hlyA by Paton and Paton (12), and primers for Stx2f-gene described by Nakao et al. (13) and Schmidt et al. (7). Apizyn (bioMérieux, Lyon, France) was used for detection of enzyme activities of the isolates.

Full-length sequence analysis of the Stx gene from the isolate can be used to confirm the gene type (Figure 1). Amplifications for the Stx2-related gene (approximately 1.5-kbp fragment) were carried out with primers, VT2F-F and VT2F-R (Table 1), using PrimeSTAR HS DNA Polymerase (Takara Bio). The amplicons were purified for sequencing using Montage PCR filters (Millipore, Billerica, Mass., USA), and sequenced with two outer primers and six inner primers (Table 1) using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (version 3.1) on a 3130xl genetic analyzer (Applied Biosystems Ltd., Carlsbad, Calif., USA).

The sequences were assembled using the SeqManII program in the Lasergene software package (DNASTAR, Madison, Wis., USA). The concatenated sequences were aligned by the ClustalW software program and a phylogenetic tree was constructed using the neighbor-joining method (Center for Information Biology and DNA Data Bank of Japan [DDBJ]) and compared to published data. Phylogenetic analyses were performed using NJplot (http://pbil.univ-lyon1.fr/software/njplot.html).

RPLA (Denka Seiken), Vero cell monolayers and Duopath Verotoxin (Merk KGaA, Darmstadt, Germany) tests were used on the supernatants of the isolates of a culture of brain heart infusion broth (BHI) (Eiken Chemical Co., Ltd., Tokyo, Japan) with and without mitomycin C (MMC) (Sigma Chemical Co., St. Louis, Mo., USA) (0.2 mg/liter) to determine the presence of the Stx. The supernatants obtained by centrifuging the cultures at 10,000 × g for 10 min were filtered through 0.2-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass., USA). Samples of the supernatants of serial twofold dilutions were applied to confluent Vero cell monolayers and to RPLA for evaluation of toxic activity (14). The Duopath Verotoxin was used on the supernatants and a polymixin B (Wako Pure Chemical Industries, Ltd., Osaka, Japan) culture extract. ATCC 43894 (E. coli O157:H7, harboring stx1 and stx2) was also examined as a reference strain in these immunological tests.

The patient sample was examined, and no diarrheagenic agents were detected except for STEC2f. Two isolates harboring eae were isolated from SS and DHL with the colony sweep PCR method. The isolates were then identified to have the gene that reacted with the stx2-specific PCR primers described in other reports (7,13). However, PCR with commercial stx3 PCR primers (EVS-1&2, EVC-1&2; Takara Bio) was negative or weakly positive against the isolates. Subsequently, after comparison between the stx3 sequence of the isolates and past studies, the isolates were identified as STEC2f (Figure 1). The sequences of the two isolates completely corresponded. New sequences were deposited to DDBJ, under accession no. AB472687.

The specific genetic and immunological characters of the isolates of STEC2f were recorded. They were eae-positive, enterogagreggative E. coli heat-stable enterotoxin 1 (EAST1)-related gene-positive, EHEC-hlyA-negative, and bfpA-negative with PCR methods. Supernatants of the isolate culture without MMC reacted negatively with RPLA for Stx1 and Stx2, and samples of the supernatants with MMC showed a titer of 64 in the test for Stx2 only, as in the previous report (7), while ATCC 43894 with MMC treatment showed titers of 16 and 8192 for Stx1 and Stx2, respectively. For the Vero cell assays with the supernatants of the isolates, the sensitivity (titer of eight) was fourfold lower than that of the reference strain, while the sensitivity was the same as that of the reference strain (titer of 16384) with MMC. The Duopath

