

## Presence and Characterization of Shiga Toxin-Producing *Escherichia coli* and Other Potentially Diarrheagenic *E. coli* Strains in Retail Meats<sup>∇</sup>

Xiaodong Xia,<sup>1</sup> Jianghong Meng,<sup>1,2\*</sup> Patrick F. McDermott,<sup>3</sup> Sherry Ayers,<sup>3</sup> Karen Blickenstaff,<sup>3</sup> Thu-Thuy Tran,<sup>3</sup> Jason Abbott,<sup>3</sup> Jie Zheng,<sup>1,4</sup> and Shaohua Zhao<sup>3</sup>

Department of Nutrition and Food Science<sup>1</sup> and Joint Institute for Food Safety and Applied Nutrition,<sup>2</sup> University of Maryland, College Park, Maryland 20742; Center for Veterinary Medicine, Food and Drug Administration, Laurel, Maryland 20708<sup>3</sup>; and Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland 20740<sup>4</sup>

Received 15 August 2009/Accepted 5 January 2010

**To determine the presence of Shiga toxin-producing *Escherichia coli* (STEC) and other potentially diarrheagenic *E. coli* strains in retail meats, 7,258 *E. coli* isolates collected by the U.S. National Antimicrobial Resistance Monitoring System (NARMS) retail meat program from 2002 to 2007 were screened for Shiga toxin genes. In addition, 1,275 of the *E. coli* isolates recovered in 2006 were examined for virulence genes specific for other diarrheagenic *E. coli* strains. Seventeen isolates (16 from ground beef and 1 from a pork chop) were positive for *stx* genes, including 5 positive for both *stx*<sub>1</sub> and *stx*<sub>2</sub>, 2 positive for *stx*<sub>1</sub>, and 10 positive for *stx*<sub>2</sub>. The 17 STEC strains belonged to 10 serotypes: O83:H8, O8:H16, O15:H16, O15:H17, O88:H38, ONT:H51, ONT:H2, ONT:H10, ONT:H7, and ONT:H46. None of the STEC isolates contained *eae*, whereas seven carried enterohemorrhagic *E. coli* (EHEC) *hlyA*. All except one STEC isolate exhibited toxic effects on Vero cells. DNA sequence analysis showed that the *stx*<sub>2</sub> genes from five STEC isolates encoded mucos-activatable Stx2d. Subtyping of the 17 STEC isolates by pulsed-field gel electrophoresis (PFGE) yielded 14 distinct restriction patterns. Among the 1,275 isolates from 2006, 11 atypical enteropathogenic *E. coli* (EPEC) isolates were identified in addition to 3 STEC isolates. This study demonstrated that retail meats, mainly ground beef, were contaminated with diverse STEC strains. The presence of atypical EPEC strains in retail meat is also of concern due to their potential to cause human infections.**

*Escherichia coli* is an important component of the intestinal microflora of humans and warm-blooded mammals. While *E. coli* typically harmlessly colonizes the intestinal tract, several *E. coli* clones have evolved the ability to cause a variety of diseases within the intestinal tract and elsewhere in the host. Those strains that cause enteric infections are generally called diarrheagenic *E. coli* strains, and their pathogenesis is associated with a number of virulence attributes, which vary according to pathotype (54). Currently, diarrheagenic *E. coli* strains are classified into six main pathotypes based on their distinct virulence determinants and pathogenic features, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (37).

Among diarrheagenic *E. coli* strains, STEC strains are distinguished by the ability to cause severe life-threatening complications, such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (30). Other symptoms of STEC infection include watery diarrhea, bloody diarrhea, and hemorrhagic colitis (HC). STEC strains that cause HC and HUS are also called EHEC. Although individ-

uals of all ages are at risk of STEC infection, children younger than 5 years of age and the elderly are more likely to suffer from severe complications (51). Outbreaks and sporadic cases of STEC infections have been reported frequently worldwide.

The pathogenesis of STEC infection in humans is not fully understood. The major virulence factors implicated in STEC infection are potent Shiga toxins, which are classified into two groups: Stx1 and Stx2 (23). Additional factors that contribute to virulence have also been described, including intimin (encoded by the *eae* gene), an outer membrane protein involved in the attachment of *E. coli* to the enterocyte, and EHEC hemolysin (encoded by EHEC *hlyA*), which acts as a pore-forming cytotoxin and causes damage to cells (41).

The first STEC O157 infections were reported in 1982, when *E. coli* O157:H7 was involved in outbreaks associated with two fast food chain restaurants in the United States (44). Since then, ever-increasing numbers of cases and outbreaks due to STEC O157 have been reported worldwide. Although non-O157 STEC strains have also been associated with human cases and outbreaks, few laboratories have been looking for them, and their potential in causing human infections may be underestimated (2). Recently, though, the significance of non-O157 STEC strains as human pathogens has become more recognized. In the United States alone, there were 23 reported outbreaks of non-O157 STEC infection between 1990 and 2007 (10).

Shiga toxin-producing *E. coli* can be transmitted through different routes, including food and water, person-to-person contact, and animal-to-person contact (9). Most human infec-

\* Corresponding author. Mailing address: Department of Nutrition and Food Science, 0112 Skinner Building, University of Maryland, College Park, MD 20742-7521. Phone: (301) 405-7976. Fax: (301) 314-3313. E-mail: jmeng@umd.edu.

