Atypical diarrheagenic Escherichia coli in milk related to a large foodborne outbreak

Shouhei Hirose¹, Kenji Ohya¹, Tomoya Yoshinari¹, Takahiro Ohnishi¹, Katsumi Mizukami²,
Tomikatsu Suzuki², Kenji Takinami², Takayoshi Suzuki³, Kenichi Lee⁴, Sunao Iyoda⁴,
Yukihiro Akeda⁴, Yuichiro Yahata⁵, Yuuki Tsuchihashi⁶, Tomimasa Sunagawa⁶,
Yukiko Hara-Kudo¹,∗

¹ Division of Microbiology, National Institute of Health Sciences, Kanagawa, Japan
² Toyama City Public Health Center, Toyama, Japan
³ Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, Kanagawa, Japan
⁴ Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan
⁵ Center for Field Epidemic Intelligence, Research and Professional Development, National Institute of Infectious Diseases, Tokyo, Japan

This is an Open Access article, distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives licence (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is unaltered and is properly cited. The written permission of Cambridge University Press must be obtained for commercial re-use or in order to create a derivative work.
*Corresponding author: Yukiko Hara-Kudo, DVM, Ph D

Division of Microbiology, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki 210-9501, Japan

Tel: +81 44 270 6571, Fax: +81 44 270 6572, E-mail: ykudo@nihs.go.jp
Abstract

A foodborne outbreak related to milk cartons served in school lunches occurred in June 2021, which involved more than 1800 cases from 25 schools. The major symptoms were abdominal pain, diarrhea, vomiting, and fever. Although major foodborne toxins and pathogens were not detected, a specific Escherichia coli strain, serotype OUT (OgGp9):H18, was predominantly isolated from milk samples related to the outbreak and most patients tested. The strains from milk and patient stool samples were identified as the same clone by core genome multilocus sequence typing and single nucleotide polymorphism analysis. The strain was detected in milk samples served for two days related to the foodborne outbreak at a rate of 69.6% and levels of less ten most probable number/100 mL but not on the days unrelated to the outbreak. The acid tolerance of the strain for survival in the stomach was similar to that of enterohemorrhagic E. coli O157:H7, and the same inserts in the chu gene cluster in the acid fitness island were genetically revealed. The pathogenicity of the strain was not clear, however, it was indicated that the causative pathogen was atypical diarrheagenic E. coli OUT (OgGp9):H18.
Introduction

A large foodborne outbreak related with milk cartons served in school lunches occurred in June 2021 in Toyama City, Japan. A public health center in the area first noticed that various schools such as elementary, junior high, and nursery schools reported numerous children with digestive symptoms on June 17th and began the initial epidemiological investigations [1]. The major symptoms observed in the patients were abdominal pain, diarrhea, vomiting, and fever.

Over 1800 cases from 25 schools with no fatalities, were reported. The common meals among these patients were the lunches served at each school, which included the pasteurized milk carton produced by the T milk factory. Furthermore, schools serving milk cartons produced by other factories did not report any patients. Therefore, the public health center determined that the milk cartons produced by the T milk factory served in school lunches on June 15th and 16th were the causative food of the outbreak. T milk factory usually produced 6,000-7,000 cartons of 200 mL, 10-20 cartons of 500 mL and 20 cartons a day from 1,600-1,800 kg raw milk by several workers. The raw milk was pasteurized at 128 °C for 2 seconds with a plate heat exchanger. The ultra high temperature pasteurization method (120-150 °C for 2-3 seconds) is most popular in Japan, and more than 90% of milk in markets was produced by the method.
Previously, outbreaks of foodborne pathogens associated with pasteurized milk in industrialized countries have been reported [2, 3]. The major pathogens were *Salmonella*, *Listeria*, enterohemorrhagic *Escherichia coli*, and *Yersinia enterocolitica*. A large outbreak of *Staphylococcus aureus* enterotoxins in milk products made from dried milk powder produced in a factory occurred in Japan, where more than 13,000 cases have been reported [4, 5]. These pathogens and the toxins were included as targets for the investigation of the outbreak that occurred in Toyama City. Representative stool samples of 64 patients from 12 schools were tested for major foodborne pathogens as follows; *Aeromonas*, *Bacillus cereus*, enterohemorrhagic *E. coli*, *Clostridium perfringens*, *Plesiomonas*, *Salmonella*, *Shigella*, *S. aureus*, *Y. enterocolitica*, *Yersinia pseudotuberculosis*, and norovirus, which were tested by culture methods or PCR assays in the public health center. At a national research institute, milk samples were tested for foodborne bacterial toxins and foodborne pathogens, leading to symptoms, other than pathogens not tested in the public health center, such as diarrheagenic *E. coli*, *Escherichia albertii*, or *Listeria*. In this study, we reported a microbiological analysis of a large foodborne outbreak, the suspected causative pathogen, and pathogen contamination of the milk cartons served in school lunches.

