Escherichia coli O8:H8 Carrying a Novel Variant of the Heat-Labile Enterotoxin LT2 Gene Caused Outbreaks of Diarrhea

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No outbreaks caused by Escherichia coli–producing heat-labile enterotoxin LT2 have been reported to date. Here, we revealed that the E. coli O8:H8 strains isolated from patients in 2 independent diarrhea outbreaks were negative for any known virulence determinants in routine microbiological tests, were very closely related, and carried a prophage-encoded gene for a novel LT2 variant (LT2d) and the genes for colonization factor antigen III. We also showed that LT2d has a cytotoxic activity similar to LT1. These data indicate the importance of E. coli strains producing LT2d as a human pathogen.

Keywords: colonization factor antigen; diarrhea outbreak; heat-labile enterotoxin; phage; scherichia coli.

Escherichia coli is a commensal intestinal inhabitant, but several strains that have acquired specific virulence factors can cause diverse diseases in healthy humans [1]. Enterotoxigenic E. coli (ETEC) causes watery diarrhea in both children and adults worldwide. ETEC is characterized by the production of heat-labile (LT) and/or heat-stable (ST) enterotoxins, along with diverse colonization factors (CFs), but includes a wide range of genetically diverse strains that express a variety of O antigens [1, 2]. LT is an AB5 toxin homologous to the cholera toxin (CT) produced by Vibrio cholerae and is genetically and antigenically divided into subtypes LT1 and LT2, both of which include several variants [3]. Although LT1 and LT2 have been shown to possess similar biological activities [4], LT2-producing strains rarely cause human disease [1], and no outbreaks caused by LT2-producing strains have been reported thus far. Known LT1 genes (elt1) are exclusively encoded on large plasmids, while LT2 genes (elt2) have been reported to be encoded by phage [5, 6]. Among the various CFs described so far (>25 variants), CF antigen I (CFA/I) and coli surface antigens 1–6 (CS1–CS6) are most prevalent in ETEC [7]. Here, we report 2 E. coli O8:H8–associated outbreaks of diarrhea and the results of the genome analysis of the isolates. Our results show that the 2 outbreaks were caused by very closely related E. coli O8:H8 strains that carry a prophage-encoded gene for a novel LT2 variant (named LT2d) and the genes for CFA/III. Unique features of the LT2d phage and the cytotoxic effect of LT2d on CHO cells are also described.

The 2 outbreaks occurred in 2 different dormitories in Oita prefecture, Japan, in April 2014 (outbreak 1: OB1) and September 2016 (OB2). Among the 300 and 120 residents who lived in each dormitory and shared food and water, 13 and 39 developed gastrointestinal symptoms in OB1 and OB2, respectively (Table 1). Most patients in both outbreaks had diarrhea (mainly watery diarrhea) and abdominal pains. Fever, vomiting, and headache were also recorded in some patients in both outbreaks. In routine microbiological tests at Oita Prefectural Institute of Health and the Environment, 8 intestinal bacterial pathogens (Campylobacter spp., Salmonella spp., Shigella spp., Vibrio spp., Yersinia spp., Staphylococcus aureus, Bacillus cereus, Clostridium perfringens) were not isolated from any stool specimens tested in both outbreaks by using selective media for each pathogen. Known virulence determinants for diarrheagenic E. coli (afaD, aggR, astA, eae, elt, estA, invE, stx1, and stx2) and norovirus were also not detected in all stool specimens tested by routine polymerase chain reaction (PCR) screening. In OB2, Aichi virus, astrovirus, and sapovirus were additionally examined by PCR, but were negative in all specimens. We did not test for diarrheagenic parasites (Cryptosporidium, Giardia, and Cyclospora). No suspected infection sources or transmission routes were epidemiologically inferred for either outbreak. However, E. coli O8 was isolated from most tested stool specimens from patients on 10 May 2016.
serotypes of the strains were both found to be O8:H8 by in silico analysis [9]. Only 49 SNPs and an 8-bp indel were detected between the 2 genomes. These results, together with the data from the PFGE analysis, indicate that the strains that caused the 2 outbreaks were very closely related, that is, very recently separated from a common clonal ancestor. The complete genome sequence determination of strain 16F5M1D1 using an Oxford Nanopore MinION sequencer revealed that the genome consisted of a 4,800,098-bp chromosome and 1 large and 2 small plasmids (Supplementary Table 1). The chromosome contained 5 prophages and 2 tandemly integrated integrative elements (Supplementary Figure 1). All sequence data generated in this study are available in the DDBJ/EMBL/GenBank BioProject database (PRJDB8539).