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TABLE 1. Oligonucleotide primers for multiplex PCR and sequencing

Assay	Target gene or primer name	Forward primer	Reverse primer	Product size (bp)	Reference
Assay 1	<i>stx</i> <sub>1</sub>	GTGGCATTAACTGAATTGTCATCA	GCGTAATCCCACGGACTCTTC	109	29
	<i>stx</i> <sub>2</sub>	GGCACTGTCTGAAACTGCTCC	TCGCCAGTTATCTGACATTCTG	255	47
Assay 2	<i>stI</i>	TCTTTCCCTCTTTTAGTCAGTC	CAGCACAGGCAGGATTAC	170	36
	<i>elt</i>	ACGGCGTTACTATCCTCTC	TGGTCTCGGTCCAGATATGTG	274	48
	<i>daaE</i>	GAACGTTGGTTAATGTGGGGTAA	TATTCACCGGTCCGGTTATCAGT	542	54
	<i>aafII</i>	CACAGGCAACTGAAATAAGTCTGG	ATTCCCATGATGTCAAGCA CTTC	378	54
Assay 3	<i>eae</i>	CTGAACCAGATCGTAACGGC	TGATAAGCTGCAGTCGAATCC	229	36
	<i>bfp</i>	AACCGTTACTGCCGGTGTGA	GTTGCCGCCTCAGCAGGAGT	450	36
	<i>ipaH</i>	CTCGGCACGTTTTAATAGTCTGG	GTGGAGAGCTGAAGTTCT CTGC	933	54
Assay 4	<i>eae</i>	CTGAACCAGATCGTAACGGC	TGATAAGCTGCAGTCGAATCC	229	36
	EHEC <i>hlyA</i>	AGCCGGAACAGTTCTCTCAG	CCAGCATAACAGCCGATGT	526	35
<i>stx</i> <sub>1</sub> sequencing	Paton 1	TCGCATGAGATCTGACC	AACTGACTGAATTGAGATG	1,470	5
	Paton 2	ATAAATCGCCATTCTGACTAC	AGAACGCCCCTGAGATCATC	180	5
	Gannon	ACACTGGATGATCTCAGTGG	CTGAATCCCCCTCCATTATG	603	5
	Vidiya	TCGCATGAGATCTGACC	AATAAGCCGTAGATTATT	448	5
<i>stx</i> <sub>2</sub> sequencing	Stx2-1	TTCTGAGCAATCGGTCACTG	CGGCGTCATCGTATACACAG	779	55
	Stx2-2	GTCACAGCAGAAGCCTTACG	ACCCACATACCACGAATCAG	714	55

tions are caused by consumption of contaminated foods (16). Domestic and wild ruminant animals, in particular cattle, are considered the main reservoir of STEC and the main source for contamination of the food supply. Retail meats derived from animals could potentially act as transmission vehicles for STEC and other diarrheagenic *E. coli* strains. However, there is limited information about STEC contamination in retail meats, and fewer data exist about the presence of other diarrheagenic *E. coli* strains in retail meats. In the present study, we investigated 7,258 *E. coli* isolates from four types of meat samples (beef, chicken, pork, and turkey) collected during 2002 to 2007 to assess STEC contamination of retail meats. In addition, the presence of other potentially diarrheagenic *E. coli* strains was examined by detecting specific virulence determinants among *E. coli* isolates collected in 2006.

#### MATERIALS AND METHODS

**Bacterial strains.** A total of 7,258 *E. coli* isolates (1,806 from ground beef, 2,106 from ground turkey, 2,179 from chicken breasts, and 1,167 from pork chops) from the retail meat program of the U.S. National Antimicrobial Resistance Monitoring System (NARMS) were investigated. Detailed information on sampling, isolation, and identification can be found at <http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm>. Briefly, retail meats were collected monthly from grocery stores in four states (Georgia, Maryland, Oregon, and Tennessee) from 2002 to 2007. For chicken and pork samples, one piece of meat was examined; for ground beef and ground turkey, 25 g of product was processed. Portions from each sample were placed in separate bags with 250 ml of buffered peptone water, and the bags were vigorously shaken. Fifty milliliters of the rinsate was mixed with 50 ml of double-strength MacConkey broth, and the contents were incubated at 35°C for 24 h. One loopful of culture was streaked onto an eosin-methylene blue (EMB) agar plate and incubated at 35°C for 24 h. The plate was examined for typical *E. coli* colonies, and one typical, well-isolated colony was streaked onto a blood agar plate and incubated at 35°C for 24 h. Indole-positive and oxidase-negative isolates were presumptively identified as *E. coli* and confirmed as *E. coli* by use of the Vitek 2 Compact microbial

identification system (bioMérieux, Hazelwood, MO). All *E. coli* isolates were stored in tryptic soy broth containing 15% glycerol at -80°C until use.

**DNA template preparation.** *E. coli* cells were recovered from frozen culture at -80°C, streaked onto blood agar, and incubated overnight at 37°C. DNA of each isolate was extracted using a previously described boiling method, with modification (46). Briefly, approximately 10 to 20 colonies were taken by use of cotton swabs and were suspended in 500 µl of distilled water. The mixture was then boiled at 100°C for 10 min. After centrifugation at 13,000 rpm for 5 min, supernatants were transferred to a new tube and stored at -20°C until used for PCR amplification.

**Identification of Shiga toxin genes.** STEC was identified by the presence of *stx*<sub>1</sub> and/or *stx*<sub>2</sub> genes. All isolates were subjected to a multiplex PCR (assay 1) which targeted *stx*<sub>1</sub> and *stx*<sub>2</sub> and most of their variants (except for *stx*<sub>1d</sub> and *stx*<sub>2f</sub>, due to their considerable sequence divergence from classical *stx*<sub>1</sub> and *stx*<sub>2</sub>, respectively) (Table 1). PCRs were performed in a 25-µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10× PCR buffer, 2 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 2.5 µl of 25 mM MgCl<sub>2</sub>, 0.25 µl (5 U) of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), and 0.5 µl (25 pmol) of each oligonucleotide primer (Invitrogen, Carlsbad, CA). The thermocycling protocol included an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 30 s), with a final extension at 72°C for 3 min. PCR products (10 µl) were resolved by electrophoresis on a 1.5% (wt/vol) agarose gel at 100 mV for 30 min. Gels were then stained with ethidium bromide, and the DNA bands were visualized and photographed under UV illumination. *E. coli* EDL 933, containing *stx*<sub>1</sub> and *stx*<sub>2</sub>, and *E. coli* K-12 were used as positive and negative controls, respectively. For initial screening, DNA templates of five isolates were pooled and mixed thoroughly, and the mixture was used as a template for PCR. When the mixture tested positive for a *stx* gene(s), DNAs of individual isolates were then tested separately to identify the *stx*-positive isolate(s).

