Epidemiological Reports

Prevalence of Diarrheagenic *Escherichia coli* in Foods and Fecal Specimens Obtained from Cattle, Pigs, Chickens, Asymptomatic Carriers, and Patients in Osaka and Hyogo, Japan

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SUMMARY: The source and routes of diarrheagenic *Escherichia coli* (DEC) remain poorly understood. To investigate the involvement of domestic animals in the dissemination of DEC, the prevalence of DEC in foods and fecal specimens from cattle, pigs, chickens, healthy carriers, and patients in Osaka and Hyogo, Japan was investigated using a multiplex real-time Polymerase Chain Reaction assay. The most abundant virulence genes were astA and eae, which had a prevalence of 46.8% and 27.4%, respectively. Additionally, stx1 (26.6%) and stx2 (45.9%) were prevalent in cattle feces, while est (8.5%) and elt (7.6%) were prevalent in pig feces. *afaB* was the second-most prevalent gene in patients and healthy carriers, and it had detection rates of 5.1% and 8.1%, respectively. In contrast, *afaB* was not detected in animal feces or foods, except for three porcine fecal samples. The *aggR* gene was more prevalent in humans than in foods or animal feces. Both Shiga toxin-producing *E. coli* and atypical enteropathogenic *E. coli* carried by cattle may be sources for diarrheal diseases in humans. Pigs may be a source for human enteroaggregative *E. coli* infections, whereas humans are expected to be the reservoir for diffusely adhering *E. coli*, enteroaggregative *E. coli*, and enteroinvasive *E. coli*.

INTRODUCTION

*Escherichia coli* is a normal inhabitant of the intestinal tract of humans and warm-blooded animals. However, certain strains cause enteric diseases in their hosts, and these are referred to as diarrheagenic *E. coli* (DEC). DEC strains have been classified into several pathotypes, including enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC). These classifications are based on epidemiological and clinical features, as well as specific virulence determinants and other characteristics that include enterotoxin production and adherence phenotypes (1).

As information on DEC accumulates, shifts in epidemiology have occurred. EPEC, which expresses *eae*, can be classified into typical EPEC (tEPEC) and atypical EPEC (aEPEC). This classification depends on the presence or absence of the bundle-forming pilus A (*bfpA*) gene (2). Furthermore, tEPEC, which is also called class I EPEC (3), is a well-recognized pathogen in developing countries (4). The reservoir for tEPEC is generally considered to be humans (5-7). In contrast, aEPEC organisms are reportedly more prevalent in both developing and developed countries, and animals are reported to be the major reservoirs of aEPEC (8).

STEC is a leading cause of food-borne infections in the U.S., representing a major public health concern (9). These pathogens are characterized primarily by their ability to produce two types of Shiga toxins (Stx), Stx1 and Stx2, which can cause severe bloody diarrhea and a life-threatening condition known as hemolytic uremic syndrome (10). Cattle have been identified as a major reservoir for STEC, and the pathogen can be transmitted to humans via the consumption of contaminated meat. However, fruits, vegetables, or contact with a contaminated environment (e.g., recreational water) have been shown to be important vehicles for infection (11).

ETEC pathogenicity is determined primarily by the production of heat-stable (ST) and labile-stable (LT) enterotoxins (12). Outbreaks due to the ETEC serogroup O169 have been increasing, and ETEC may be an emerging cause of food-borne diseases in Asia, Europe, and the U.S.A. (13-17).

EAEC, which harbors the transcriptional activator-encoding *aggR* gene and its regulon, is a major cause of acute and persistent diarrhea in the small intestine of children and adults worldwide. This includes industrialized countries (18). STEC O104:H4, an
human patients after enrichment culture. EIEC, which carries an invasion plasmid harboring the regulatory gene virB, causes dysentery-like symptoms (12). However, the number of patients that contract EIEC is small. Thus, EIEC is no longer found in developed countries.

DAEC comprises a heterogeneous group of organisms with variable virulence (20). We have suggested that the subgroup of DAEC that possesses the afimbrial adhesin B (afaB) gene and/or induces high levels of IL-8 secretion in epithelial cells may play a role in causing sporadic diarrheal illnesses, particularly in pediatric patients (21-23).

The astA gene, which encodes EAEc heat-stable enterotoxin 1 (EAST1), was initially detected in EAEc (24). It has subsequently been detected in other DEC pathotypes, including EPEC, ETEC, and STEC (25-27). Although the role of EAST1 in human disease is unknown (28), we have designated E. coli that possess astA but no other identifiable pathogenic properties as EAST1EC. This constitutes the 7th DEC pathotype examined in this study.