Phylogenetic analysis of strain 16F5M1D1 based on the core genes identified by Roary [10] revealed that 16F5M1D1 belongs to phylogroup B1 and was most closely related to the EPEC O156:H8 strain 13E0767 in the E. coli reference strain set used (Figure 1A; Supplementary Table 2). In the public database (accessed on 16/07/2019), the genome information of 3 E. coli O8:H8 strains isolated from spinach in 2011 in the United States (PSU_0120 to PSU_0122) was available. These strains had the O8:H8 serotype, as confirmed by in silico analysis [11], but were phylogenetically distinct from 16F5M1D1 (Figure 1B). We therefore propose a designation of LT2d for the LT2 variant found in 16F5M1D1. By PCR analysis using newly designed elt2d-specific primers (elt2d-F: 5'-CTTTTTTCTCTGATCTTCCAG-3'; and elt2d-R: 5'-CAGAAGCACAGGCGAATC-3'), elt2d was detected in all O8 strains isolated in the 2 outbreaks. LT2d is encoded by a lambda-like phage integrated into the prfC gene on the 16F5M1D1 chromosome (Figure 1C). The LT2d phage genome shows interesting similarity to Shiga toxin (Stx)-transducing phages; the early region of the LT2d phage showed the highest similarity to that of the Stx2d phage of the Stx-producing E. coli (STEC) strain 1720a_02, whereas the late region was most similar to that of the Stx2a phage of STEC cq9. This genetic organization suggests that the elt2d gene is under the control of the late gene promoter; thus, its expression is induced by phage-inducing agents, such as mitomycin C (MMC), as observed for stx2 genes [13]. As expected, in the CHO cell elongation assay [14], clear elongation of CHO cells was induced by the lysate prepared from the 16F5M1D1 culture treated with MMC but not by the lysate of the untreated 16F5M1D1 culture. In contrast, clear elongation was induced by the lysates of an LT1-producing ETEC strain, O6:HNT, irrespective of MMC treatment (Figure 1D). This result indicates that LT2d has a cytotoxic activity similar to LT1 and LT2d production is dependent on phage induction. For further confirmation, we constructed an elt2d deletion mutant of strain 16F5M1D1 by the Wanner method [15] and its derivative, complemented with the Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible elt2d gene, using a low copy number plasmid, pCL1920 [16], and performed the CHO cell elongation assay. CHO cell elongation was not induced by the lysate prepared from the elt2d deletion mutant even with MMC treatment. In contrast, that of the IPTG-induced complemented strain induced CHO cell elongation, confirming that the cytotoxic effect on CHO cells observed was directly related to LT2d.

In the phylogenetic analysis of the A and B subunit genes of elt2, the 16F5M1D1 gene belonged to the elt2 cluster but formed a branch distinct from known elt2 variants (elt2a, b, and c) (Figure 1B). We therefore propose a designation of LT2d for the LT2 variant found in 16F5M1D1. By PCR analysis using newly designed elt2d-specific primers (elt2d-F: 5'-CTTTTTTCTCTGATCTTCCAG-3'; and elt2d-R: 5'-CAGAAGCACAGGCGAATC-3'), elt2d was detected in all O8 strains isolated in the 2 outbreaks. LT2d is encoded by a lambda-like phage integrated into the prfC gene on the 16F5M1D1 chromosome (Figure 1C). The LT2d phage genome shows interesting similarity to Shiga toxin (Stx)-transducing phages; the early region of the LT2d phage showed the highest similarity to that of the Stx2d phage of the Stx-producing E. coli (STEC) strain 1720a_02, whereas the late region was most similar to that of the Stx2a phage of STEC cq9. This genetic organization suggests that the elt2d gene is under the control of the late gene promoter; thus, its expression is induced by phage-inducing agents, such as mitomycin C (MMC), as observed for stx2 genes [13]. As expected, in the CHO cell elongation assay [14], clear elongation of CHO cells was induced by the lysate prepared from the 16F5M1D1 culture treated with MMC but not by the lysate of the untreated 16F5M1D1 culture. In contrast, clear elongation was induced by the lysates of an LT1-producing ETEC strain, O6:HNT, irrespective of MMC treatment (Figure 1D). This result indicates that LT2d has a cytotoxic activity similar to LT1 and LT2d production is dependent on phage induction. For further confirmation, we constructed an elt2d deletion mutant of strain 16F5M1D1 by the Wanner method [15] and its derivative, complemented with the Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible elt2d gene, using a low copy number plasmid, pCL1920 [16], and performed the CHO cell elongation assay. CHO cell elongation was not induced by the lysate prepared from the elt2d deletion mutant even with MMC treatment. In contrast, that of the IPTG-induced complemented strain induced CHO cell elongation, confirming that the cytotoxic effect on CHO cells observed was directly related to LT2d.

The CFA/III gene cluster was found in the 103-Kb plasmid in 16F5M1D1 (Supplementary Figure 2). It has been reported that CFA/III was identified in 8% of the ETEC strains isolated from patients with travelers’ diarrhea in Japan [17] and detected in several strains in a long-term global distribution study of ETEC [2]. This plasmid contained the repFIB replicon and a set of genes for conjugational transfer but no additional known virulence genes.

In conclusion, we identified 2 outbreaks of diarrhea caused by very closely related clones (strains) of E. coli O8:H8 that carry a gene for a novel variant of LT2, named LT2d, and the genes for CFA/III. The elt2d gene and the CFA/III gene cluster are encoded by a prophage and a large plasmid, respectively. Our findings indicate that more attention should be paid to infections by E. coli strains producing LT2d with colonization factors.
The phylogenetic analyses, genetic structure of the LT2d-encoding phage, and CHO cell elongation assay. A, A core gene–based maximum likelihood (ML) tree of *Escherichia coli* O8:H8 strain 16F5M1D1 and an *E. coli* reference strain set. A cryptic *Escherichia* clade I strain TW15838 was included as an outgroup. The tree was constructed based on 225,254 SNP sites located on 2569 core genes. Phylogroups, pathotypes, and serotypes are indicated. B, Neighbor-joining (NJ) trees based on the nucleotide sequences of the A and B subunit genes of the LT2d phage genome. C, The results of the CHO cell elongation assay. CHO cells (2×10⁵ cells/well/500 µL) in a 24-well plate were treated with 100-fold diluted bacterial cell lysates for 48 hours at 37°C and visualized under a light microscope (×100). Cell lysates were prepared by sonicating bacterial cultures incubated with the presence or absence of 500 ng/mL of mitomycin C or 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours at 37°C. Enterotoxigenic *E. coli* O6:HNT (LT1-positive) and *E. coli* K-12 MG1655 were used as positive and negative controls, respectively. Untreated: CHO cells untreated with bacterial lysate.
Supplementary Data
Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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