**DNA sequence and phylogenetic analysis of *stx* genes.** Shiga toxin genes were amplified and sequenced using the primers listed in Table 1. The *stx*<sub>1</sub> genes were amplified with primer Paton 1 to generate a 1,470-bp product which covered both the A and B subunits of *stx*<sub>1</sub>. Two overlapping DNA fragments (779 and 714 bp), which together cover the entire *stx*<sub>2</sub> gene, were amplified with primers Stx2-1 and Stx2-2, respectively. The PCR products were purified using 96-well multiscreen filter plates (Millipore Corp., Billerica, MA), and purified amplicons were sequenced on both strands. Sequencing reactions were performed using a Big Dye Terminator cycle sequencing ready reaction DNA sequencing kit and electro-

phoresed on an AB3730 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequence data were compiled and analyzed using DNA Sequencher 4.0 software (Gene Codes Corp., Ann Arbor, MI). Nucleotide phylogenetic trees were generated by multiple sequence alignment and neighbor-joining analysis of the alignment, using the MAFFT program (<http://align.bmr.kyushu-u.ac.jp/mafft/software/>). Reference DNA and amino acid sequences for Shiga toxins were obtained from GenBank and are indicated in the phylogenetic tree by their accession numbers.

**Vero cell cytotoxicity assay.** The STEC isolates were examined for cytotoxicity on Vero cells according to previously published protocols (20, 55). First, 96-well microtiter plates were seeded with approximately  $10^4$  Vero cells/well and incubated at 37°C for 24 h in the presence of 5% CO<sub>2</sub>. The tissue culture medium (Eagle's minimum essential medium [EMEM] containing 10% fetal bovine serum; ATCC, Manassas, VA) was aspirated and replaced with 100 µl of fresh medium prior to the addition of bacterial supernatant dilutions. The bacterial isolates were inoculated into 5 ml LB broth and incubated overnight at 37°C with shaking. The cell concentration of the overnight bacterial culture was adjusted with LB broth to approximately  $10^9$  CFU/ml (optical density at 600 nm [OD<sub>600</sub>] = 1). The culture was centrifuged at 10,000 × g for 10 min, and the supernatant was filtered through a 0.45-µm-pore-size membrane filter. The filtrate was serially diluted (1:5) in tissue culture medium. One hundred microliters of each dilution was inoculated into triplicate wells of the 96-well microtiter plate with Vero cells. Control wells, which refer to wells containing cells not inoculated with toxin, were included on each plate for a nonintoxicated cell background. After incubation at 37°C in a 5% CO<sub>2</sub> atmosphere for 48 h, detached cells, medium, and toxin were removed by vigorous shaking. Remaining Vero cells were fixed with 2% formalin in 0.067 M phosphate-buffered saline (pH 7.2) for 1 min, the fixative was removed, and the plate was stained with 0.13% crystal violet in 5% ethanol for 30 min. Excess stain was removed by rinsing, and the plates were air dried. For quantification, stain was eluted with 200 µl of 50% ethanol, and the color intensity of each well was measured with an Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT) at a 600-nm wavelength. The color intensity was proportional to the number of viable, attached cells in the well. The absorbance values were then plotted against the log of the toxin dilution. To determine the toxin dilution resulting in 50% cell detachment (CD<sub>50</sub>), the zero-detachment dye absorbance value was obtained from control wells with nonintoxicated cells on each plate, and the CD<sub>50</sub> value was determined by extrapolating one-half of this value to the log scale of toxin dilution. *E. coli* EDL933 and *E. coli* K-12 were used as positive and negative controls, respectively. All assays were conducted in triplicate and independently repeated three times.

***eae* and EHEC *hlyA* genes.** The STEC isolates were further tested for the presence of *eae* and EHEC *hlyA* genes, using a multiplex PCR (assay 4) (Table 1). The PCR conditions used were the same as those described above.

**Genomic DNA fingerprinting using PFGE.** Genomic DNA fingerprints of the STEC isolates were further determined using pulsed-field gel electrophoresis (PFGE) according to a standard protocol developed by PulseNet for *E. coli* O157:H7 (19). Briefly, agarose-embedded DNA was digested with 50 U of XbaI for 3 h in a water bath at 37°C. DNA fragments were separated by electrophoresis in 0.5× Tris-borate-EDTA buffer at 14°C for 18 h on a CHEF-III Mapper electrophoresis system, with a pulse time of 2.2 s to 54.2 s. The gels were stained with ethidium bromide, and images were taken under UV transillumination. The images were analyzed with Bionumerics software by using Dice coefficients and the unweighted-pair group method to achieve dendrograms with a 1.5% band position tolerance.

**Identification of virulence determinants of other diarrheagenic *E. coli* strains.** To assess the presence of other potentially diarrheagenic *E. coli* strains in retail meats, 1,275 *E. coli* isolates recovered from retail meats in 2006 were chosen and examined using two multiplex PCRs (assays 2 and 3) (Table 1) to detect the following virulence gene markers: *eae* for intimin of EPEC, *bfp* for the bundle-forming pilus of EPEC, *elt* and *stI* for the heat-labile and heat-stable enterotoxins of ETEC, respectively, *ial* for the invasion-associated locus of EIEC, *aafII* for aggregative adherence fimbriae II in EAEC, and *daaE* for F1845 fimbriae in DAEC. Each of the two PCR assays was performed in a 25-µl reaction mixture containing 2 µl of template DNA, 2.5 µl of 10× PCR buffer, 2 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 4 µl of 25 mM MgCl<sub>2</sub>, 0.25 µl (5 U) of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), and 0.5 µl of each oligonucleotide primer (Invitrogen, Carlsbad, CA). The thermocycling conditions were as follows: 95°C for 12 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min, with a final 10-min extension at 72°C. PCR products (10 µl) were examined in a 2% (wt/vol) agarose gel at 120 mV for 30 min. *E. coli* strains ATCC 35401 (containing *elt* and *stI*), ATCC 43893 (*ipaH*), ATCC 43887 (*eae bfp*), 042 (*aafII*), and F1845 (*daaE*) were used as positive controls, while *E. coli* K-12 served as a negative control.

