

Original Article

An Outbreak of Foodborne Illness Caused by Enteroaggregative *Escherichia coli* in a High School in South Korea

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SUMMARY: In June 2013, a diarrheal outbreak occurred among high school students in Incheon, South Korea. We investigated the outbreak to identify the pathogen and mode of transmission. A case-control study using a self-administered questionnaire was conducted by local authorities and the Korean Centers for Disease Control and Prevention. Bacterial cultures of stool samples, environmental samples, and samples of preserved food items were prepared. PCR, serotyping, and pulsed-field gel electrophoresis (PFGE) were used to identify and characterize the outbreak-related pathogen. We identified 54 cases of gastroenteritis, with symptoms including diarrhea, abdominal pain, and nausea. None of the food items served in the high school cafeteria were significantly associated with illness, although the odds ratio for kippered trotters mixed with vegetables was relatively high (odds ratio: 2.92, 95% confidence interval: 0.62–13.69). Enteraggregative *Escherichia coli* (EAEC) was isolated from this item and the stool samples from 22 symptomatic students and 4 asymptomatic food handlers. The PFGE patterns of EAEC isolated from these sources were indistinguishable. This outbreak was caused by EAEC, and kippered trotters mixed with vegetables, perhaps contaminated by asymptomatic food handlers, were linked to the outbreak. This case-control study highlights the importance of safe food preparation.

INTRODUCTION

Escherichia coli are generally commensal organisms, although six major pathotypes cause diarrhea: enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteraggregative (EAEC), enteroinvasive (EIEC), and diffuse-adherent *E. coli* (1). EAEC strains are the most recently identified and are characterized by heterogeneous clinical symptoms and virulence genes (2). EAEC strains attach to human epithelial cells in a distinctive manner termed the aggregative adherence (AA) or “stacked-brick” pattern (3). However, because adhesion testing is difficult to perform (4), EAEC strains are detected by using a PCR-based method to identify the presence of specific EAEC genes. These genes include those encoding an anti-aggregation protein transporter (pCVD432 or the AA probe) (5,6), an enteraggregative heat stable toxin (*astA*) (7,8), aggregative adherence fimbria I (*aggA*), aggregative adherence fimbria II (*aafA*), the secreted protein dispersin (*aap*), and a transcriptional activator (*aggR*) (9).

EAEC is a major cause of persistent diarrhea among children in developing countries (10), individuals in-

fectured with human immunodeficiency virus (11), and international travelers. Several foodborne outbreaks of EAEC in Japan, the UK, and Italy have been reported (12–14), although the sources of infection were rarely identified.

In June 2013, an outbreak of diarrheal illness occurred at a high school in Incheon, South Korea. An EAEC strain was isolated from students and a food item (kippered trotters mixed with vegetables), served in the school cafeteria. In this report, we describe the epidemiologic investigation that determined the extent of the outbreak and identified the causative pathogen and the possible source of the outbreak.

MATERIALS AND METHODS

Epidemiologic investigation: On June 28, 2013, a school nurse notified local health authorities of an increased incidence of diarrheal illness among the students. An epidemiologic investigation was conducted by the local health authorities and the Korean Centers for Disease Control and Prevention (KCDC) immediately after the outbreak was reported to the KCDC. A case-control study was performed to identify the causative factors of the outbreak. A case was defined as gastroenteritis with diarrhea (≥ 3 times in any 24-h period) that developed after consuming school cafeteria food during the relevant time period (June 24 to June 27, 2013). Asymptomatic students were used as controls and randomly matched in a 2:1 ratio to symptomatic students, according to grade and class. We used self-administered questionnaires to collect demographical and clinical information, as well as histories of food and water con-

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sumption from June 24 to June 27. Food handlers were asked about the food preparation procedures and to report any clinical symptoms that had occurred in the 2 weeks prior to June 24. The cafeteria and kitchen were inspected for the presence of the pathogens.