Methods
Milk samples

Milk carton (200 mL) samples served on June 14th, 15th, and 16th were stored in freezers at -20 °C in schools for investigations of food poisoning or any other accidents. Milk cartons scheduled to serve at the lunches on June 17th were stored in refrigerators. The cartons were collected by the investigators of Toyama City Public Health Center, and some of them were provided to National Institute of Health Sciences (NIHS) for microbiological tests. The milk cartons served on June 14th, 15th, 16th, and 17th were manufactured at the T milk factory in Toyama city, Japan on June 11th, 14th, 15th, and 16th, respectively.

Detection of staphylococcal enterotoxins, B. cereus enterotoxin and the emetic toxin (cereulide), and C. perfringens enterotoxin in milk

Eleven milk samples were initially collected by Toyama City Health Center; two milk samples from two schools served on June 14th suggested no relation with the food poisoning by epidemiological investigation, two milk samples from two schools on June 15th, two milk samples from two schools on June 16th suggested the relations, and five milk samples from one school stored in the schools for lunches on June 17th but not served, were tested for investigation on bacterial toxins. Additionally, five milk samples from one school stored for lunches on June 17th, but not served, were tested for the presence of bacterial toxins as described in Supplementary Methods.
Detection of L. monocytogenes, S. aureus, B. cereus, Escherichia albertii, and diarrheagenic E. coli in milk

The milk samples were also tested to investigate other foodborne pathogens. Because the main symptoms of patients were abdominal pain, diarrhea, vomiting, and fever, and the results of test in Toyama City Public Health Center were referred, L. monocytogenes, S. aureus, B. cereus, E. albertii, and diarrheagenic E. coli were targeted to detect by enrichment and isolation as described in Supplementary Methods. In addition, E. albertii and diarrheagenic E. coli were tested by PCR; E. albertii specific gene [6] for E. albertii; stx [7] and eae [8] for enterohemorrhagic E. coli (EHEC); heat-labile enterotoxin (LT) [9] and heat-stable toxin (ST) [9] for enterotoxigenic E. coli (ETEC); eae [8] and bfpA [10] for enteropathogenic E. coli (EPEC); aggR [11] for enteroaggregative E. coli (EAEC); astA [11] for enteroaggregative E. coli heat-stable enterotoxin 1 (EAST1) producing E. coli; invE (QuickPrimer InvE gene; Takara Co., Shiga, Japan) and ipaH (QuickPrimer IpaH gene; Takara Co.) for enteroinvasive E. coli (EIEC).

Isolation of E. coli from milk

Enrichment cultures of 11 initially collected milk samples in mEC or CT-mEC were streaked onto CHROMagar STEC and DHL agar, and then incubated at 37 °C for 24 h. E.
coli colonies suspected to be mauve or blue on CHROMagar STEC and red on DHL agar were tested for biochemical characteristics using TSI (Oxoid) and LIM (Eiken Chemical Co., Ltd, Tokyo, Japan) agars. Representative colonies showing E. coli biochemical characteristics were tested for O- and H-serotyping and genotyping, as described below.

Thirty-one additional milk samples (9, 10, 9, and 3 samples of June 14th, 15th, 16th, and 17th, respectively; 25 g) were cultured in 225 mL CT-mEC at 42 °C, streaked onto CHROMagar STEC and DHL agar, and incubated at 37 °C for 24 h. Representative colonies coincident with E. coli biochemical characteristics were tested for O- and H-serotyping and genotyping as described below.

Isolation of E. coli from patient feces

Colonies suspected to be E. coli on DHL agars cultured of 64 patient stool samples by Toyama City Public Health Center were tested for biochemical characteristics with TSI and LIM agars. Colonies positive for E. coli biochemical characteristics were tested for O- and H-serotyping and O-genotyping, as described below.

