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An outbreak of enterohemorrhagic *Escherichia coli* O157:H7 infection associated with minced meat cutlets in Kanagawa, Japan

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Summary

An outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection occurred in October 2016 in Kanagawa, Japan. A total of 61 patients and 17 asymptomatic cases were confirmed to be infected with EHEC O157:H7 by laboratory testing. Among these cases, 24 patients were hospitalized and four patients developed hemolytic-uremic syndrome. An epidemiological investigation found that this outbreak of EHEC O157:H7 infection was associated with the consumption of uncooked minced meat cutlets that were sold frozen at branches of a supermarket chain. The implicated uncooked meat cutlets were made of a mixture of minced beef, pork, onions and eggs. All 40 meat cutlets tested from one particular lot were positive for EHEC O157:H7. The patterns observed on pulsed-field gel electrophoresis of strains isolated from cases and meat cutlets were identical. The bacterial counts of EHEC O157:H7 and *E. coli* in meat cutlets ranged from 2.3 to 110 MPN/g and from 240 to 4600 MPN/g, respectively. There are currently no national regulatory standards to ensure the safety of these types of meat products in Japan. Consumers should ensure that such products are cooked thoroughly and that safe food handling procedures are used to prevent infection.
Introduction

*Escherichia coli* O157 is recognized as a major causative agent of enterohemorrhagic *E. coli* (EHEC) infection which has been categorized as a category III notifiable disease under the Infectious Diseases Control Law in Japan. Approximately 4000 cases of EHEC infection, consisting of 2500 symptomatic patients and 1500 asymptomatic carriers, are reported annually in Japan (1–4). Among the EHEC isolates analyzed in 2016, *E. coli* O157 accounted for 53%, followed by serogroups O26 (32%) and O103 (3.6%) (4). During 2013 and 2016, 13–25 outbreaks of foodborne EHEC infection were reported, with the number of patients involved in these outbreaks ranging from 105 to 766 cases (4).

In October 2016, an outbreak of EHEC O157:H7 infection occurred in Kanagawa, Japan. The outbreak was associated with the consumption of uncooked minced meat cutlets contaminated with EHEC O157:H7 that were sold frozen at branches of a supermarket chain. We report the results of the epidemiological investigation conducted in Kanagawa, detail the analyses of the causative EHEC O157:H7 isolates, and suggest control measures that may help prevent similar outbreak in the future.

Materials and methods

Outbreak investigation

Cases of EHEC O157:H7 infection were identified through the notification system
under the Infectious Diseases Control Law. When cases of EHEC O157:H7 infection were diagnosed, a physician notified the Health Center (HC) of EHEC O157:H7 infection. The HC conducted an epidemiological investigation to identify the source of the infection, and interviewed all of the cases.

Case definition

For the outbreak studied, a symptomatic case was defined as a resident of Kanagawa with diarrhea or bloody diarrhea and at least one of the following additional symptoms: abdominal cramps, vomiting or fever, along with a stool sample that tested positive for EHEC O157:H7. An asymptomatic case was defined as a resident of Kanagawa without gastrointestinal symptoms with a stool sample that tested positive for EHEC O157:H7.

Isolation of EHEC O157:H7 from fecal samples

Fecal samples from individuals who had been in contact with cases were cultured for EHEC O157:H7 at the Prefectural and Municipal Institutes of Public Health, according to the published procedure (5). Suspected colonies were characterized biochemically using standard microbiological methods. EHEC O157:H7 was confirmed by an agglutination test with O and H antisera (Denka Seiken, Tokyo, Japan). Strains were also tested for the presence of the stx genes (stx1 and stx2) by PCR.
Enumeration of EHEC O157 in meat cutlets

Frozen minced meat cutlets stocked in six supermarket branches were submitted to the HC for laboratory testing and transported to the Kanagawa Prefectural Institute of Public Health. The meat cutlets were stored at -35°C prior to use.

EHEC O157 in cutlets was enumerated by the conventional three-tube multiple dilution most-probable-number (MPN) procedure using four dilution series. A 25-g portion of each meat cutlet was mixed with 225 ml of modified EC broth (Oxoid, Tokyo, Japan) in a stomacher bag. Each sample was homogenized using the stomacher (Masticator: GSI Creos, Tokyo, Japan) for 1 min. Then, 10-ml of aliquots of the homogenates were added to three empty tubes, and three 10-fold dilutions were performed in modified EC broth. The tubes were incubated at 36°C for 20 h. A loopful sample from all tubes was inoculated onto a CT-SMAC agar plate (Oxoid). After incubation at 36°C for 20 h, suspected colonies were confirmed to be EHEC O157 using an E. coli O157 latex test kit: UNI (Oxoid) and PCR (6). The MPN was determined by the positive and negative PCR results and the latex test. EHEC O157 isolates from meat cutlets and from case fecal samples were further analyzed by pulsed-field gel electrophoresis (PFGE).