Laboratory investigation:

Sample collection and culture: Stool samples were collected from students with diarrheal illness and food handlers for standard bacterial and viral assessment. In South Korea, foodservice establishments are legally required to preserve portions of all served food items in freezers for 144 h; consequently, we were able to obtain samples of the items prepared on June 24 to June 27. Environmental samples from the kitchen (e.g., knives, chopping boards, and dishcloths) were also collected. Routine testing of the drinking water and tap water at the school was performed by the Incheon Research Institute of Public Health and Environment (IRIPE). All samples were cultured on individual selective agar plates (e.g., MacConkey agar plates for *E. coli*) to isolate the relevant microorganism(s). Isolates were cultured overnight in Luria Broth at 37°C for molecular analysis.

Detection of pathogenic *E. coli* virulence genes via PCR: We performed multiplex PCR assays using a kit (Kogene, Seoul, Korea) developed by us and containing primers recognizing the most highly conserved *E. coli* genes, *stx1* and *stx2* for EHEC; *lt*, *sth*, and *stp* for ETEC; *eaeA* and *bfpA* for EPEC; *aggR* for EAEC; and *ipaH* for EIEC (patent no. 10-1156719 in Korea; registration date, June 8, 2012). Single colonies of *aggR*-positive bacteria were examined as previously described for the presence of the pCVD432 (15), *aggA* (16), *aafA* (17), *aap* (18), and *astA* (19) genes (Table 1). After incubating bacteria overnight at 37°C, the enriched broth cultures were centrifuged at 13,000 rpm (Sorvall Biofuge Pico; Thermo Scientific, Surrey, UK) for 1 min, and the pellets were heated at 100°C for 10 min. Supernatants were collected via centrifugation and used as PCR templates. PCR reactions contained 2 U DNA Taq polymerase (Ex Taq; Takara Bio, Shiga, Japan) in a total volume of 50 µl and were carried out in a thermal cycler (PTC-100; MJ Research, Watertown, MA, USA) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final cycle at 72°C for

5 min. Amplified PCR products were electrophoretically separated in 2% agarose gels, stained with ethidium bromide, visualized via ultraviolet illumination, and imaged using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA, USA).

Serotyping of *E. coli*: The O antigens of the *E. coli* strain were identified by agglutination with O antisera (O1–O181; Universidad de Santiago de Compostela, Lugo, Spain) (20). H antigens were identified via PCR-restriction fragment length polymorphism analysis of the *fliC* gene which encodes flagellar proteins (21). The presence of an H antigen was confirmed by using Denka *Escherichia coli* Antisera Set2 (Denka Seiken, Tokyo, Japan).

Pulsed-field gel electrophoresis analysis (PFGE): PFGE was performed according to the PulseNet standard protocol (<http://www.pulsenetinternational.org/protocols/Pages/default.aspx>). The restriction enzyme used was *Xba*I, and the PFGE profiles of the outbreak-related strain isolated from different sources were compared.

Statistical analysis: Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to assess the association between illness and exposure to food items and water. Fisher's exact test was also conducted, and a P value (*P*) < 0.05 was considered statistically significant. All statistical analyses were carried out using SAS 9.2 software (SAS Institute, Cary, NC, USA). This investigation was initiated in response to a public health emergency and legally mandated by the government of Korea; hence, institutional review board approval and informed consent were not required.

RESULTS

Epidemiologic investigation: Among the 889 students, 54 experienced symptoms, with an attack rate of 6.1%. Symptoms included diarrhea (100%), abdominal pain (74.1%), and nausea (31.5%) and were generally mild; none of the patients were hospitalized. The median incubation time beginning at lunchtime on June 24 was 45 h (range: 9–97 h). Dates of illness onset ranged from June 24 to June 28 with the peak of the epidemic curve on June 25, 2013 (Fig. 1). All food handlers de-

