Jpn. J. Infect. Dis., 70, 464–469, 2017

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

Lili Wang[1,2], Shaobo Zhang[2], Dongming Zheng[2], Sami Fujihara[3], Akiyo Wakabayashi[4], Kazuyuki Okahata[4], Masakazu Suzuki[4], Atsunori Saeki[5], Hiromi Nakamura[6], Yukiko Hara-Kudo[7], Eriko Kage-Nakadai[2,8], and Yoshikazu Nishikawa[2]*

[1]School of Life Science and Biotechnology, Dalian University of Technology, Dalian, China; [2]Graduate School of Human Life Science, Osaka City University, Osaka; [3]National Hospital Organization Osaka Minami Medical Center, Osaka 586-8521; [4]Hyogo Prefecture Tajima Meat Hygiene Inspection Office, Hyogo 667-0112; [5]Osaka Municipal Meat Inspection Center, Osaka 559-0032; [6]Osaka City Institute of Public Health and Environmental Sciences, Osaka 543-0026; [7]National Institute of Health Sciences, Tokyo 158-8501;and [8]The OCU Advanced Research Institute for Natural Science and Technology, Osaka City University, Osaka 558-8585, Japan

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 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 enterotoxigenic *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

Received November 4, 2016. Accepted February 20, 2017.
J-STAGE Advance Publication March 28,2017.
DOI: 10.7883/yoken.JJID.2016.486
*Corresponding author: Mailing address: Graduate School of Human Life Science, Osaka City University,Osaka 558-8585, Japan. Tel: +81-6-6605-2883, Fax: +81-6-6605-2883, E-mail: nisikawa@life.osaka-cu.ac.jp

EAEC that acquired a Stx2-encoding gene, emerged in Germany during an outbreak in which 3,816 people were infected and 54 were killed (19).

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 (Gentra 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 EC8212; EIEC strain E35990; EAggEC 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 carrier, 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 est and elt 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

Table 1. Detection of diarrheagenic *E. coli* by multiplex real-time PCR in foods and fecal specimens of cattle, pigs, chickens, healthy asymptomatic individuals (carriers), and patients with diarrheagenic *E. coli*

	Year	No.	EPEC	STEC		ETEC		EAEC	DAEC	EAST1EC
			eae	*stx1*	*stx2*	*elt*	*est*	*aggR*	*afaB*	*astA*
Foods	2005-2008	333[1)]	25(7.5)[d,2),3)]	2 (0.6)[c]	2 (0.6)[b]	2 (0.6)[c]	1 (0.3)[b]	1 (0.3)[a,b]	0 (0)[b]	65 (19.5)[c]
Fish		136	5 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0 (0)	7 (5.1)
Fruits & Vegetables		66	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	8 (12.1)
Ready-to-eat foods		51	2 (3.9)	0 (0)	0 (0)	1 (2.0)	0 (0)	0 (0)	0 (0)	4 (7.8)
Beef		32	2 (6.3)	1 (3.1)	2 (6.3)	0 (0)	0 (0)	0 (0)	0 (0)	10 (31.3)
Pork		28	6 (21.4)	1 (3.6)	0 (0)	0 (0)	1 (3.6)	0 (0)	0 (0)	16 (57.1)
Poultry		20	10 (50.0)	0 (0)	0 (0)	1 (5.0)	0 (0)	0 (0)	0 (0)	20 (100.0)
Cattle feces	2006-2007	109	82 (75.2)[a]	29 (26.6)[a]	50 (45.9)[a]	0 (0)[d]	0 (0)[b]	1 (0.9)[a,b]	0 (0)[b]	103 (94.5)[a]
Pigs feces	2006-2007	118	69 (58.5)	12 (10.2)	0 (0)	4 (3.4)	8 (6.8)	0 (0)	1 (0.8)	94 (79.7)
	2013	580	274 (47.2)	6 (1.0)	4 (0.7)	49 (8.4)	51 (8.8)	0 (0)	2 (0.3)	463 (79.8)
	Subtotal	698	343 (49.1)[c]	18 (2.6)[b]	4 (0.6)[b]	53 (7.6)[a]	59 (8.5)[a]	0 (0)[b]	3 (0.4)[b]	557 (79.8)[b]
Chicken feces	2013	358	224 (62.6)[b]	1 (0.3)[c]	1 (0.3)[b]	10 (2.8)[b]	0 (0)[b]	0 (0) [b]	0 (0)[b]	351 (98.0)[a]
Animal feces		1,165	649 (55.7)	48 (4.1)	55 (4.7)	63 (5.4)	59 (5.1)	1 (0.1)	3 (0.3)	1,011 (86.8)
Healthy carriers	2009	119	8 (6.7)	1 (0.8)	1 (0.8)	0 (0)	0 (0)	2 (1.7)	22 (18.5)	30 (25.2)
	2012-2013	313	15 (4.8)	0 (0)	0 (0)	0 (0)	0 (0)	4 (1.3)	12 (3.8)	49 (15.7)
	2014	50	3 (6.0)	0 (0)	0 (0)	1 (2.0)	1 (2.0)	0	5 (10.0)	20 (40.0)
	Subtotal	482	26 (5.4)[d,e]	1 (0.2)[c]	1 (0.2)[b]	1 (0.2)[c]	1 (0.2)[b]	6 (1.2)[a]	39 (8.1)[a]	99 (20.5)[c]
Patients	2012-2013	595	24 (4.0)	2 (0.3)	2 (0.3)	4 (0.7)	2 (0.3)	11 (1.8)	31 (5.2)	46 (7.7)
	2014	75	2 (2.7)	0 (0)	0 (0)	0 (0)	1 (1.3)	1 (1.3)	3 (4.0)	18 (24.0)
	Subtotal	670	26 (3.9)[e]	2 (0.3)[c]	2 (0.3)[b]	4 (0.6)[c]	3 (0.4)[b]	12 (1.8)[a]	34 (5.1)[a]	64 (9.6)[d]
Total		2,650	726 (27.4)[B,4)]	53 (2.0)[C]	60 (2.3)[C]	70 (2.6)[C]	64 (2.4)[C]	20 (0.8)[D]	76 (2.9)[C]	1,239 (46.8)[A]