**Serotyping.** All STEC and other potentially diarrheagenic *E. coli* isolates identified in the study were sent to the *E. coli* Reference Center at Pennsylvania State University to determine their O antigen and H antigen.

**Statistical analysis.** Differences in percentage data were analyzed by the chi-square test, using SPSS software (version 12.0; SPSS Inc., Chicago, IL). A *P* value of <0.05 was considered significant for all comparisons.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the complete *stx*<sub>1</sub> operons from isolates 20177, 22813, N2688, N11354, N11355, N13844, and N15018 were submitted to GenBank and given accession numbers GQ429154 to GQ429160. The nucleotide sequences of the complete *stx*<sub>2</sub> operons from isolates N22813, 23765, N2688, N2743, N2746, N4854, N5545, N5578, N11354, N11355, N11682, and N15432 and a partial *stx*<sub>2</sub> operon from isolate N5789 were also deposited in the GenBank database under accession numbers GQ429161 to GQ429173.

## RESULTS

**Presence of STEC in retail meats.** Among the 7,258 *E. coli* isolates recovered from retail meats collected in four states from 2002 to 2007, 17 (0.23%) tested positive for the presence of a *stx* gene(s) and were considered STEC (Table 2). Almost all STEC isolates (*n* = 16) were from ground beef, accounting for 0.89% of 1,806 *E. coli* isolates from this product. One STEC isolate (0.09%) was identified among 1,167 *E. coli* isolates from pork chops. No STEC strains were found in ground turkey or chicken breast. Five STEC isolates were detected among 1,306 *E. coli* isolates collected in 2005, followed by three STEC isolates each in 2004, 2006, and 2007, two STEC isolates in 2002, and one in 2003. The percentages of STEC isolates among the *E. coli* isolates ranged from 0.08% to 0.38% annually during the 6-year period, but there was no significant difference in percentage by year. Nearly 60% of the STEC (*n* = 10) isolates were identified among *E. coli* isolates from Maryland, where a higher percentage (0.57%) of STEC strains was observed than those for other states (0.05 to 0.22%) (*P* < 0.05).

**Characterization of STEC.** Serotyping results showed that H antigens were successfully typed for all 17 STEC isolates, whereas the O antigens of 6 STEC isolates could not be determined (Table 2). Only five serotypes were found for 11 typeable isolates. Many STEC isolates (7 of 11 isolates) belonged to serotype O83:H8, whereas the other four typeable isolates exhibited different serotypes: O8:H16, O15:H16, O15:H27, and O88:H38. Different H types were observed in six O-nontypeable isolates, among which isolates N5577 and N5578 reacted with the same H antiserum (H46).

Digestion of genomic DNAs from 17 STEC isolates by use of the XbaI restriction enzyme and analysis using PFGE revealed 14 distinct profiles (Fig. 1). Although isolates of the same serotypes tended to cluster together, polymorphism of the genome sequence was also observed between some isolates of the same serotype, according to different PFGE patterns. Three pairs of STEC isolates (N11354 and N11355, N2743 and N2746, and N11354 and N11355) showed identical PFGE profiles and serotypes. Isolates within each pair were recovered from the same food source (i.e., ground beef) and the same geographic locale. Presumably, they were the same clones. All other isolates had their own specific PFGE profiles, with similarity indexes ranging from 67% to 84%.

**STEC virulence genes and Vero cell cytotoxicity.** Among the 17 STEC isolates, 5 contained both *stx*<sub>1</sub> and *stx*<sub>2</sub> genes, whereas 2 contained *stx*<sub>1</sub> only and 10 contained *stx*<sub>2</sub> only (Table 2).

TABLE 2. Characteristics of STEC and atypical EPEC isolates from retail meats<sup>a</sup>

Isolate	Serotype	Presence of gene					Source	State	Year
		<i>stx</i> <sub>1</sub>	<i>stx</i> <sub>2</sub>	<i>eae</i>	EHEC <i>hlyA</i>	<i>bfp</i>			
22813	O83:H8	+	+	-	+		Ground beef	MD	2002
N2688	O88:H38	+	+	-	+		Ground beef	MD	2004
N11354	O83:H8	+	+	-	+		Ground beef	GA	2006
N11355	O83:H8	+	+	-	+		Ground beef	GA	2006
N15018	O15:H27	+	+	-	-		Ground beef	GA	2007
20177	O8:H16	+	-	-	-		Ground beef	TN	2002
N13844	ONT:H51	+	-	-	-		Pork chop	OR	2007
N5577	ONT:H46	-	+	-	+		Ground beef	MD	2005
N5578	ONT:H46	-	+	-	+		Ground beef	MD	2005
N5789 <sup>b</sup>	O15:H16	-	+	-	+		Ground beef	MD	2005
23765	ONT:H2	-	+	-	-		Ground beef	OR	2003
N2743	O83:H8	-	+	-	-		Ground beef	MD	2004
N2746	O83:H8	-	+	-	-		Ground beef	MD	2004
N4854	ONT:H10	-	+	-	-		Ground beef	MD	2005
N5545	ONT:H7	-	+	-	-		Ground beef	MD	2005
N11682	O83:H8	-	+	-	-		Ground beef	MD	2006
N15432	O83:H8	-	+	-	-		Ground beef	OR	2007
N11475	O154:H9			+		-	Chicken breast	GA	2006
N11537	O2:H27			+		-	Chicken breast	GA	2006
N11573	O26:H9			+		-	Chicken breast	GA	2006
N11575	O123:H51			+		-	Chicken breast	GA	2006
N12563	ONT:H7			+		-	Chicken breast	TN	2006
N11452	ONT:H8			+		-	Ground beef	GA	2006
N12148	ONT:H8			+		-	Ground beef	OR	2006
N12174	O15:H2			+		-	Ground beef	OR	2006
N12475	O10:H2			+		-	Ground beef	TN	2006
N11710	O18:H16			+		-	Pork chop	MD	2006
N12051	O81:H7			+		-	Pork chop	OR	2006

<sup>a</sup> Common virulence genes include *stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae*, and EHEC *hlyA* for STEC and *eae* and *bfp* for EPEC.