O- and H-serotyping and O-genotyping of E. coli

E. coli isolates were tested for E. coli O and H antigen agglutination with antisera according to the manufacturer’s protocols (Denka, Tokyo, Japan) and Statens Serum Institute.
Whole genome sequencing and bioinformatics analysis of E. coli OUT (OgGp9):H18

Whole genome sequencing of E. coli OUT (OgGp9):H18 using the DNBSEQ-G400 Instrument (MGI Tech, Shenzhen, China) was performed for core genome (cg) multilocus sequence typing (MLST) [13] and single nucleotide polymorphism (SNP) analysis[14, 15]. The detail procedures of each experiment and analysis are described in Supplementary Methods. The presence of the above virulence factors in the genomes of E. coli OUT (OgGp9):H18 strain isolated from a milk sample (ESC818) and a patient (ESC828) were searched by virulence factor database [16] and BLASTN program [17] with default settings.

Acid tolerance of E. coli OUT (OgGp9):H18

E. coli OUT (OgGp9):H18 strains (ESC818 and ESC 828), E. coli K-12 (NBRC 3301; National Institute of Technology and Evaluation Biological Resource Center, Chiba, Japan), and EHEC O157:H7 (EC7, a strain from a patient from an outbreak in Sakai City, 1996) [18] cells were cultured in TSB at 37 °C for 18 h. The cultures were diluted in buffered peptone water (BPW, pH 7.0; Nissui) to 10⁶ CFU/mL. The bacterial dilutions (0.1 mL) were inoculated into each 0.9 mL BPW at pH 2.5, 3.0, 4.0, and 7.0 prepared with 1 N HCl.
BPW-inoculated *E. coli* strains were incubated at 37 °C for 3 h. After incubation, the cultures were diluted to $10^{-6}$ and $10^{-7}$ in phosphate-buffered saline (PBS), and the dilutions (0.1 mL) were inoculated onto TSA in duplicates. After culturing at 37 °C for 18 h, colonies were counted to confirm the populations in BPW at various pH. The acid tolerance test was performed in triplicates.

**Comparative analysis of acid fitness island**

The acid fitness island (AFI) [19] in *E. coli* K-12, *E. coli* OgGp9:H18 (ESC818), and *E. coli* O157:H7 (Sakai) genomes was browsed and extracted using Artemis [20]. Alignments between each locus were generated using the BLASTN program [17] with default settings and then analyzed and visualized using Easyfig [21].

**Freezing resistance of *E. coli* OUT (OgGp9):H18**

*E. coli* OUT (OgGp9):H18 strain (ESC818) was cultured in TSB at 37 °C for 18 h, and the culture (40 µL) was inoculated into four tubes containing 40 mL milk purchased in Tokyo. To confirm the number of bacteria inoculated, the bacterial culture was diluted to $10^{-6}$ and $10^{-7}$ in PBS, and then the dilutions (0.1 mL) were inoculated onto TSA in duplicates. After culturing at 37 °C for 18 h, colonies were counted. The inoculated milk samples were stored in a refrigerator at -28 °C. Immediately after and at one, three, and seven days, the milk
inoculated with the strain was thawed. The strain population was determined using the most probable number (MPN) method [22]. In brief, 10 mL, 1 mL, and 0.1 mL of milk were inoculated into 10 mL CT-mEC, in triplicates. After incubation at 42 °C for 22 h, the cultures were streaked on Chromagar STEC and incubated at 37 °C for 22 h. The colonies suspected to be *E. coli* were confirmed as OgGp9 by O-genotyping. The freezing resistance test was performed in triplicates.

**Quantification of *E. coli* OUT (OgGp9):H18 and bacterial population in milk**

Nineteen milk samples that tested positive for *E. coli* OUT (OgGp9):H18 contamination were quantitatively assessed for the level of contamination by the pathogen using the MPN method described above. The milk samples were also quantitatively tested to measure the bacterial contamination level by the MPN method, using TSB. After incubation at 37 °C for 18 h, the cultures were observed for turbidity and streaked on TSA to determine bacterial growth. Additionally, a real-time PCR was performed targeting 16s rRNA [23] to estimate the bacterial population. *E. coli* (NBRC3972, NITE Biological Resource Center, National Institute of Technology and Evaluation, Tokyo, Japan) cultures in TSB were serially diluted with PBS to $10^{-1}$ to $10^{-9}$. DNA was extracted from the dilutions using a QIAamp DNA mini kit (Qiagen) for real-time PCR. A portion (0.1 mL) of the $10^{-6}$ dilution was plated onto TSA in quintuplets and incubated at 37 °C for 24 h to confirm the number of bacteria. A standard
curve was constructed using Ct and CFU values, and the bacterial population in the milk was estimated.