Table 1. Primers used for amplification assays

Target gene (Reference)		Primer sequence (5' to 3')	Product size (bp)
pCVD432 (15)	F ¹⁾	CTGGCGAAAGACTGTATCAT	630
	R ¹⁾	AATGTATAGAAATCCGCTGTT	
<i>aggR</i> (16)	F	GTATACACAAAAGAAGGAAGC	254
	R	ACAGAATCGTCAGCATCAGC	
<i>aggA</i> (16)	F	TTAGTCTTCTATCTAGGG	457
	R	AAATTAATTCCGGCATGG	
<i>aafA</i> (17)	F	TGCGATTGCTACTTTATTAT	242
	R	ATTGACCGTGATTGGCTTCC	
<i>aap</i> (18)	F	CTTGGGTATCAGCCTGAATG	310
	R	AACCCATTCGGTTAGAGCAC	
<i>astA</i> (19)	F	CCATCAACACAGTATATCCGA	111
	R	GGTCGCGAGTGACGGCTTTGT	

¹⁾: Orientation. F, forward; R, reverse.

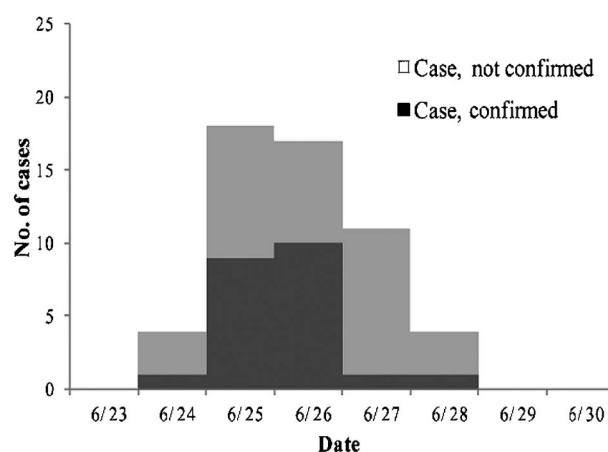


Fig. 1. Epidemic curve of the enteroaggregative *E. coli* (EAEC) outbreak.

Table 2. Association of illness with food items on June 24 (Monday)

Food item	Symptomatic case (n = 54)		Control (n = 107)		Odds ratio (95% CI ⁴⁾)	P-Value ²⁾
	eater/not-eater ¹⁾	(%)	eater/not-eater	(%)		
Lunch						
Beef soup with radish	46/5 ³⁾	90.2	91/13	87.5	1.31 (0.44–3.91)	0.79
Kipperd trotters mixed with vegetables	51/2	96.2	96/11	89.7	2.92 (0.62–13.69)	0.22
Grilled seaweed	49/4	92.5	96/11	89.7	1.40 (0.42–4.64)	0.77
Cabbage kimchi	28/26	51.9	48/49	49.5	1.10 (0.56–2.14)	0.87
Dinner						
Chopped noodle soup with chicken	28/23	49.0	68/39	63.6	0.70 (0.35–1.38)	0.30
Salmon cutlet	22/29	43.1	62/44	58.5	0.54 (0.27–1.06)	0.09
Spicy dried squid	20/29	69.0	54/48	52.9	0.61 (0.31–1.22)	0.17
Fresh radish kimchi	16/36	44.4	40/62	39.2	0.69 (0.34–1.40)	0.38
Drinking water						
Cafeteria	47/7	87.0	89/14	86.4	1.06 (0.40–2.80)	1.00
Purifier (2nd floor)	29/25	53.7	44/60	42.3	1.58 (0.82–3.06)	0.18
Purifier (3rd floor)	12/41	22.6	17/81	17.3	1.39 (0.61–3.19)	0.52
Purifier (4th floor)	7/47	13.0	10/90	10.0	1.34 (0.48–3.75)	0.60
Purifier (5th floor)	2/52	3.7	10/93	9.7	0.36 (0.08–1.69)	0.22

¹⁾: Number of students who have taken each item/number of students who have not taken each item.

²⁾: Fisher's exact test was conducted.

³⁾: Total number of symptomatic case in each item varied, because unknown exposure to individual food item was excluded from the number.

⁴⁾: CI, confidence interval.

nied having had any symptoms during the previous 2 weeks. The school served lunch and dinner during to pupils on weekdays, and there was no major school event after June 1 that might have caused the outbreak.