<sup>b</sup> This strain was negative for Vero cell cytotoxicity, but whether it could produce Stx was not tested by other assays. It was tentatively called STEC here based on the presence of *stx*.

None of the STEC isolates in this study carried the *eae* gene, while seven (41%) STEC isolates were EHEC *hlyA* positive. Cytotoxicities of the STEC isolates were examined on Vero cells. Sixteen isolates were considered toxic to Vero cells compared to *E. coli* K-12. Only one isolate (N5789) showed no cytotoxicity to Vero cells. For the cytotoxic STEC isolates, the CD<sub>50</sub>s ranged from 10<sup>-1.2</sup> to 10<sup>-3.9</sup> (Fig. 2). Overall, STEC isolates with both *stx*<sub>1</sub> and *stx*<sub>2</sub> displayed greater toxicity (CD<sub>50</sub> < 10<sup>-3.2</sup>) than did those with only one of the *stx* genes, with the exception of N2743/N2746 and N13844.

***stx* gene sequence and phylogenetic analysis.** All *stx*<sub>1</sub> genes from seven STEC isolates were successfully amplified and sequenced. A portion of the *stx*<sub>2</sub> gene from N5789 could not be amplified despite repeated efforts. In addition, chromatograms of *stx*<sub>2</sub> sequences amplified from isolates N5577 and N15018 exhibited two peaks at several positions, suggesting the presence of more than one allele of *stx*<sub>2</sub>. Shiga toxin 2 gene sequences from these two isolates were not determined further in this study and were excluded from phylogenetic analysis. Two previously described *stx*<sub>1</sub> sequences (one classical *stx*<sub>1</sub> and one *stx*<sub>1c</sub> sequence) and seven *stx*<sub>1</sub> sequences determined in this study were aligned and used to construct a phylogenetic tree (Fig. 3A). Sequences were aligned from the start codon of *stx*<sub>1a</sub> to the stop codon of *stx*<sub>1b</sub>. Most (6 of 7 sequences) of the *stx*<sub>1</sub> sequences in this study were closely related to classical *stx*<sub>1</sub>, whereas *stx*<sub>1</sub> from N15018 was very similar to *stx*<sub>1c</sub>. Isolates N2688 and N13844 shared an identical *stx*<sub>1</sub> gene sequence. Isolates 22813, N11354, and N11355 also shared an identical

*stx*<sub>1</sub> gene sequence, but it was different from the *stx*<sub>1</sub> gene sequence found in N2688 and N13844.

Six previously described sequences of *stx*<sub>2</sub> and its variants and the 12 *stx*<sub>2</sub> sequences determined in this study were aligned (Fig. 3B). None of the *stx*<sub>2</sub> sequences determined in this study were close to *stx*<sub>2e</sub> or *stx*<sub>2g</sub> (< 93% similarity) (data not shown). Isolates 22813 and N11354 shared an identical *stx*<sub>2</sub> gene sequence, as did N2743, N2746, and N11682. Putative amino acid sequence analysis revealed that Stx toxins from five isolates (N2743, N2746, N4854, N11682, and N15432) possessed two amino acid substitutions compared to classical Stx2, namely, Ser291 and Glu297 in the Stx2A<sub>2</sub> subunit (data not shown), which are characteristic of mucus-activatable Stx2d.

**Presence of virulence genes specific for other diarrheagenic *E. coli* strains.** In addition to 3 STEC isolates identified among the 1,275 *E. coli* isolates collected in 2006, 11 *E. coli* isolates were *eat*<sup>+</sup> *bfp*<sup>-</sup> and were classified as atypical EPEC (Table 2). The positive rates of atypical EPEC strains among *E. coli* isolates from different types of meat were 1.2% for chicken breast (5/415 isolates), 1.4% for ground beef (4/293 isolates), 1.1% for pork chop (2/180 isolates), and 0% (0/387 isolates) for ground turkey. With the exception of three atypical EPEC isolates whose O serogroups could not be determined, none of the eight typeable EPEC isolates belonged to the same serotype (Table 2). No virulence genes specific for ETEC, EIEC, EAEC, or DAEC were detected among the *E. coli* isolates.