Determination of the E. coli count and the total bacterial count in the meat cutlets

A 25-g portion of each meat cutlet was mixed with 225ml of phosphate-buffered
saline (pH 7.0) in a stomacher bag, and each sample was homogenized using a stomacher for 1 min. Then, 10-ml of aliquots of the homogenates were used to inoculate the first three tubes containing 10 ml of double-strength EC broth (Oxoid) with a Durham tube, followed by three 10-fold dilutions in single-strength EC broth. The tubes were incubated at 44.5°C for 24 h, and gas production was visually examined. A loopful of sample from each of the tubes for which gas production was detected was inoculated onto a Chromocult coliform agar plate (Merck, Tokyo, Japan). After incubation at 36°C for 20 h, colonies with β-D-galactosidase and β-D-glucuronidase positive characteristics were counted as *E. coli*. The MPN was determined based on the number of *E. coli* positive tubes.

The standard pour plate count method was used to determine the total bacterial count using plate count agar (Eiken Chemical, Tokyo, Japan). The same serially-diluted samples of the meat cutlets used for the *E. coli* counts were used for the total bacterial counts. After plates were incubated at 36°C for 72 h, the total bacterial count was determined.

PFGE analysis

The PFGE profiles of EHEC O157:H7 were determined using a previously reported protocol (7). The DNA from EHEC O157:H7 cells embedded in agarose plugs was digested with 40 U of *XbaI* (Takara Bio Inc., Shiga, Japan) at 37°C for 5 h. *Salmonella Braenderup* H9812 was used as the control strain. PFGE was performed on a 1% agarose
gel using the CHEF MAPPER apparatus (Bio-Rad, Tokyo, Japan). The gel stained with ethidium bromide was observed under UV transillumination.

Statistical analysis

The F-test and Welch’s t-test used to assess differences between means. Differences in mean of total bacterial counts between lots of meat cutlets were analyzed using Welch’s t-test. All statistical analyses were performed using SPSS for windows software version 17.0 (SPSS Inc., Chicago, USA).

Result

Characteristics of the outbreak

Among the symptomatic and asymptomatic cases from October and November 2016, we identified 98 individuals who had been in contact with other cases and confirmed by laboratory testing 61 patients and 17 asymptomatic cases of EHEC O157:H7 infection, each with a history of consuming the implicated meat cutlets. The age of the patients and asymptomatic cases ranged from 1 to 79 years (Fig.1), and from 4 to 73 years, respectively, with median ages of 21 and 42 years, respectively. Among these 78 cases, 30 of the patients and 10 of the asymptomatic cases were female. The dates of onset of symptoms ranged from October 12 to November 17 (Fig. 2). Based on the dates of consumption of the meat cutlets and the dates of symptom onset in 39 patients, the incubation period of
38 of 39 patients ranged from 1 to 8 days (Fig. 3), and the median incubation period was 4 days. The clinical features of the illness included diarrhea, bloody diarrhea, abdominal pain, vomiting, and fever (Table 1). Four patients (one 5-year-old male and three female of 6, 8, and 63 years of age) developed hemolytic-uremic syndrome. Among the 61 patients, 24 were hospitalized, including 10 female patients (6–72 years old, median age: 21) and 14 male patients (5–78 years old, median age: 14.5). All of the patients recovered. The longest period of hospitalization was 94 days, for an 8-year-old girl with hemolytic-uremic syndrome who did not have any underlying medical conditions.