Among the food items served for lunch on June 24, kippered trotters mixed with vegetables had a high OR (2.92, 95% CI; 0.62–13.69), but this result was not statistically significant. No food item served on June 24 (Monday) or June 25 (Tuesday) was significantly associated with illness, nor was the purified drinking water in the cafeteria and the purifiers in the school building (Table 2). Tap water was only used for cooking in the cafeteria, and the symptomatic students did not report consuming any snacks in addition to the cafeteria food. During the investigation, we observed proper food handling and hygienic practices among all food handlers.

Laboratory investigation:

Pathogen identification from the outbreak samples: The IRIPE tested 26 stool samples from the symptomatic students and 7 stool samples from the food handlers for 10 species of bacteria (*E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Bacillus cereus*) and 5 species of virus (norovirus, rotavirus, adenovirus, astrovirus, and sapovirus). *E. coli* strains were detected on MacConkey agar plates in 22 of 26 (84.6%) stool samples from students and 4 of 7 (57.1%) stool samples from asymptomatic food handlers. Among the preserved food items, only the kippered trotters mixed with vegetables served on June 24 were positive for *E. coli* on MacConkey agar plates. All kitchen environment samples were negative for bacterial pathogens, and all drinking water samples from the cafeteria were negative for general bacteria including *E. coli* in routine tests.

The KCDC received 27 *E. coli* isolates from the IRIPE to test for the presence of virulence genes. PCR detected the same 3 EAEC-related virulence genes

(*aggR*, pCVD432, and *aap*) in all isolates.

Serotyping and PFGE analysis of *E. coli*: Serotyping of O and H antigens revealed that the serotype of all tested EAEC strains was O:H-. The PFGE patterns of all tested isolates were indistinguishable after digestions the DNA with the restriction enzyme *Xba*I. There was no difference in the PFGE patterns of the EAEC O:H- strains isolated from the kippered trotters mixed with vegetables and the stool samples (Fig. 2).

DISCUSSION

The present study describes a foodborne gastroenteritis outbreak caused by EAEC O:H- in South Korea. The main symptoms of the outbreak were diarrhea and abdominal pain, with a median incubation period of 45 h. EAEC was isolated from both stool samples of symptomatic students and a food item sieved to students (kippered trotters mixed with vegetables), and the isolates were indistinguishable in a PFGE analysis. Samples of the other preserved food items served to students were negative for bacterial pathogens including *E. coli*. These epidemiological and microbiological results suggest that the kippered trotters mixed with vegetables were the most likely vehicle of the pathogen. In this school, food is served only on weekdays (June 24 was a Monday), and the epidemic peaked on June 25. Therefore, we assumed that the food or water that caused this outbreak was consumed on June 24, which is the day the kippered trotters mixed with vegetables, which were positive for EAEC, were served.

Kipperd trotters mixed with vegetables are prepared by mixing pre-cooked pig's trotters with seasoned vegetables without heating. In contrast, all other food items served on June 24 were heated. Interestingly, when cooked at high temperatures, the number of *E. coli* in food is significantly reduced (22,23). The ingredients of the kippered trotters mixed with vegetables were