Ethical Statement
The study design and the protocol for the use of feces in patients were approved by the Institutional Review Board of the NIHS, Japan (approval no. 355).

Results
Detection of staphylococcal enterotoxins, B. cereus enterotoxin and the emetic toxin (cereulide), and C. perfringens enterotoxin in milk
Although contamination with staphylococcal enterotoxins, B. cereus enterotoxin, cereulide, and C. perfringens enterotoxin in two milk samples served on June 14\textsuperscript{th}, 15\textsuperscript{th}, 16\textsuperscript{th}, and 17\textsuperscript{th} (two samples each) were tested using commercially available test kits or LC-MS/MS analysis, these toxins were not detected in any of the samples.

Detection of Listeria, S. aureus, B. cereus, E. albertii, diarrheagenic E. coli, and other E. coli in milk
Listeria, S. aureus, E. albertii, and diarrheagenic E. coli were not detected in any of the milk samples that were tested. Cereulide-producing strains of B. cereus were isolated from
milk samples initially collected by Toyama City Public Health Center served on June 14\textsuperscript{th}, 15\textsuperscript{th}, and 16\textsuperscript{th}.

\textit{E. coli} other than diarrheagenic \textit{E. coli} described above were isolated from milk samples served on June 16\textsuperscript{th} and not served on June 17\textsuperscript{th} (one sample each), but not from those served on June 14\textsuperscript{th} (two samples), June 15\textsuperscript{th} (two samples), June 16\textsuperscript{th} (one sample), and not served on June 17\textsuperscript{th} (4 samples). The O-antigens of most \textit{E. coli} isolates were untypable (OUT) by agglutination testing with anti-serum. The O-genotype of OUT strains was typed to OgGp9 composed of O-genotype O17, O44, O73, and O106 \cite{12}. Even though OgGp9 strains were tested for anti-O17, O44, O73, and O106 sera, positive reactions for agglutination were not observed. Because the H-antigen detected for OgGp9 strains was H18, the serotype of \textit{E. coli} strain was determined as OUT (OgGp9):H18. \textit{E. coli} OUT (OgGp9):H18 formed mauve and red colonies on CHROMagar STEC and DHL agars, respectively.

\textit{Isolation of \textit{E. coli} OUT (OgGp9):H18 from milk}

\textit{E. coli} OUT (OgGp9):H18 was isolated from the milk samples served on June 15\textsuperscript{th} (50.0\%, 6/12 samples from 12 schools) and 16\textsuperscript{th} (90.9\%, 10/11 samples from 11 schools) (Table 1). Thus, \textit{E. coli} OUT (OgGp9):H18 was isolated from milk samples served on both days of the foodborne outbreak at a rate of 69.6\% (16/23). From the milk samples scheduled but not
served on June 17th, *E. coli* OUT (OgGp9):H18 was isolated (25%; 2/8 samples from 3 schools) but not from the milk served on June 14th (0%; 0/11 samples from 11 schools).

**Isolation of *E. coli* OUT (OgGp9):H18 from feces in patients**

Most colonies from 64 patients' feces on DHL agar were suspected as *E. coli*. The colonies coincident with *E. coli* biochemical characteristics according to the results of TSI and LIM agars were tested for O- and H-serotyping and genotyping, and *E. coli* OUT (OgGp9):H18 was isolated from 61 of 64 patients (95%). *E. coli* O18 and O68 were isolated from the three other patients.

**Virulence factors of *E. coli* OUT (OgGp9):H18 from milk and patients**

In the genomes of *E. coli* OUT (OgGp9):H18 from a milk sample (ESC818) and a patient (ESC828), typical virulence factors as diarrheagenic *E. coli* that were tested in milk samples; *stx* and *eae* for EHEC; ST and LT for ETEC; *eae* and *bfpA* for EPEC; *aggR* for EAEC; *astA* for EAST1 producing *E.coli*; *invE*, and *ipaH* for EIEC were not found by the BLAST search.