In an epidemiological investigation by the Prefectural and Municipal HCs in Kanagawa, all laboratory-confirmed symptomatic and asymptomatic cases were interviewed, and stated that they purchased uncooked minced meat cutlets from one of 12 branches of a supermarket chain. The implicated meat cutlets contained ground beef and pork mixed with onion and eggs, and were breaded. They were manufactured in a plant in Shizuoka Prefecture. Four pieces of meat cutlet (approximately 90 g each) were packaged in a plastic packet, and were sold frozen. On the packaging, consumers were recommended to fry the meat cutlets in oil at 170–175°C for 6 min prior to eating. Some patients or their family members stated in the interview that they have not checked the temperature of the oil during frying, and had instead relied on the cooking time or the surface color of the cutlets to determine when they were cooked. Some also stated that the cutlets appeared pink inside during consumption.
The HCs of Kanagawa Prefecture where the seller was located, and those of Shizuoka Prefecture where the meat cutlets were manufactured, conducted a traceback investigation to identify potential sources of EHEC O157:H7, including possible fecal contamination. The manufacturer produced 2010 packages of lot 1 meat cutlets, and shipped 1910 packages to 17 branches of a supermarket chain in Kanagawa. However, the HCs were unable to determine the sources of contamination. After a recall of the meat cutlets was issued, 595 packages (31.2%) of lot 1 cutlets were recovered. The majority of the packages (410: 21.5%) were returned by consumers, and the reminder (9.7%) were removed from circulation by supermarket branches.

Over the course of the outbreak, EHEC O157:H7 outbreak strains from 61 patients were submitted from hospitals to the Prefectural and Municipal Institutes of Public Health. Isolates were detected from 17 asymptomatic cases, and were confirmed as EHEC O157:H7 at the Institutes of Public Health. All EHEC O157:H7 strains possessed the stx2 gene.

Subtyping by PFGE

A total of 78 isolates from 61 patients and 17 asymptomatic cases, and two isolates of EHEC O157:H7 from meat cutlets, were analyzed using PFGE. The PFGE patterns of all 80 isolates were identical (Fig.4).
Detection and enumeration of EHEC O157

A total of 68 pieces of meat cutlet consisting of 40 pieces from lot 1, eight pieces from lot 2, and 20 pieces from lot 3, were subjected to enumeration of *E. coli* O157 (Table 2). EHEC O157 was detected in all 40 pieces from the lot 1 meat cutlets. The number of EHEC O157 in the lot 1 meat cutlets ranged from 2.3 to 110 MPN/g, and the genomic mean was 9.3 MPN/g. The geometric mean of each package ranged from 4.3 to 30.8 MPN/g. EHEC O157 was not isolated from the 28 pieces of cutlet from lots 2 and 3.

Enumeration of *E. coli* and the total number of bacteria

The number of *E. coli* and the total bacterial count were determined as indicators of fecal contamination in 48 cutlets (Table 2). *E. coli* was detected in all of the cutlets from lot 1, with numbers ranging from 240 to 4600 MPN/g. The MPN values for *E. coli* in the meat cutlets from lots 2 and 3 were lower than the detection limit, with the exception of one piece of meat cutlets from lot 2 for which an MPN value of 0.36 MPN/g was detected.

The total bacterial counts for all of the meat cutlets tested are shown in Table 2 and ranged from $2.7 \times 10^4$ to $2.6 \times 10^6$ CFU/g. The geometric mean of total bacterial count for the lot 3 meat cutlets was statistically significantly higher than that of the lot 1 meat cutlets ($p < 0.01$).

**Discussion**
In the outbreak of EHEC O157:H7 infection that occurred in October 2016 in Kanagawa, Japan, 61 symptomatic and 17 asymptomatic cases were identified. All patients and asymptomatic cases detected reported having consumed minced meat cutlets. Laboratory analysis revealed that all pieces of meat cutlet in a particular lot were highly contaminated with *E. coli* and EHEC O157:H7, and the PFGE patterns of EHEC O157:H7 isolated from cases and from meat cutlets were identical. These results suggested that fecal contamination of the meat cutlets had occurred, although the source of the contamination was not identified by an epidemiological investigation, and that the outbreak was associated with the consumption of meat cutlets contaminated with EHEC O157:H7. Meat, especially beef, is recognized as an important source of EHEC O157:H7 infection. Contamination of EHEC O157:H7 in retail beef has previously been reported (8-10), and although the prevalence of such contamination is low, outbreaks associated with EHEC O157:H7-contaminated beef have occurred (11-13). Contamination of pork with EHEC O157:H7 has also been reported (14), and the consumption of contaminated pork has been found to be the source of an outbreak of EHEC O157:H7 infection (15).