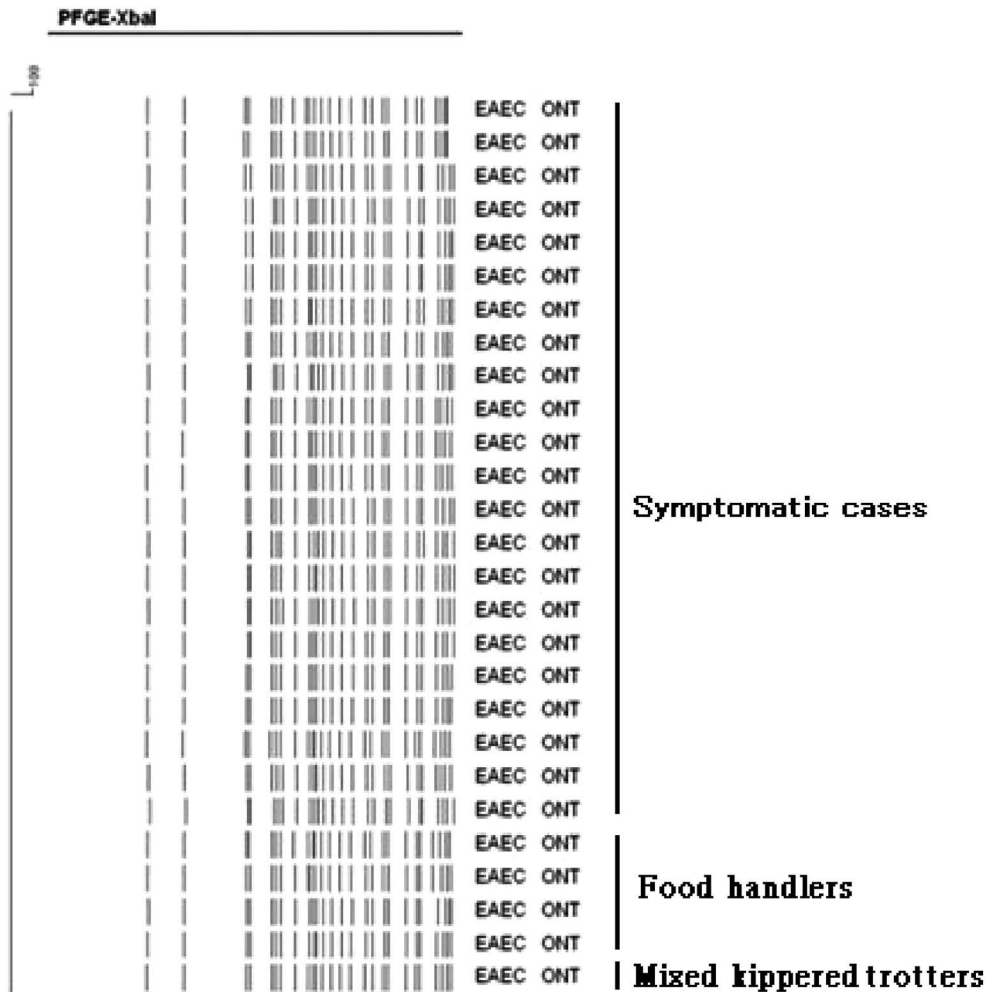


Fig. 2. Pulsed-field gel electrophoresis patterns of the enteroaggregative *E. coli* (EAEC) strains isolated from human stool samples and kippered trotters mixed with vegetables.

smoked pigs' trotters, soy sauce, vinegar, and vegetables, including cabbage, fresh-cut leafy greens, and onions. The smoked pigs' trotters were supplied by "company A" as a finished product and were used after unpacking and heating. According to the school's Critical Control Point record, the trotters were heated in boiling water, and the core temperature was found to be 96.2°C. Thus, it is unlikely that the contaminated pigs' trotters per se contributed to this outbreak, as they were fully heated after unpacking. After heating the prepared trotters, the food handlers mixed them with vegetables supplied by "company B". No other food items served during the outbreak period involved mixing processes. Although vegetables are common food vehicles for diarrheal illness outbreaks caused by *E. coli* (24,25), no such outbreaks were reported by other schools using foods supplied by "company B". The possibility of waterborne contamination is also low, as the tap water samples were negative for *E. coli*.

Even though all food handlers were reportedly asymptomatic, 4 of the 7 (57.1%) food handlers tested positive for EAEC, which is much higher than the attack rate in the students (6.1%). The food handlers who were positive for EAEC were confirmed to have participated in the mixing of the kippered trotters and mixed vegetables. Therefore, they may have been the source of

the contamination that participated the outbreak.