**Genetic relationship among *E. coli* isolates from milk and a patient and other representative *E. coli* strains**
In cgMLST analysis, the genome IDs of all 2513 loci identified from ESC818 and ESC828 were identical. Additionally, in the cgSNP analysis, only one SNP was detected among the 3,388,601 bp of core genome in our analysis. These results indicate that isolates from ESC818 and ESC828 were the same clones (Fig. 1A and B). Upon conducting the MST analysis based on cgMLST, these isolates were classified into the same branch as uropathogenic \( E. \ coli \) O17:H18 (UMN026) and EAEC O44:H18 (042) (Fig. 1A). Similarly, the phylogenetic tree based on cgSNP indicated that these isolates were relatively more related to UPEC (UMN026) and EAEC (042) than other strains (Fig. 1B).

**Acid tolerance of \( E. \ coli \) OUT (OgGp9):H18**

The mean populations of \( E. \ coli \) OUT (OgGp9):H18 strains (ESC818 and ESC 828), EHEC O157:H7 (EC7), and \( E. \ coli \) K-12 in BPW at various pH values were 3.9 log CFU/ mL, 4.0 log CFU/ mL, 3.9 log CFU/ mL, and 3.9 log CFU/ mL, respectively. All the strains grew to 6.2–7.0 (ca 6.7) log CFU/mL at pH 7.0 (Fig. 2). However, \( E. \ coli \) OUT (OgGp9):H18 strains and EHEC O157:H7 grew slightly at pH 2.5, 3.0, and 4.0. In addition, \( E. \ coli \) K-12 grew slightly at pH 4.0, although the inoculation level was maintained at pH 3.0 and decreased to 2.9 log CFU/ mL at pH 2.5.

**Comparative analysis of AFI**
In the OUT (OgGp9):H18 genome, the AFI, a cluster of genes responsible for acid tolerance of widespread *E. coli* from non-pathogenic K-12 to EHEC, such as *gadA* glutamate decarboxylase [19] was observed (Fig. 3). Additionally, the island of the OUT (OgGp9):H18 genome and O-island 140 consists of genes involved in iron uptake, such as *chuA* [24], similar to the O157:H7 genome. O-island 140 was also present in the AFI of the EAEC (042) genome (Fig. 3).

**Freezing resistance of *E. coli* OUT (OgGp9):H18**

The mean populations of *E. coli* OUT (OgGp9):H18 in inoculated milk sample was 413 CFU/40 mL. The mean populations of *E. coli* OUT (OgGp9):H18 were 600, 1033, 813, and 600 MPN/100 mL after 0, 1, 3, and 7 days of storage, respectively (Table 2). No large decrease was observed during the storage period.

**Quantification of *E. coli* OUT (OgGp9):H18 and bacterial populations in milk samples**

The mean populations of *E. coli* OUT (OgGp9):H18 in six milk samples from six schools served on June 15th, five samples from five schools served on June 16th, and one sample from one school scheduled but not served on June 17th were 8.4 MPN/100 mL, 7.0 MPN/100 mL, and 9.2 MPN/100 mL, respectively (Table 3). The mean bacterial populations in milk samples on June 14th, 15th, 16th, and 17th were 710 MPN/100 mL, 299 MPN/100 mL, 610
MPN/100 mL, and 19367 MPN/100 mL, respectively. The mean populations were also estimated by the standard curve analysis of real-time PCR targeting 16s rRNA and were 3.8 log CFU/mL, 3.8 log CFU/mL, 5.0 log CFU/mL, and 5.5 log CFU/mL, respectively.

Discussion

After Toyama City Public Health Center detected the foodborne outbreak, patient samples were immediately analyzed at the center. Tests conducted for many major foodborne bacteria and norovirus were negative. A portion of the milk samples was also analyzed at NIHS. Any bacterial toxins and pathogenic bacteria investigated in the study, other than *B. cereus*, were not detected. Although cereulide-producing strains of *B. cereus* were isolated, it was determined that the strains were not related to food poisoning because they were detected in all tested milk samples, including those irrespective of the outbreak. Notably, *E. coli* was predominantly isolated from milk cartons that were served in school lunches on the days that caused the foodborne outbreak, but not in those scheduled to be served on other days. The serotype was determined to be OUT (OgGp9):H18. The major symptoms of the patients like abdominal pain and diarrhea were consistent with those of diarrheagenic *E. coli* infection. In addition, *E. coli* was detected in most of the fecal samples of patients. The *E. coli* OUT (OgGp9):H18 strain from milk was similar to those from patients, as assessed by cgMLST and cgSNP analyses. These results suggest that the intake of *E. coli* OUT (OgGp9):H18...
contaminated milk induced the foodborne outbreak. Additional microbiological tests and epidemiological information indicated that *E. coli* OUT (OgGp9):H18 was the causative bacterium of this outbreak.