We determined that the number of EHEC O157:H7 present in the contaminated meat cutlets (each approximately 90 g) ranged from 2.3 to 110 MPN/g. Thus, each cutlet contained approximately 210 to 9900 MPN of EHEC O157:H7 in total. These values agreed with those of previous reports, with EHEC O157:H7 counts ranging from 0.9 to 4.3 CFU/g, from <10 to 6200 CFU/g, from < 0.3 to 15 MPN/g, and from < 0.3 to 2300
MPN/g in meat samples implicated in previous outbreaks (11,16–18). Estimation of the EHEC O157:H7 infectious dose based on the enumeration results from raw beef products is challenging, because consumers cook the products before consumption, thereby reducing the number of bacteria. The results of this study and previous studies therefore suggest that the infectious dose required to cause disease is lower than that of the EHEC O157:H7 count in the raw meat product.

The number of *E. coli* in the implicated lot of meat cutlets ranged from 240 to 4600 MPN/g, whereas the *E. coli* counts for the other two lots were under the detection limit. However, the total bacterial count for lot 3 exceeded that of lot 1. The results of this study suggested that *E. coli* could be an indicator of the presence of EHEC O157:H7. This is an agreement with previous studies (19, 20), showing that indicator organisms such as *E. coli* can be used to assess the hygienic status of processing plants and the performance of antimicrobial interventions, and can thus be used to predict the potential presence of pathogenic bacteria such as EHEC O157:H7 (19, 20). *E. coli* is an appropriate indicator organism for EHEC O157:H7, because it is likely disseminated in the same manner as EHEC O157:H7 (21). However, Tuttle and coworkers found that coliform counts were statistically significantly associated with the presence of EHEC O157:H7, whereas *E. coli* counts and aerobic plate counts were not related (11). The conflicting results of these studies may be attributed to methodological differences in enumeration of *E. coli* count.

In this study, we tested 40 pieces of outbreak-associated cutlet lot (lot 1) for EHEC
O157:H7 and 20 of these 40 pieces of lot 1 cutlets for *E. coli*. Because these numbers of meat cutlets were equivalent to 0.5% and 0.25%, respectively, of the 2010 packages of lot 1 meat cutlets produced (8040 individual pieces in total), it was difficult to estimate the magnitude of contamination among the lot 1 meat cutlets. However, the homogeneity in EHEC O157:H7 counts and *E. coli* counts among the lot 1 meat cutlets tested, as well as the unique PFGE pattern of the isolates derived from all patients and meat cutlets suggested that a single contamination event had occurred during the production process.

This particular outbreak of EHEC O157:H7 was characterized to be associated with uncooked frozen meat cutlets. The shelf life of these cutlets was four months, and they could be stocked in household freezers. Because frozen food products have a long shelf life in consumers’ homes, they represent a particular risk to the public as possible vehicles of infection (12). Therefore, the occurrence of an outbreak of foodborne illness associated with frozen food should be announced to the public as soon as possible to prevent further infection. Samples of the implicated meat cutlets were brought to the Kanagawa Prefectural Institute of Public Health by HC staff on October 27, and the microbiological examination started immediately. The outbreak was announced late the night of October 31. The recall of meat cutlets was issued on November 1, immediately after the announcement of the outbreak of EHEC O157:H7 infections, through the mass media, websites, and a warning message about meat cutlets contaminated with EHEC O157:H7 was displayed at supermarket branches. This warning message appeared to be effective.
as the last reported case occurred in Kanagawa on November 17. The process of warning at-risk persons not to eat the implicated food has been discussed (22) and its effectiveness appears to be dependent on reaching the at-risk population and conveying the seriousness of the illness.

In this outbreak, persons between 5 and 14 years of age were the age group most affected by the illness. These results were attributed to the age distribution of male patients. The number of female patients (49.2%) was similar to that of male patients (50.8%), but the age distribution differed between the genders. Whereas female patients were fairly evenly distributed among the age groups in this outbreak, male patients were predominantly distributed in the < 20 and > 49 age groups, and the number of male patients was slightly larger than that of female patients in the < 20 age group. Reports from the United States and Japan showed that the proportion of affected males under 12 or 20 years was higher than that of females in the same age group, and the proportion of affected males over or equal to 12 or 20 years was lower than that of females over or equal to 12 or 20 years of age (23, 24). Further studies should be conducted to elucidate reasons that might account for differences in the proportion of affected individuals by age and gender.

Cutlets are categorized as an uncooked side dish that are produced by adding seasoning to meat, fish and/or vegetables, and are sold frozen. There are no national regulatory standards to ensure the safety of this type of food product in Japan, while food
products categorized in “frozen food” need to meet certain regulatory standards: Specifications and Standards for Foods, Food Additives, etc. under the Food Sanitation Act. Uncooked side dish including meat cutlets should also be covered by regulatory standards. Moreover, good hygiene practices to prevent the contamination of food with pathogens should be implemented in the food industry. In addition, consumers should be advised on appropriate procedures for ensuring that food is cooked thoroughly, thereby decreasing the risk of infection from contaminated products, as in our study, inadequate cooking of meat products in the home was reported. To prevent foodborne diseases, including *E. coli* O157:H7 infection, food safety education is an important preventive measure that should be provided to consumers.