Classically, humans have been presumed to be the reservoir of human DEC. However, cattle previously have been recognized as carriers of STEC. Therefore, the reservoirs and pathways for all pathotypes of DEC need to be reinvestigated using sensitive methods. Otherwise, target-oriented preventions will never be available. Previously, we developed a multiplex real-time Polymerase Chain Reaction (PCR) protocol that facilitates highly sensitive detection of DEC (29). To determine the possible sources and routes of transmission for DEC, we used this method to detect DEC in a variety of foods and fecal specimens from pigs, cattle, chickens, healthy human carriers, and human patients after enrichment culture.

MATERIALS AND METHODS

Specimens: A total of 333 food samples (136 fish, 66 fruit and vegetables, 51 ready-to-eat foods, 32 beef, 28 pork, and 20 poultry samples) were obtained at local retail markets or at the Osaka Municipal Central Wholesale Market from 2005 to 2008. Samples were transported in cooler bags and examined immediately after arrival at the laboratory. Fecal samples from 109 cattle were collected at the Osaka Municipal Meat Inspection Center (MMIC) from June 2006 to September 2007. Additionally, 358 chicken fecal samples were collected at the Hyogo MMIC in 2013, and 698 pig fecal samples were obtained at either the Osaka or Hyogo MMICs from 2006 to 2013. Finally, 482 human fecal samples were collected from healthy adults and cultured at the Osaka City Institute of Public Health and Environmental Sciences from 2009 to 2014, and 670 fecal samples from patients experiencing diarrhea (age 0 to 99 years; average 53.8 years; median 65 years) were collected at the Department of Clinical Laboratory of the Osaka Minami Medical Center from 2012 to 2014. Campylobacter spp., Salmonella spp., Norovirus, and Rotavirus were found in 22, 2, 13, and 4 patients’ stools, respectively. Two astA-positive samples were discarded because these specimens tested positive for Campylobacter or Norovirus. In total, 2,650 samples were examined for DEC.

Enrichment culture of food and fecal specimens: Individual food samples (10 g each) from the local retail markets were homogenized in 90 ml of brain heart infusion broth (BHI; Nissui Pharmaceutical Company, Tokyo, Japan) using a Masticator (IUL Instrument, Barcelona, Spain). Each homogenate was decanted into a 200-ml Erlenmeyer flask through a paper strainer attached to a Stomacher bag and then incubated for 3 h at 37°C to resuscitate damaged cells. Each cultured homogenate was subsequently transferred to a 500-ml flask and mixed with an equal amount of double-strength tryptone phosphate broth prepared according to the U.S. Food and Drug Administration BAM manual. Several portions of food samples from the Osaka Municipal Central Wholesale Market were enriched with Brilliant Green Lactose Bile (Nissui). Each mixture was incubated for 20 h at 44°C in a water bath, and 0.5 ml of each sample was obtained for PCR screening. Fecal samples were cultured in BHI for 20 h at 42°C to enrich for bacteria. After the enrichment culture, the samples were stored in a freezer until evaluation.

DNA isolation from bacterial cells: Bacterial DNA was obtained from the enrichment broth cultures. Specifically, 0.5-ml aliquots of the enrichment broth samples (prepared as described above) were centrifuged at 15,000 × g for 5 min. Pelleted bacteria were subjected to DNA extraction using a Genomic DNA Purification Kit (Genta Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. The purified DNA was used as a template for real-time PCR.

Multiplex real-time PCR for detection of DEC: Duplex or triplex PCR reactions were prepared as previously reported (29). Briefly, genes encoding intimin (eae), Stx (stx1 and stx2), LT (elt), ST types h and p (est-h and est-p, respectively), EAEC transcription factor (aggR), EAEc EAST1 (asta), EIEC transcription factor (virB), and DAEC adhesin (afaB) were targeted for amplification. Triplex PCR was performed to simultaneously detect eae in EPEC, in addition to stx1 and stx2 in STEC. The other triplex reaction was for ETEC (elt, est-h, est-p). Two duplex PCRs were performed for simultaneous detection of EAEC (aggR) and EAST1EC (asta), in addition to EIEC (virB) and DAEC (afaB). When stx-positive samples were detected by triplex PCR, subsequent duplex PCR was performed to distinguish stx1 and stx2. The genes est-h and est-p also were distinguished by duplex PCR. All PCR reactions were performed using the Quantitect Multiplex PCR solution (Qiagen, Hilden, Germany) with the ABI PRISM® 7000 Sequence Detection System or StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR cycling conditions were as follows: one cycle of denaturation at 95°C for 15 min followed by 40 cycles of 95°C for one min and 60°C for one min.