Gastrointestinal pathogens must survive the acidic conditions in the stomach to infect the host; thus, acid tolerance is related to virulence [25]. We observed that the acid tolerance of *E. coli* OUT (OgGp9):H18 was similar to that of EHEC O157:H7 (Fig. 2). The populations of *E. coli* OUT (OgGp9):H18 strains and EHEC O157:H7 were maintained at 4.7–4.9 log CFU/mL under acidic conditions (pH ranging between 2.5 and 4.0). Acid tolerance of *E. coli* OUT (OgGp9):H18 strain was genetically analyzed. Although genes responsible for acid tolerance in *E. coli* such as *gad* and *hde* cluster in the AFI and are conserved from the non-pathogenic K-12 to EHEC [24], their transcriptomic responses differ at varying pH conditions, resulting in differences in acid tolerances of the *E. coli* strains [26, 27]. In the AFI of the EHEC genome, a *chu* gene cluster, involved in iron uptake, designated O-island 140, is inserted, implying that O-island 140 contributes to the enhanced acid tolerance of EHEC compared to K-12 [24]. Iron is an essential cofactor of several enzymes, and it has been reported that acidic pH enhances the expression of genes involved in iron uptake in K-12 cells [28]. In the above transcriptional studies, upregulation of EHEC-specific genes involved in iron uptake, such as *chu* genes, was observed in acid-treated EHEC [26, 27]. Additionally, enhanced expression of *chu* genes along with acid stress response in EHEC treated with spinach root
exudates was observed, but it was not observed in EHEC treated with spinach leaf extracts, which may be attributed to acidic conditions in root exudates [29]. Considering the pathogenicity of EHEC, upregulation of *chu* genes in O-island 140 along with genes in the AFI in EHEC within human macrophages contributed to the survival of EHEC in macrophages [30]. These reports imply that *chu* genes in the O-island 140 play a vital role in acidic conditions and contribute to acid tolerance and pathogenicity of EHEC. In the OUT (OgGp9):H18 genome, O-island 140 was also inserted within the AFI, similar to the EHEC genome, which suggests genetic and phenotypic features such as acid tolerance and pathogenesis of this strain. *E. coli* OUT (OgGp9):H18 strains can survive in the stomach, similar to EHEC O157:H7, and the ability to survive would be virulent.

The pathogenicity of *E. coli* OUT (OgGp9):H18 has been analyzed vigorously in another study. The strain showed adhesive properties in cultured cells and lethality in mice (Hara-Kudo, Y. *et al.*, personal communication), implying that the strain was pathogenic. The strain was phylogenetically closely related to some strains of EAEC (042) and UPEC (UMN026). Previously, EAEC OgGp9:H18 strains harboring *aggR* were also isolated from patients with gastrointestinal symptoms [31]. However, the *E. coli* OUT (OgGp9):H18 strains in our study did not possess typical EAEC virulence factors such as *aggR*, suggesting the presence of other atypical virulence factors in the strains. The results of microbiological tests and epidemiological information indicate that the *E. coli* OUT (OgGp9):H18 strain was the
cause of the outbreak, although the details of the pathogenesis are not clear at the present. In another study, the virulence factors of *E. coli* OUT(OgGp9):H18 have been analyzed and will be revealed in the future.

We investigated the proportion of contamination in milk cartons. Although some of the samples were tested in this study, it appeared that 50% of the milk cartons served on June 15th were contaminated with *E. coli* OUT (OgGp9):H18 (Table 1). In addition, milk cartons served on June 16th and scheduled but not served on June 17th were also contaminated with *E. coli* OUT (OgGp9):H18 at rates of 90.9% and 25% (Table 1), respectively. It was indicated that contamination of *E. coli* OUT (OgGp9):H18 to milk occurred on June 14th and continued for the next two days. The lack of cleaning of manufacturing lines or sanitary work could be considered one of the causes of this continuous contamination.