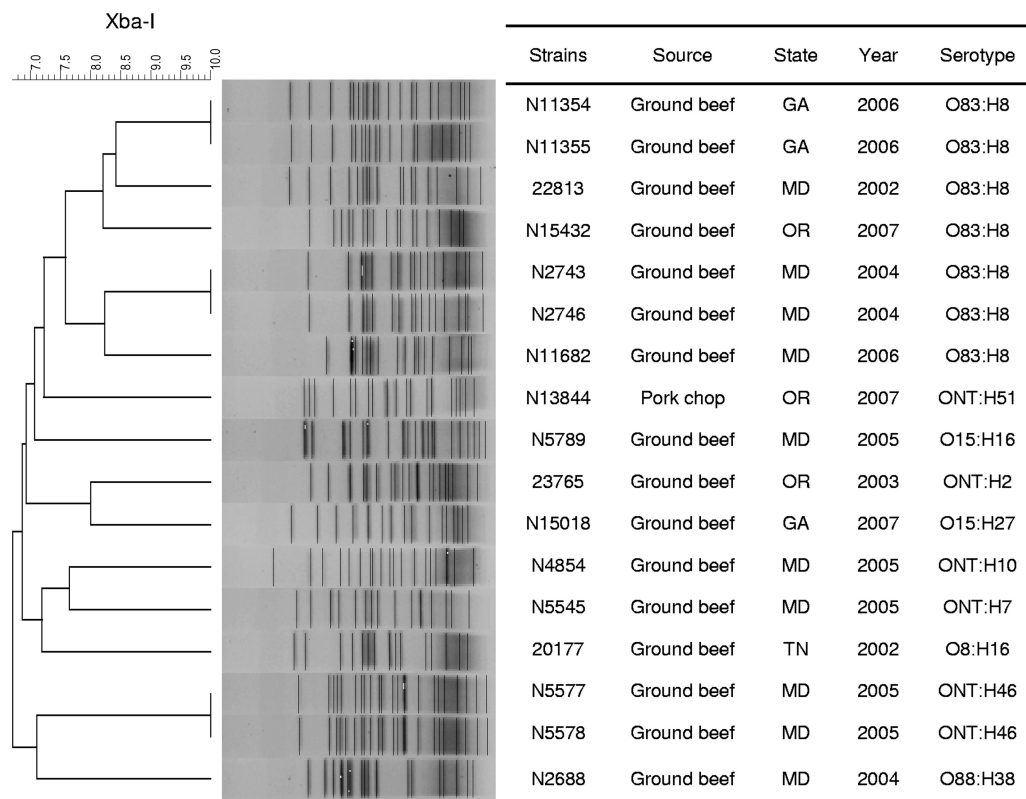


FIG. 1. Dendrogram of PFGE profiles obtained by XbaI digestion for 17 STEC isolates from retail meat. Similarities of PFGE profiles were calculated using the Dice algorithm, with a 1.5% tolerance level.

**DISCUSSION**

In this study, we analyzed 7,258 *E. coli* isolates recovered from retail meats collected by the NARMS program for the presence of *stx* genes, and 1,275 *E. coli* isolates recovered in 2006 were further examined for virulence determinants of other diarrheagenic *E. coli* strains. To our knowledge, this was the largest survey of virulence factors in *E. coli* isolates non-selectively recovered from retail meats. Only a small number

(17) of the *E. coli* isolates were identified as STEC. No DAEC, EIEC, or ETEC strains were detected in the 2006 collection of *E. coli* isolates, although 11 atypical EPEC isolates were identified.

Shiga toxin-producing *E. coli* strains are mostly commensal bacteria in animals, with a high potential for food-borne transmission to humans (9). Ruminants, primarily cattle, are the predominant reservoir of STEC, and beef products serve as one of the most important sources of food-borne STEC infections (9). This consensus was supported by the result in the present study that almost all STEC isolates were recovered from ground beef. Contamination of beef by STEC has been examined by other researchers around the world. In the United States, a recent study by Samadpour et al. reported STEC in 3.5% of 1,750 retail ground beef samples collected from stores in Seattle, WA (45). In other countries, STEC was detected in 4% of beef samples in France (43) and 3% of raw beef samples in Australia (11). Fantelli and Stephan reported STEC in 1.75% of minced beef samples in Switzerland (17), and Lee et al. found STEC in 1.5% of beef samples in Korea (32). Many factors, such as geographical location and sampling, isolation, and testing methods, make comparisons of different studies difficult. In a study using a protocol similar to ours, Lee et al. detected a higher rate of STEC in beef in Korea, possibly indicating the influence of regional differences or different processing technologies in different countries (32). Culture confirmation is also an important factor affecting the results from different studies. In some research, samples were consid-

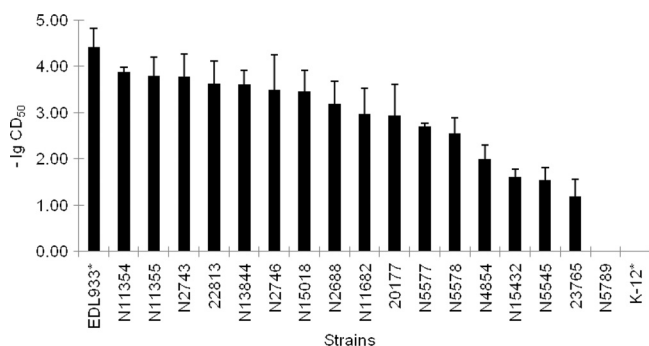


FIG. 2. Vero cell cytotoxicity of Shiga toxin-producing *E. coli* isolates from retail meats. The CD<sub>50</sub> is expressed as the toxin dilution that causes 50% Vero cell detachment compared with untreated cells (control). The value on the y axis indicates the log of the reciprocal of the CD<sub>50</sub>. The data shown are averages for three independent assays. \*, strains EDL933 and K-12 served as positive and negative controls, respectively.