### Table 1. Primer sequences for sequence Stx2 related gene

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence 5'-3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT2F-F</td>
<td>ACT TCT TGC GAG GTA TTA TTC</td>
<td>Present study</td>
</tr>
<tr>
<td>VT2F-R</td>
<td>GTA TGG CCT TAA GGG TAA AC</td>
<td>Present study</td>
</tr>
<tr>
<td>AJ010730_305-323RC</td>
<td>ATC TCT CGC TAT ATG GCT C</td>
<td>Present study</td>
</tr>
<tr>
<td>528-1</td>
<td>AGA TGG GGC TTC ATT CAC TGG TTG</td>
<td>(7)</td>
</tr>
<tr>
<td>528-2</td>
<td>TAC TTT AAT GGC CGC CTC GTC TCC</td>
<td>(7)</td>
</tr>
<tr>
<td>G3-F</td>
<td>TTT ACT GTG CAT TTC TCT TCG C</td>
<td>(13)</td>
</tr>
<tr>
<td>G3-R</td>
<td>TCA GTA AGA TCC TGA GGC TTG</td>
<td>(13)</td>
</tr>
<tr>
<td>AJ010730_892-910</td>
<td>CCA AAA ACA GAA AAC AGA A</td>
<td>Present study</td>
</tr>
</tbody>
</table>
Verotoxin test was negative for Stx with both polymixin B extract of the isolates culture and the supernatants with MMC. STEC<sub>2f</sub> (O115:HNM) with eae were isolated from a symptomatic patient in Fukuoka Prefecture, Japan. It was atypical for STEC to have such a short incubation period, about 13 h (15). However, no other diarrhea-causing agents were detected. Additionally, the patient and his associate, who showed the same symptoms, had eaten together for the first time in a week. Therefore, it is possible that the vehicle of the agent was food and not pigeons.

The first description of a STEC<sub>2f</sub> isolate of serotype O115:HNM including its characteristics and its host’s epi-

sode is noteworthy. According to other reports, in Japan or Europe, STEC<sub>2f</sub> consists of O serogroup O63:HNM, O63:H6, O128:HNM, O128ab:HNM, O128:H2, O132:H34, O145:H34 and O178:H7 from humans (3,6,9,16-19), O15, O18ab:HNM, O20, O25:H7, O45, O45:HNM, O66:HNM, O75:HNM, O128:H2, O132, O135:HNM, O152:HNM, OUT:HNM, OUT:HUT and Rough:HNM from pigeons and doves (10,16, 20,21), and O147 from other wild birds (20); O115:HNM here was found in humans for the first time. But the previ-

ous and the present isolates of STEC<sub>2f</sub> showed the typical characteristics: the presence of eae and the absence of the EHEC-\textit{hlyA} (3,6,9,10,16-19,21,22). The present STEC<sub>2f</sub> iso-

lates harboring the EAST1-related gene were the same as in some reports (3,19), but differ from those in other reports (9,16). Most previous STEC<sub>2f</sub> isolates from humans have been from infants (Japan [9,19], UK [6] and Germany [3,16]). Only two adult cases who had other illnesses have been reported (The Netherlands [18]). However, the isolates in the present study were isolated from an adult patient (23 years old) who had no underlying illnesses.

Due to the importance of emerging pathogens in public health, it is hoped that this study will assist in the further development of detection methods for STEC<sub>2f</sub>. The oc-

currences of STEC<sub>2f</sub> have clearly been higher than prior expecta-

tions (3,18). For example, using primers and probes adapted to \textit{stx}<sub>2f</sub>, van Duynhoven et al. (18) reported that 3.3% (7/211) of human stool specimens with STEC suspicion were positive for STEC<sub>2f</sub>. Some previous reports have also described ident-

ifying STEC<sub>2f</sub> from atypical enteropathogenic \textit{E. coli} (EPEC) retrospectively (3) because common \textit{stx}<sub>2f</sub> PCR did not detect the \textit{stx}<sub>2f</sub> (7,10).

Thus, using \textit{Stx}2f-detecting primers for PCR is important for routine testing of enteropathogens among clinical samples, and it is important for stocked atypical EPEC isolates to be tested with these primers. In addition to PCR, application of a suitable antigen that reacts with \textit{Stx}2f can be helpful for detection of the enteropathogens with immune kits.

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REFERENCES


ton D.C.
18. van Duynhoven, Y.T., Friesema, I.H., Schuurman, T., et al. (2008): Preva-


445.