Because of the intake of 200 mL milk carton per patient, the ingestion dose of *E. coli* OUT (OgGp9):H18 was estimated at 15–18 based on the quantitative data of pathogen contamination in milk by the MPN method. The milk samples were stored at -20 °C for a few days. Freezing might have affected the survival rate of *E. coli* OUT (OgGp9):H18. Thus, we investigated decrease of *E. coli* OUT (OgGp9):H18 populations in milk by freezing, and then them were minimally affected by freezing for 7 days (Table 2). Therefore, the estimated ingestion dose was reasonable. Usually, infectious doses of EHEC has been estimated less than 10 to several hundreds [32, 33] and this trait is attributed to acid tolerance [25]. Since
major symptoms were abdominal pain and diarrhea but not severe such as bloody diarrhea

and hemolytic uremic syndrome (Suzuki, T. et al, personal communication), pathogenicity of

the *E. coli* OUT (OgGp9):H18 strains in this study were considered to be lower than that of

EHEC. However, it is consistent that infectious dose of the strains is at the same level with

EHEC, because the strains can survive at low pH, as same as EHEC.

In this study, viable bacterial populations in milk samples were analyzed using the MPN

method, and the populations in milk cartons served on June 15th (mean 299 MPN/100 mL)

and June 16th (mean 610 MPN/100 mL) were similar to those on June 14th (mean 710

MPN/100 mL) (Table 3). Large differences were not observed among the viable bacterial

populations in milk cartons from June 14th, 15th, and 16th. This indicated that the

pasteurization of milk served on June 15th and 16th was similar to that on June 14th. In

addition, it was shown that the bacterial number of *E. coli* OUT (OgGp9):H18 strain

decreased by 5 log CFU at 65°C for 1 min, similar to those of *E. coli* K-12 and EHEC

O157:H7, and therefore heat resistance of the strain was not observed (Hara-Kudo, Y. et al,

personal communication). These results indicate that contamination by *E. coli* OUT(OgGp9):H18 might be occurred after pasteurization steps of milk. For example, a surge

tank for pasteurized milk or milk packing in cartons would be contaminated. T factory

usually produced 6,000-7,000 cartons (200 mL) a day and actually produced 6,851 cartons on

June 11th though 7,840 cartons were produced on June 14th. It might be led some problems.
After the foodborne outbreak, the T milk factory was inspected to identify critical points leading to *E. coli* OUT (OgGp9):H18 contamination, and measures for preventing such recurrence was recommended by the Toyama City Government and the Ministry of Labour, Health and Welfare. Although the origin of *E. coli* OUT (OgGp9):H18 and the factors for contamination of milk cartons and continuous contamination were not clarified, it appeared that cross-contamination of pasteurized milk with raw milk by unsanitary handling, insufficient cleaning of raw milk tanks and milk cartons packing equipment, failure on temperature control of pasteurized milk, or structural defect of surge tanks for pasteurized milk were suspected as various aspects with potential risk for bacterial contamination and the growth (Suzuki, T. *et al*, personal communication). Along with them, the Japan Dairy Industry Association provided technical advice to the T milk factory for improving their manufacturing process.

**Acknowledgments**

This study was partially supported by a Health and Labour Sciences Research Grant (No. 21KA1006) from the Ministry of Health, Labour, and Welfare, Japan.

**Conflict of interest**
There are no conflicts of interest to report.

Data availability statement

The draft genomes of *E. coli* OUT (OgGp9):H18 from milk (ESC818) and a patient (ESC828) were deposited at GenBank/EMBL/DDBJ under BioProject number PRJDB15309.

References


23. Fratamico PM, et al. (2011) Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin-producing Escherichia coli serogroups
27, O45, O103, O111, O121, and O145 in ground beef. *Foodborne Pathogens and Disease*; 8: 601-607.


Responses on Exposure to Spinach and Lettuce Extracts. *Frontiers in Microbiology*; 7: 1088.