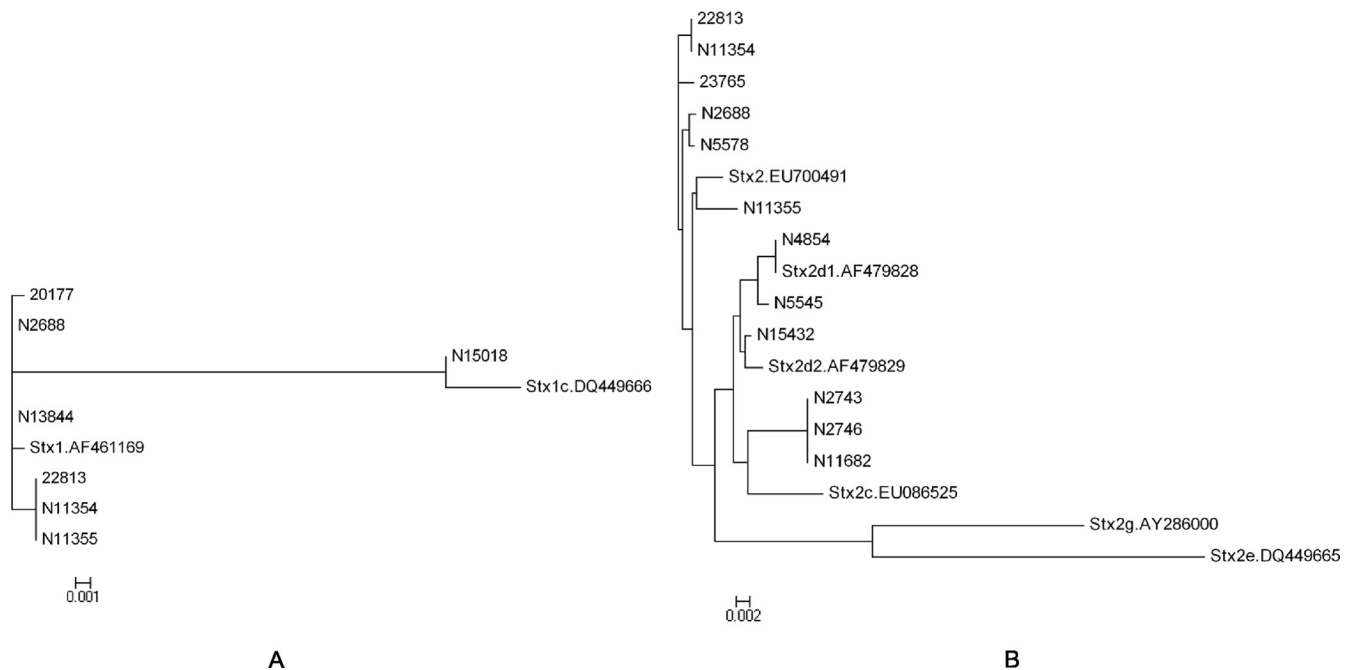


FIG. 3. Phylogenetic trees of *stx*<sub>1</sub> sequences (A) and *stx*<sub>2</sub> sequences (B) determined in this study and sequences of previously described *stx* genes and their variants. *stx*<sub>1d</sub> and *stx*<sub>2f</sub> were not included due to their considerable sequence divergence from classical *stx*<sub>1</sub> and *stx*<sub>2</sub>, respectively. The horizontal bar indicates 0.001 (A) and 0.002 (B) nucleotide substitution per site. Reference DNA sequences for *stx* genes were obtained from GenBank and are identified in the phylogenetic trees by their accession numbers.

ered to contain STEC based only on positive PCR results for enrichment broth (43, 45), while in other studies, culture confirmation was performed to assess the real occurrence of STEC in meat samples (6, 17). Such variations could lead to a big difference in results, since isolation of STEC from *stx*-positive samples was found to be relatively difficult due to the small number of bacterial cells or the occurrence of free *stx*-carrying phages in meat samples (43, 45). In terms of isolation method, instead of picking multiple colonies randomly from one plate and testing each of them (17, 26), colony hybridization with a *stx* probe would more conveniently detect any STEC in all colonies on each plate and yield more accurate results (18, 45). The level of STEC contamination (<1%) in the NARMS ground beef appeared low compared to reports from other investigators, but caution should be exercised in interpreting this low prevalence. The NARMS program was designed to determine the prevalence of generic *E. coli*, not STEC. Moreover, only one *E. coli* isolate was picked from each *E. coli*-positive meat sample in the NARMS program. Consequently, studies specifically designed to determine the prevalence of specific pathotypes, such as STEC, would likely detect a much greater prevalence. Nevertheless, the screening of over 7,000 randomly selected *E. coli* isolates from four states over a 6-year period provides useful data on the presence of STEC in retail meats against the background of generic *E. coli* populations. Moreover, the analysis of a large number of samples enables us to estimate the extent of STEC contamination in different meats.

There is a paucity of data on contamination by STEC in retail meats other than beef. In this study, only one STEC isolate was found among 1,168 *E. coli* isolates from pork chops,

whereas none of the *E. coli* isolates from chicken ( $n = 2,181$ ) or turkey ( $n = 2,106$ ) was identified as STEC. Swine has been suggested as a potential reservoir of STEC strains, and the presence of STEC in pork has been reported before (18). In a study by Samadpour et al., 9 of 51 pork samples collected in local grocery stores in the Seattle area were positive for STEC (45). In New Zealand, Brooks et al. detected one STEC isolate in 35 pork samples (6). In contrast to swine, poultry is generally not considered a source of STEC. The lack of STEC in poultry meat from the NARMS program was consistent with reports from Schroeder et al., who failed to detect STEC in retail chicken (51 ground chicken samples and 212 whole chickens) and turkey (50 ground turkey samples and 194 turkey breasts) samples from the Washington, DC, area (46). Similarly, Brooks et al. failed to isolate STEC from 36 chicken samples in New Zealand (6, 46), and Heuvelink et al. did not find STEC O157 in raw chicken ( $n = 744$ ) and poultry products ( $n = 75$ ) in The Netherlands (26). However, Samadpour et al. recovered 4 STEC isolates from 33 chicken breasts and 1 STEC isolate from 15 turkey samples (12), and Doyle and Schoeni found STEC O157 in 4 of 263 poultry products (15). These isolations were previously thought to possibly be due to exposure to infected ruminants, but recent isolation of STEC from laying hens indicated that poultry may be a source of contamination (14). As discussed above, contamination rates in different studies could be affected by many factors, and attention needs to be paid in comparing data from different studies.