Bacterial strains: The following bacterial strains were obtained: EPEC strain E2348/69; ETEC strains E5798 and 97-245-244; STEC strains 99-140-A, V-831, EC7225, and E8212; EIEC strain E35990; EAegEC strain E59152; and EAST1EC strain 96-127-23. DAEC strain V-64 was used as a positive control.
**Statistics:** Statistical differences in the prevalence of diarrheagenic *E. coli* detected from the different sources were determined by performing a chi-squared test with Yates’ continuity correction or Fisher’s exact probability test.

**RESULTS**

**Detection of the intimin gene eae in EPEC and STEC:** The EPEC/STEC-associated gene *eae* was detected in 726 food and fecal samples (Table 1). This virulence gene was more prevalent in cattle feces (75.2%; *P* < 0.05) than in other samples, followed by poultry feces and pig feces. No significant differences were observed between the healthy human carrier feces and human patient feces.

**Detection of stx genes:** The STEC-associated genes *stx1* and *stx2* were more prevalent in cattle feces (26.6% and 45.9%, respectively) than in other sources examined (*P* < 0.05) (Table 1). Pig feces showed a higher detection rate for *stx1* (*P* < 0.05) compared to food, chicken, healthy carriers, and patient samples. Compared to the high detection rate for *stx2* in cattle feces (50/109 positive, 45.9%), few positive samples were found among the other 2,541 samples examined (*n* = 10, 0.4%).

**Detection of enterotoxin genes from ETEC:** The detection rates for the enterotoxin genes *elt* and *est* differed significantly, and depended on the type of samples assayed (Table 1). Pig feces had higher detection rates for *elt* and *est* compared to all other samples (*P* < 0.05). Chicken feces (*P* < 0.05) also showed a higher detection rate for *elt* than samples from food, healthy human carriers, and human patients.

**Detection of EAEC and DAEC virulence genes:** The *aggR* and *afaB* genes were most predominant in human fecal samples (Table 1). Among the 20 samples testing positive for *aggR*, only 2 were from non-human fecal specimens, specifically, food and bovine feces. The detection rates for *aggR* were significantly higher in fecal samples from healthy carriers (*P* < 0.05) and patients (*P* < 0.05) than in pig and chicken feces. The detection rate of *afaB* was also significantly higher in feces from both healthy human carriers (*P* < 0.05) and patients (*P* < 0.05). Additionally, no food, cattle fecal samples, or chicken fecal samples tested positive for *afaB*, while only three pig fecal samples were *afaB*-positive.

**Detection of EAST1 gene:** In this study, the most prevalent virulence gene in each sample group was *astA*, which was detected in a total of 1,241 samples (46.8%) (Table 1). Chicken feces exhibited the highest detection rate of 98.0%, followed by 94.5% and 79.8% for cattle and pig feces, respectively. The *astA* gene was more prevalent in cattle, pig, and chicken feces (*P* < 0.05) compared to food and fecal samples from healthy carriers and patients. Patient samples showed the lowest detection rate of 9.9%, which was significantly lower (*P* < 0.05) than other sample groups.

**DISCUSSION**

Frequent outbreaks caused by STEC O157 have resulted in bacteriologists performing more ecological studies. These investigations revealed that STEC is...
harbored in the digestive tracts of domestic animals, particularly ruminants (30). Unfortunately, the natural reservoirs for other strains of DEC and the pathways required for them to infect humans remain unclear. However, humans are believed to be a reservoir for other types of DEC. In the present study, foods and fecal specimens were screened using a multiplex real-time PCR protocol. Our experiments revealed that enterovirulence genes, especially astA and eae, were more common in cattle, pigs, and chickens than has been previously reported in studies using culture methods (31). Domestic animals are thought to be natural carriers of EAST1EC and EPEC.

In 1996, EAST1EC O166:H15 was detected as the sole etiological agent causing outbreaks in Japan among adult patients (28). In addition, EAST1EC was often detected in children suffering from diarrhea (32). However, in the present study, the detection rate (20.5%) in healthy carriers was significantly higher (P < 0.05) than that in patients (9.9%). The prevalence of astA among humans may simply reflect the higher prevalence of EAST1EC in domestic animals and their feces. The astA gene may have some role in the survival of E. coli in these herbivorous or omnivorous animals, which have a more developed large intestine than humans. Thus, fewer E. coli in carnivores may harbor the astA gene.

EPEC is the main cause of infantile diarrhea in developing countries (4), and it may be a cause of diarrhea in industrialized countries (33). Humans are considered to be the sole reservoir of EIEC (7). However, in the present study, the detection rates of eae were lower in both healthy carriers (5.4%) and patients (3.9%) compared to cattle and chickens, which is in agreement with findings from previous studies (12). The eae gene detected in the current study should primarily derive from aEPEC, as reported in our previous report (27). A similar result was described by Cabal et al. (12), who reported that eae was highly prevalent (>90%) in bfpA-negative fecal samples, indicating the presence of aEPEC.