Figure legends

Fig. 1. Phylogenetic analysis of OUT (OgGP9):H18 isolates and other *E. coli* strains with serotype and pathotype information. The *E. coli* isolates, in this case, are shown in red. (A) MST based on cgMLST allelic distance of *E. coli* isolates and other strains. The colors of the circles indicate *E. coli* pathotypes. The length between the two circles reflects the genetic distance. (B) Maximum likelihood phylogenetic tree based on 3,593 SNP sites in the genome backbone of *E. coli* isolates and other strains. The scale bar indicates the number of substitutions per site. K-12: a model strain of *E. coli* (non-pathogenic), EHEC: enterohemorrhagic *E. coli*, UPEC: uropathogenic *E. coli*, EAEC: enteroaggregative *E. coli*, ETEC: enterotoxigenic *E. coli*, EPEC: enteropathogenic *E. coli*, APEC: avian pathogenic *E. coli*, NMEC: neonatal meningitis *E. coli*, AIEC: adherent invasive *E. coli*.
Fig. 2. Comparison of acid tolerance of *E. coli* strains at different pH in buffered peptone water. Acid tolerance of ESC818-*E. coli* OUT (OgGp9):H18 isolated from milk samples, ESC828-*E. coli* OUT (OgGp9):H18 isolated from patient feces, EHEC O157:H7-enterohemorrhagic *E. coli* derived from the foodborne outbreak, K-12- a model strain of *E. coli* (non-pathogenic) at pH 2.5, 3.0, 4.0 and 7.0 in buffered peptone water are shown. Error bars indicate standard deviation (n = 3).
Fig. 3. Comparative analysis of the acid fitness island (AFI) in K-12, OgGp9:H18 (ESC818), EHEC O157:H7 (Sakai), and EAEC O44:H18 (042) genomes. Sequence alignment of the AFI (between gadA and slp) in K-12, OgGp9:H18 (ESC818), EHEC (Sakai), and EAEC (042) genomes is shown. Vertical boxes between each sequence indicate similarity according to BLASTN (red for matches in the same direction). Blue arrows with annotation are coding sequences except for genes in the O-island 140 (inserted between yhiF and yhiD, yellow arrows).
Table 1. Qualitative analysis of *Escherichia coli* OUT (OgGp9):H18 contamination in milk carton served in school lunch

<table>
<thead>
<tr>
<th>Milk served date</th>
<th>Milk produced date</th>
<th>Served school* of positive and negative milk carton for <em>E. coli</em> OUT (OgGp9):H18 (number of carton)</th>
<th>Positive</th>
<th>Negative</th>
<th><em>E. coli</em> OUT (OgGp9):H18 positive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 14th</td>
<td>June 11th</td>
<td>No samples</td>
<td>D (1), E (1), F (1), J (1), K (1), L (1), B or C (2), G or H or I (3)</td>
<td>0/11 (0)</td>
<td></td>
</tr>
<tr>
<td>June 15th</td>
<td>June 14th</td>
<td>A (1), D (1), E (1), J (1), K (1), O (1)</td>
<td>F (1), B or C (2), G or H or I (3)</td>
<td>6/12 (50)</td>
<td></td>
</tr>
<tr>
<td>June 16th</td>
<td>June 15th</td>
<td>A (1), D (1), E (1), F (1), J (1), K (1), B or C (1), G or H or I (3)</td>
<td>B or C (1)</td>
<td>10/11 (90.9)</td>
<td></td>
</tr>
<tr>
<td>June 17th</td>
<td>June 16th</td>
<td>M (1), M or N (1)</td>
<td>A (1), M (4), M or N (1)</td>
<td>2/8 (25)</td>
<td></td>
</tr>
</tbody>
</table>

*a total 15 schools (A - O)*
Table 2. Survival of *Escherichia coli* OUT(OgGp9):H18 in frozen milk

<table>
<thead>
<tr>
<th>Freezing period (day)</th>
<th>Bacterial population (MPN/100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>600 ± 365*</td>
</tr>
<tr>
<td>1</td>
<td>1033 ± 971</td>
</tr>
<tr>
<td>3</td>
<td>813 ± 405</td>
</tr>
<tr>
<td>7</td>
<td>600 ± 365</td>
</tr>
</tbody>
</table>

*mean±SD
Table 3. Quantitative analysis of *Escherichia coli* OUT (OgGp9):H18 contamination in milk carton served in school lunch

<table>
<thead>
<tr>
<th>Milk served date</th>
<th>Milk produced date</th>
<th>No. of tested milk carton</th>
<th>No. of positive milk carton</th>
<th>E. coli OUT (OgGp9):H18</th>
<th>MPN value of viable bacteria (MPN/100 mL)</th>
<th>Bacterial count by real-time PCR for 16S rRNA (Log CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>June 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>Mean 3.8 SD 0.3</td>
</tr>
<tr>
<td>June 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>June 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>6</td>
<td>8.4</td>
<td>7.5</td>
<td>Mean 3.8 SD 0.8</td>
</tr>
<tr>
<td>June 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>June 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>5</td>
<td>7.0</td>
<td>3.1</td>
<td>Mean 5.0 SD 0.3</td>
</tr>
<tr>
<td>June 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>June 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>1</td>
<td>9.2</td>
<td>NA</td>
<td>Mean 5.5 SD 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Delivered to schools but no served.