Several outbreaks of gastroenteritis due to EAEC have been previously reported. These include 3 foodborne outbreaks in Japan. The first was a massive outbreak caused by the EAEC O126:H10 strain in 1993, in which more than 2,500 students from 16 schools developed gastrointestinal illness after eating contaminated school lunches (12). The second and third outbreaks were associated with EAEC O126 and O111, and involved junior high school students and adults who attended a party, respectively (26). Four outbreaks of EAEC occurred in the UK, at restaurants, Christmas dinners, and a conference (13), but the sources of the infections were for the most part not identified. In Italy, two successive foodborne outbreaks associated with EAEC O92:H33 occurred, with cheese made using unpasteurized sheep milk as the possible source (14). The presence of EAEC in food handlers has been noted, but whether asymptomatic carriers transmit the infections is unclear (13,14,27).

The attack rate in this outbreak and the prevalence of asymptomatic food handlers, was 6.1% and 57.1%, respectively. Compared with the previously reported EAEC attack rates of 42% and 58% in Italy (14), 40% in Japan (12), and 47% and 91% in the UK (13), our attack rate is very low. It is possible that the number of

EAEC cases was under-reported in our study: stool samples were only collected from symptomatic students owing to limited human resources, and some students might not have reported their symptoms to avoid stool sampling.

In the past, the *astA* gene was considered characteristic of EAEC strains (7), despite its detection, in only a subgroup of EAEC strains, and its extensive distribution among other *E. coli* strains (28,29). In our study, none of the EAEC isolates harbored the *astA* gene. They did, however, contain both the *aggR* and *aap* genes. Because these genes reside within the same genetic locus (30), they are often found together in EAEC isolates (11,31). The AggR protein is a transcriptional activator of the gene encoding AraC/XylS, an aggregative adherence regulator, and the *aap* gene is under the control of AggR. The *aap* gene encodes an anti-aggregation protein (dispersin), which acts to distribute bacteria on the epithelial surface to establish new foci of infection (30). Benitez et al. suggested that in *Vibrio cholera*, mutation of 'hap mucinase', which has a similar function as dispersin, increases the density of bacterial colonization and attenuates its virulence (32).

In Italy, an outbreak of *aggR*- and *aap*-positive EAEC occurred in 2008 (14). Moreover, the EHEC O104:H4 strains that caused bloody diarrhea and hemolytic-uremic syndrome (HUS) in Germany, in May and June 2011, were also *aggR*- and *aap*-positive (33–35). The German outbreak was involved 2 types of pathogenic *E. coli*, EAEC and EHEC (36,37), and transfer of the HUS-associated gene that encodes the Shiga toxin from EHEC to EAEC has been described. The acquisition of virulence determinants through successive horizontal gene transfers is a major factor in the evolution and diversification of pathogenic bacteria and is more common than modification of existing DNA (38). However, a specific genomic background may be required for the integration, retention, and expression of foreign DNA (38,39), and the evolution of pathogenic bacteria often exhibits a strong lineage dependency. Therefore, ongoing surveillance and characterization of EAEC strains are needed.

Our study has a few limitations. First, the kippered trotters mixed with vegetables were not significantly associated with illness, despite isolation of EAEC from this food item. Because the attack rate was low, the pathogenicity of the EAEC strain was also presumably low, which might have affected the statistical analysis, as control students who had eaten the kippered trotters mixed with vegetables might have only exhibited minor symptoms. Second, we were only able to conduct a case-control study, owing to limited human resources. Thus, the number of cases might have been under-estimated, as students with minor symptoms might not have reported them to the public health authorities.

Our study confirms that the outbreak of diarrheal illness in a South Korean high school was due to EAEC O111:H-. Kippered trotters mixed with vegetables were identified as the probable source of infection via microbiology testing, the EAEC strains in this food item and the stool samples of symptomatic students were indistinguishable in a PFGE analysis. However, the results of statistical analysis did not agree with those of the microbiology tests. In this outbreak, it is possible that asymp-

tomatic food handlers transmitted the pathogen during food preparation. Improved surveillance and active investigation of EAEC infection in asymptomatic food handlers is needed in schools.

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Conflict of interest None to declare.

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