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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* ($STEC_{2f}$) with *eae*. This is the first report of the serotype O115:HNM possessing stx_{2f}. We discuss the necessity of routinely using stx_{2f}-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, 2c, 2dact, 2-O118 (former Stx2d-Ount), 2e, 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* ($STEC_{2f}$) were identified among *eae*-harboring *E. coli* from feral pigeons in Europe (7).

It is important to record cases involving $STEC_{2f}$ to further our understanding of $STEC_{2f}$ infection because there have been contradictory observations about the occurrence of $STEC_{2f}$ in human beings. On the one hand, $STEC_{2f}$ is very rarely associated with human infections; it has been thought that $STEC_{2f}$ might be a pigeon-adapted Stx variant with a limited impact on human diseases (8). On the other, an increasing number of $STEC_{2f}$ isolates from humans have been observed among clinical isolates (3). In some previous cases involving $SETC_{2f}$, the pathogen was retrospectively identified as $SETC_{2f}$ (3,9), because common stx_2 PCR never detected the stx_{2f} (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). Apizym (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).

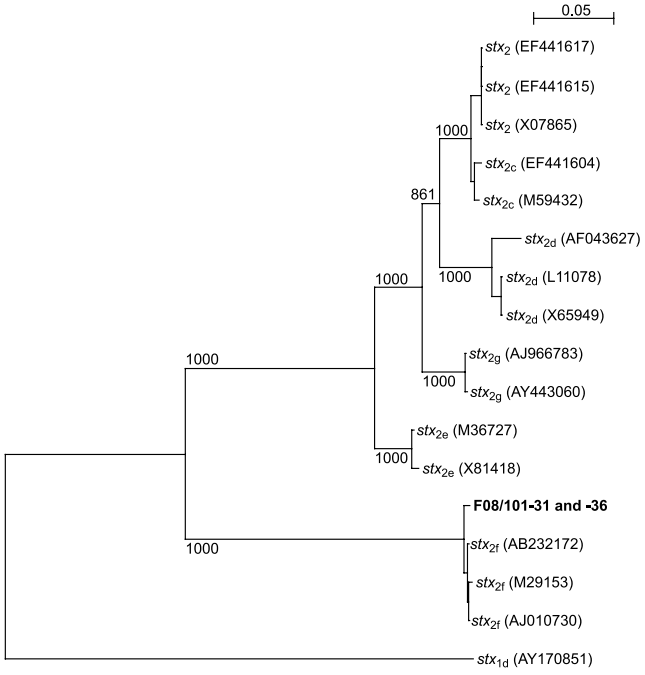

Fig. 1. Phylogenetic tree showing nucleotide sequence clusters of stx_2 of *E. coli* and the isolates in the present study, F08/101-31 and 101-36, shown in boldface, belong to the branch of stx_{2f} genogroups, based on about 1.23-kbp length from the start codon of subunit A to the stop codon of subunit B. The numbers indicate bootstrap values from 1000 replicates. The sequences of the two isolates were completely corresponding. stx_{1d} was added as an outgroup. Accession numbers for reference sequences are in parentheses. The scale bar indicates the number of nucleotide substitutions per site. New sequences were deposited to DDBJ, under accession no. AB472687.

RPLA (Denka Seiken), Vero cell monolayers and Duopath Verotoxin (Merck 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 stx_1 and stx_2) was also examined as a reference strain in these immunological tests.

The patient sample was examined, and no diarrheagenic agents were detected except for $STEC_{2f}$. 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 stx_{2f}-specific PCR primers described in other reports (7,13). However, PCR with commercial stx_2 PCR primers (EVS-1&2, EVC-1&2; Takara Bio) was negative or weakly positive against the isolates. Subsequently, after comparison between the stx_2 sequence of the isolates and past studies, the isolates were identified as $STEC_{2f}$ (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 $STEC_{2f}$ were recorded. They were *eae*-positive, enteroaggregative *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

Primer name	Nucleotide sequence 5′-3′	Reference
VT2f-F	ACT TCT TGC GAG GTA TTA TTC	Present study
VT2F-R	GTA TTG CCT TAA GGG TAA AC	Present study
AJ010730_305-323RC	ATC TCT CGC TAT ATG GCT C	Present study
128-1	AGA TTG GGC GTC ATT CAC TGG TTG	(7)
128-2	TAC TTT AAT GGC CGC CCT GTC TCC	(7)
G3-F	TTT ACT GTG GAT TTC TCT TCG C	(13)
G3-R	TCA GTA AGA TCC TGA GGC TTG	(13)
AJ010730_892-910	CCA AAA ACA GAA AAC AGA A	Present study

Verotoxin test was negative for Stx with both polymixin B extract of the isolates culture and the supernatants with MMC.

$STEC_{2f}$ (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 $STEC_{2f}$ isolate of serotype O115:HNM including its characteristics and its host's episode is noteworthy. According to other reports, in Japan or Europe, $STEC_{2f}$ 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. Both the previous and the present isolates of $STEC_{2f}$ showed the typical characteristics: the presence of *eae* and the absence of the EHEC-*hlyA* (3,6,9,10,16-19,21,22). The present $STEC_{2f}$ isolates 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 $STEC_{2f}$ 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 $STEC_{2f}$. The occurrences of $STEC_{2f}$ have clearly been higher than prior expectations (3,18). For example, using primers and probes adapted to stx_{2f}, van Duynhoven et al. (18) reported that 3.3% (7/211) of human stool specimens with STEC suspicion were positive for $STEC_{2f}$. Some previous reports have also described identifying $STEC_{2f}$ among atypical enteropathogenic *E. coli* (EPEC) retrospectively (3) because common stx_2 PCR did not detect the stx_{2f} (7,10).

Thus, using Stx2f-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 Stx2f can be helpful for detection of the enteropathogens with immune kits.

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