[1)]: Total no. of samples.
[2)]: No. in brackets indicate the positive no. of diarrheagenic *E. coli* from each sample group.
[3)]: Values with different lowercase letters (a-e) in the same column are significantly different ($P < 0.05$).
[4)]: Values with different capital letters (A-D) in the total row are significantly different ($P < 0.05$).

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 tEPEC (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 corroborates 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,

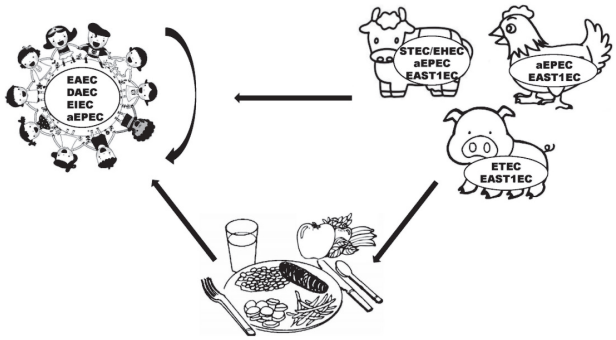

Fig. 1. Hypothesis of diarrheagenic *E. coli* reservoirs. The reservoirs of DAEC, EAEC, and EIEC should be humans. Domestic animals harbor not only well-known STEC, but also aEPEC, EAST1EC, and ETEC, although the etiological significance of EAST1EC is still controversial. aEPEC carried by cattle is expected to be a causative agent of human diarrheal diseases. Porcine aEPEC appears to circulate among pigs. Similarly, aEPEC detected in healthy carriers is expected to be indigenous, since the organisms are distinct from patient strains. It remains to be elucidated if poultry aEPEC can infect humans. Pigs may be the source of human ETEC infections.

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. Although aEPEC was detected in poultry samples, it remains unclear whether these microbes can infect humans. Pigs are expected to be the source of human ETEC infections, whereas the reservoir for DAEC and EAEC, like that of EIEC, should be humans (5).

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 declaire.