Cattle are considered to be a major source of diarrheagenic aEPEC, as described in our molecular epidemiological studies (24, 31). Specifically, patient strains were closer to bovine strains, while aEPEC from healthy carriers could be discriminated from the other aEPEC, including patient strains. Although a high detection rate (49.1%) for eae in pig feces was observed in those studies, pig-specific EPEC exhibited properties distinct from those of human diarrheagenic strains (27, 34). The high detection rate (62.6%) of eae in chicken feces corroborated the results from other studies that similarly reported a high prevalence of EPEC in avian species, such that all EPEC strains isolated from chickens, ducks, and pigeons were identified to be aEPEC (35). Alonso et al. (35) reported that aEPEC strains from chickens exhibited a wide variety of serotypes, some of which have been isolated in other animal species (O2:H40, O5:H40) and in children with diarrhea (O8:H-). Most of the strains encode intimin β, which was the most common intimin subtype of aEPEC strains identified in patients in our previous study (27). These results indicate that cattle, chickens, and cattle- and chicken-derived food products may be important sources of the aEPEC strains that cause human disease (27, 35).

STEC virulence genes were detected at significantly high rates in cattle feces (P < 0.05), and they were found in beef samples, as well. These findings suggest that cattle may be a source of STEC worldwide, as previously reported (36). In addition, pig fecal samples showed significantly higher detection rates for stx genes compared to other groups. However, in the present study, stx1 and stx2 were separately detected in only 18 and 4 of the 698 total pig samples, respectively. These findings are insufficient to support the hypothesis that pigs serve as a reservoir for human STEC, as cattle do. These detection rates (0.6–2.6%) were lower than the 14% detection rate in pigs in a national surveillance study conducted from 1999 to 2001 in Japan (37). STEC possessing stx2e, a variant of stx2, is a well-known swine pathogen causing edema. In the national surveillance study, stx2e (which our PCR could not detect) was dominant, and none of the STEC harbored eae (37). Presumably, STEC with low pathogenicity for humans was also included in that study. However, stx2e-expressing STEC are known to infect humans, albeit rarely.

Enterotoxin genes of ETEC were detected most frequently (P < 0.05) in pig feces compared to the other samples. Some ETEC can cause diarrheal diseases in newborn and post-weaning pigs. The variants that possessed est and/or elt that were detected by our multiplex PCR assay might be porcine pathogens. However, Ban et al. recently found that the ETEC O169:H41 strain possesses genes encoding proteins homologous to the colonization factor K88 found in pig ETEC (38). Since ETEC O169:H41 is an emerging DEC in both Japan and the United States (13, 39), ETEC originating from pigs may be transmitted to humans via meat and foods, just as STEC carried by cattle is transmitted via beef products and water polluted by feces.

Among the 7 pathotypes of DEC, EAEC (detected with a PCR for aggR) is the 4th most prevalent in both healthy humans and patients (33, 40). Although EAEC is relatively rare among humans, this pathotype is still significantly more prevalent in human fecal samples compared to those of animals. Our results are consistent with the findings of Akiyama et al. (31), who reported that aggR was not detected in any of the cattle-derived DEC examined. The reservoir for EAEC is likely humans. The afaB gene in DAEC was also frequently isolated from human fecal specimens regardless of enteric symptoms (33, 40). The present study indicates that afaB from DAEC is not commonly present in foods (0%) or animals (0–0.8%). Therefore, the natural reservoir for DAEC also must be humans, as domestic animals reportedly do not harbor DAEC (41). Although DAEC was expected to preferentially colonize young children instead of adults (20), no significant correlation was found between DAEC infection and the age of afaB-positive patients. Tanih et al. (42) reported that EIEC was not detected in cattle or pigs in South Africa. No virB from EIEC has been detected in our studies, suggesting that the risk of EIEC outbreaks should be negligible in Japan, as we have reported before (33, 40).

In conclusion, domestic animals harbor not only well-known STEC, but also aEPEC, EAST1EC,
and ETEC (Fig. 1). However, EAST1EC is unlikely to be an etiological agent. Our previous molecular epidemiological study revealed that both STEC and aEPEC carried by cattle may cause diarrheal diseases in humans (27, 34). Porcine aEPEC does not seem to be a causative agent of human disease. Additionally, the aEPEC found among healthy human carriers appears be indigenous, given that the organisms were distinct from patient strains. It remains to be elucidated if poultry aEPEC can infect humans. Pigs may be the source of human ETEC infections.

Acknowledgments We are grateful to the staff of the Osaka City Institute of Public Health and Environmental Science, and the Municipal Meat Inspection Centers of Osaka and Hyogo for their technical assistance. This study was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None to declare.

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Reservoirs of Diarrheagenic *Escherichia coli*