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**Conflict of interest**  None to declare.
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1. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare, Japan. Enterohemorrhagic


5. National Institute of Infectious Diseases. EHEC diagnosis and laboratory test, Pathogen


Figure legends

Fig. 1 Age and gender of confirmed symptomatic cases in an outbreak of EHEC O157:H7 infection in Kanagawa.

Fig. 2 Date of symptom onset of confirmed cases in an outbreak of EHEC O157:H7 infection in Kanagawa.

Fig. 3 Incubation period of 39 confirmed cases in an outbreak of EHEC O157:H7 infection in Kanagawa.

Fig. 4 Pulsed-field gel electrophoresis pattern of EHEC O157:H7 isolates cleaved with restriction enzyme XbaI. Lane 1–3, isolates from patients with EHEC O157:H7 infection; lane 4, an isolate from lot 1 meat cutlet; lane M, DNA size standard Salmonella Braenderup H9812.
Table 1 Characteristics of laboratory-confirmed symptomatic cases in an outbreak of EHEC O157:H7 infection in Kanagawa

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All cases (n=61)</th>
<th>Hospitalized cases (n=24)</th>
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</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>21 (1-79)</td>
<td>18 (5-78)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>30 (49.2)</td>
<td>10 (41.7)</td>
</tr>
<tr>
<td>No. of reported signs and symptoms (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>54 (88.5)</td>
<td>22 (91.7)</td>
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<tr>
<td>Bloody diarrhea</td>
<td>41 (67.2)</td>
<td>24 (100)</td>
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<tr>
<td>Abdominal cramps</td>
<td>52 (82.5)</td>
<td>23 (95.8)</td>
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<tr>
<td>Vomiting</td>
<td>9 (14.8)</td>
<td>5 (20.8)</td>
</tr>
<tr>
<td>Fever</td>
<td>22 (36.1)</td>
<td>13 (54.2)</td>
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<tr>
<td>HUS</td>
<td>4 (6.6)</td>
<td>4 (16.7)</td>
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<tr>
<td>Renal failure</td>
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<td>4 (16.7)</td>
</tr>
<tr>
<td>Cardiac enlargement</td>
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<td>1 (4.2)</td>
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<td>Condition</td>
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<td>Value 2</td>
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<td>-----------------</td>
<td>---------</td>
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<tr>
<td>Pancreatitis</td>
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<tr>
<td>Hepatic failure</td>
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<td>1 (4.2)</td>
</tr>
<tr>
<td>Ascites</td>
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<td>Hematuria</td>
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Table 2  The number of EHEC O157, *E. coli* and total bacterial count in 3 lots of meat cutlets

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Package number</th>
<th>Expiration date</th>
<th>No. of pieces tested/package*</th>
<th>EHEC O157 (MPN/g)</th>
<th>E. coli (MPN/g)</th>
<th>Total bacterial count (CFU/g)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean**</td>
<td>Range</td>
<td>Mean**</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>26 Feb. 2017</td>
<td>4</td>
<td>5.6</td>
<td>2.3–24.0</td>
<td>884.6</td>
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<td>2</td>
<td>26 Feb. 2017</td>
<td>4</td>
<td>4.9</td>
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ND  not done,   NA  not applicable,   * Meat cutlets were packaged with 4 pieces per package,   ** Geometric mean,   ***  *E. coli* was detected from one piece of meat cutlet
Fig. 1 Age and gender of confirmed symptomatic cases in an outbreak of EHEC O157:H7 infection in Kanagawa.
Fig. 2 Date of symptom onset of confirmed cases in an outbreak of EHEC O157:H7 infection in Kanagawa.
Fig. 3 Incubation period of 39 confirmed cases in an outbreak of EHEC O157:H7 infection in Kanagawa.
Fig. 4  Pulsed-field gel electrophoresis pattern of EHEC O157:H7 isolates cleaved with restriction enzyme XbaI. Lane 1–3, isolates from patients with EHEC O157:H7 infection; lane 4, an isolate from lot 1 meat cutlet; lane M, DNA size standard Salmonella Braenderup H9812.