REFERENCES

1. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123-40.
2. Trabulsi LR, Keller R, Tardelli Gomes TA. Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis. 2002;8:508-13.
3. Nataro JP, Baldini MM, Kaper JB, et al. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J Infect Dis. 1985;152:560-5.
4. Campos LC, Franzolin MR, Trabulsi LR. Diarrheagenic *Escherichia coli* categories among the traditional enteropathogenic *E. coli* O serogroups--a review. Mem Inst Oswaldo Cruz. 2004;99:545-52.
5. Murase M. Enteroinvasive *Escherichia coli* (EIEC). In: Sakazaki R, editor. Food- and Water-borne Infection and Food Poisoning. Tokyo: Chuohouki; 2000. p. 222.Japanese.
6. Kai A. Enterotoxigenic *Escherichia coli* (ETEC). In: Sakazaki R, editor. Food- and Water-borne Infection and Food Poisoning. Tokyo: Chuohouki; 2000. p. 231.Japanese.
7. Tamura K, Sakazaki R. Enteropathogenic *Escherichia coli* (EPEC). In: Sakazaki R, editor. Food- and Water-borne Infection and Food Poisoning. Tokyo: Chuohouki; 2000. p. 245.Japanese.
8. Ochoa TJ, Barletta F, Contreras C, et al. New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Trans R Soc Trop Med. Hyg. 2008;102:852-6.
9. Singh P, Sha Q, Lacher DW, et al. Characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. Front Cell Infect Microbiol. 2015;5:29.
10. Riley LW, Remis RS, Helgerson SD, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med. 1983;308:681-5.
11. Chekabab SM, Paquin-Veillette J, Dozois CM, et al. The ecological habitat and transmission of *Escherichia coli* O157:H7. FEMS Microbiol Lett. 2013;341:1-12.
12. Cabal A, García-Castillo M, Cantón R, et al. Prevalence of *Escherichia coli* Virulence Genes in Patients with Diarrhea and a Subpopulation of Healthy Volunteers in Madrid, Spain. Front Microbiol. 2016;7:641.
13. Nishikawa Y, Helander A, Ogasawara J, et al. Epidemiology and properties of heat-stable enterotoxin-producing *Escherichia coli* serotype O169:H41. Epidemiol Infect. 1998;121:31-42.
14. Beatty ME, Bopp CA, Wells JG, et al. Enterotoxin-producing *Escherichia coli* O169:H41, United States. Emerg Infect Dis. 2004;10:518-21.
15. Cho SH, Kim J, Oh KH, et al. Outbreak of enterotoxigenic *Escherichia coli* O169 enteritis in schoolchildren associated with consumption of kimchi, Republic of Korea, 2012. Epidemiol Infect. 2014;142:616-23.
16. Harada T, Itoh K, Yamaguchi Y, et al. A foodborne outbreak of gastrointestinal illness caused by enterotoxigenic *Escherichia coli* serotype O169:H41 in Osaka, Japan. Jpn J Infect Dis. 2013;66:530-3.
17. MacDonald E, Møller KE, Wester AL, et al. An outbreak of enterotoxigenic *Escherichia coli* (ETEC) infection in Norway, 2012: a reminder to consider uncommon pathogens in outbreaks involving imported products. Epidemiol Infect. 2015;143:486-93.
18. Flores J, Okhuysen PC. Enteroaggregative *Escherichia coli* infection. Curr Opin Gastroenterol. 2009;25:8-11.
19. Frank C, Werber D, Cramer JP, et al. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. N Engl J Med. 2011;365:1771-80.
20. Servin AL. Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli*. Clin Microbiol Rev. 2005;18:264-92.
21. Arikawa K, Meraz IM, Nishikawa Y, et al. Interleukin-8 secretion by epithelial cells infected with diffusely adherent *Escherichia coli* possessing Afa adhesin-coding genes. Microbiol Immunol. 2005;49:493-503.
22. Meraz IM, Arikawa K, Ogasawara J, et al. Epithelial cells secrete interleukin-8 in response to adhesion and invasion of diffusely adhering *Escherichia coli* lacking Afa/Dr genes. Microbiol Immunol. 2006;50:159-69.
23. Meraz IM, Arikawa K, Nakamura H, et al. Association of IL-8-inducing strains of diffusely adherent *Escherichia coli* with sporadic diarrheal patients with less than 5 years of age. Braz J Infect Dis. 2007;11:44-9.
24. Savarino SJ, Fasano A, Robertson DC, et al. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J Clin Invest. 1991;87:1450-5.
25. Kameyama M, Yabata J, Nomura Y, et al. Biochemical features and virulence gene profiles of non-O157/O26 Enterohemorrhagic *Escherichia coli* strains from humans in the Yamaguchi Prefecture, Japan. Jpn J Infect Dis. 2015;68:216-20.
26. Sirikaew S, Patungkaro W, Rattanachuay P, et al. Enterotoxigenic *Escherichia coli* O169:HUT from a diarrheal patient: Phylogenetic group and antimicrobial susceptibility. Southeast Asian J Trop Med Public Health. 2014;45:1376-84.
27. Wang L, Wakushima M, Aota T, et al. Specific properties of Enteropathogenic *Escherichia coli* isolates from diarrheal patients and comparison to strains from foods and fecal specimens from cattle, swine, and healthy carriers in Osaka City, Japan. Appl Environ Microbiol. 2013;79:1232-40.
28. Zhou Z, Ogasawara J, Nishikawa Y, et al. An outbreak of gastroenteritis in Osaka, Japan due to *Escherichia coli* serogroup O166:H15 that had a coding gene for enteroaggregative *E. coli* heatstable enterotoxin 1 (EAST1). Epidemiol Infect. 2002;128:363-71.
29. Hidaka A, Hokyo T, Arikawa K, et al. Multiplex real-time PCR for exhaustive detection of diarrheagenic *Escherichia coli*. J Appl Microbiol. 2009;106:410-20.

30. Beutin L, Geier D, Steinruck H, et al. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J Clin Microbiol. 1993;31:2483-8.
31. Akiyama Y, Saito E, Futai H, et al. Comprehensive Study of Pathogenic Genes Distributed in *Escherichia coli* Isolated from Cattle. Shokuhin Eiseigaku Zasshi. 2015;56:118-22.
32. Patzi-Vargas S, Zaidi MB, Perez-Martinez I, et al. Diarrheagenic *Escherichia coli* carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis. 2015;9:e0003510.
33. Nishikawa Y, Zhou Z, Hase A, et al. Diarrheagenic *Escherichia coli* isolated from stools of sporadic cases of diarrheal illness in Osaka City, Japan between 1997 and 2000: prevalence of enteroaggregative *E. coli* heat-stable enterotoxin 1 gene-possessing E. coli. Jpn J Infect Dis. 2002;55:183-90.
34. Wang L, Nakamura H, Kage-Nakadai E, et al. Comparison by multilocus variable-number tandem repeat analysis and antimicrobial resistance among atypical enteropathogenic *Escherichia coli* strains isolated from foods and human and animal faecal specimens. J Appl Microbiol. 2017;122:268-78.
35. Alonso MZ, Sanz ME, Irino K, et al. Isolation of atypical enteropathogenic *Escherichia coli* from chicken and chicken-derived products. Br Poult Sci. 2016;57:161-4.
36. Jaros P, Cookson AL, Reynolds A, et al. Nationwide prevalence and risk factors for faecal carriage of *Escherichia coli* O157 and O26 in very young calves and adult cattle at slaughter in New Zealand. Epidemiol Infect. 2016;144:1736-47.
37. Kijima-Tanaka M, Ishihara K, Kojima A, et al. A national surveillance of Shiga toxin-producing *Escherichia coli* in food-producing animals in Japan. J Vet Med B Infect Dis Vet Public Health. 2005;52:230-7.
38. Ban E, Yoshida Y, Wakushima M, et al. Characterization of unstable pEntYN10 from enterotoxigenic *Escherichia coli* (ETEC) O169:H41. Virulence. 2015;6:735-44.
39. Devasia RA, Jones TF, Ward J, et al. Endemically acquired foodborne outbreak of enterotoxin-producing *Escherichia coli* serotype O169:H41. Am J Med. 2006;119:168.e7-10.
40. Fujihara S, Arikawa K, Aota T, et al. Prevalence and properties of diarrheagenic *Escherichia coli* among healthy individuals in Osaka City, Japan. Jpn J Infect Dis. 2009;62:318-23.
41. Lalioui L, Le Bouguénec C. *afa*-8 Gene cluster is carried by a pathogenicity island inserted into the $tRNA^{Phe}$ of human and bovine pathogenic *Escherichia coli* isolates. Infect Immun. 2001;69:937-48.
42. Tanih NF, Sekwadi E, Ndip RN, et al. Detection of pathogenic *Escherichia coli* and *Staphylococcus aureus* from cattle and pigs slaughtered in abattoirs in Vhembe District, South Africa. Scientific World Journal. 2015;2015:195972.