Although O157:H7 is the most common STEC serotype that causes human illness in the United States, there is growing concern over the emergence of highly virulent non-O157 STEC serotypes that are globally distributed, several of

which are associated with outbreaks and/or severe human illness, such as HUS and HC (2, 7). Although *E. coli* O157 was reported to be present in 0.7% of ground beef samples in a USDA study and 1.1% of beef samples in another study in Washington (38, 45), none of the STEC isolates in the present study belonged to serotype O157, which was in agreement with another study conducted in the United States (50). Among the typeable STEC isolates, it is interesting that there was relatively common recovery of serotype O83:H8, which was not reported previously for food or animals. Although the seven isolates belonged to the same serotype, they did not have identical PFGE profiles and *stx* genotypes, except for N11354 and N11355, indicating a nonclonal spread. The fact that STEC O83 has been associated with human illness (2) raises the possibility that O83:H8 (with diverse genotypes and cytotoxicities) might be transmitted to humans via meat products. Additionally, isolate N15018 belonged to O15:H27, a serotype which has also been implicated in human illness (42). The failure to determine the O type for several STEC isolates suggests the complexity of STEC isolates present in retail meats. Since many STEC isolates were nontypeable by serotyping, it is important to improve the current typing scheme and to develop new typing technologies to encompass serogroups that are untypeable at present.

Since not all STEC strains are equally pathogenic to humans, evaluation of virulence-associated factors is necessary to assess an individual isolate's potential to cause human illness. Our results showed that none of the STEC isolates carried the *eae* gene, which is consistent with findings of other studies, in which no *eae* carriage was observed among non-O157 STEC isolates from beef (24, 43). Studies have shown that most STEC isolates from healthy cattle do not carry the *eae* gene (22, 27), and since beef products are generally derived from healthy cattle, this may explain the absence of *eae* in STEC isolates from NARMS beef samples. Although *eae* is considered important for causing attaching and effacing lesions in human intestinal epithelial cells, it may not be essential for STEC pathogenicity, since *eae*-negative STEC strains have also been reported to cause severe human infections (39). It was postulated that *eae*-negative STEC may utilize additional adherence factors, such as Saa (an autoagglutinating adhesin), Iha (an adherence-conferring protein), Efa1 (an EHEC factor for adherence), and LP (the long polar fimbria protein), in the disease process (52). Interestingly, the EHEC *hlyA* gene was observed frequently (41% [7 of 17 isolates]) in the STEC isolates in the present study. Similar frequencies (40 to 51%) of this gene were observed by Slanec et al., in STEC strains isolated from food samples (40%), and by Aidar-Ugrinovich et al., in STEC isolates from calves (51%) (1, 49). EHEC *hlyA* is located in a large plasmid which many human-pathogenic STEC strains harbor. The gene product may contribute to pathogenesis by acting as a pore-forming cytolysin on eukaryotic cells. The presence of this gene may enhance the virulence potential of STEC isolates from retail meats.

Shiga toxins differ in toxicity, toxin receptor, and amino acid sequence (33). Nucleotide sequence analysis of *stx*<sub>1</sub> and *stx*<sub>2</sub> genes present in our STEC isolates confirmed this finding. *stx*<sub>2</sub> gene sequences exhibited much more sequence diversity than did *stx*<sub>1</sub> sequences (Fig. 3). Three *stx*<sub>2</sub> sequences were identical to a previously published *stx*<sub>2</sub> gene sequence

(GenBank accession no. AY443058.1) from a human isolate, and two of them were identical to an *stx*<sub>2</sub> allele in a bovine isolate (AY443054.1). No other *stx*<sub>2</sub> sequences were identical at the nucleotide level. All mucus-activatable *stx*<sub>2d</sub> sequences clustered together with two previously described activatable *stx*<sub>2d</sub> sequences (*stx*<sub>2d1</sub> and *stx*<sub>2d2</sub>), but with some sequence difference (Fig. 3B). *stx*<sub>2c</sub> and *stx*<sub>2</sub> from N5545 were also included in the cluster, but they did not have the characteristic amino acid substitutions common in activatable *stx*<sub>2d</sub>. There were two isolates (N5577 and N15018) whose *stx*<sub>2</sub> chromatograms showed two peaks at multiple sites, which indicates that these two strains may carry more than one allele, an interesting phenomenon that was also observed by other researchers (3). All STEC isolates were shown to be toxic to Vero cells, except for one isolate (N5789). The *stx*<sub>2</sub> sequence could not be obtained for this isolate, since it could not be amplified by the first pair of sequencing primers. Several primers were tried to amplify a fragment which would cover the Stx2A subunit of the gene, but part of the gene could never be amplified. It is possible that a large intervening insertion may be present that prevents successful PCR amplification. It is also possible that this insertion abolishes the activity of Stx2A and consequently renders the isolate less toxic than other STEC strains.

Shiga toxin types were suggested to correlate with the clinical symptoms of STEC infection (28). Mucus-activatable Stx2d is associated with high virulence and the ability to cause HUS (4, 21, 28). The particular attribute of this variant is that it can be cleaved by elastase in the intestinal mucosa, causing an increase in cytotoxicity of up to 1,000-fold (31). This character is attributed to two amino acid substitutions relative to the sequence of classical Stx2, namely, Ser291 and Glu297. Based on predicted amino acid sequence analysis, 5 of 15 *stx*<sub>2</sub>-containing isolates in this study harbored mucus-activatable *stx*<sub>2d</sub>. The relatively large number of mucus-activatable *stx*<sub>2d</sub> genes found in STEC strains isolated from retail meat deserves attention. Studies found that *eae*-negative, mucus-activatable Stx2d-producing STEC strains were involved in sporadic and outbreak cases of HC and HUS (28, 40). It was also shown that although *eae*-negative STEC strains are normally isolated from persons with no or mild disease, most *eae*-negative STEC strains associated with severe symptoms harbor mucus-activatable *stx*<sub>2d</sub> as the sole *stx* gene (4). The pathogenic potential of the five isolates with mucus-activatable *stx*<sub>2d</sub> should not be underestimated. Currently, very limited data are available concerning the presence of STEC strains harboring mucus-activatable *stx*<sub>2d</sub> in food or livestock sources. Zheng et al. identified seven STEC isolates carrying activatable *stx*<sub>2d</sub> among 153 STEC strains isolated from food, cattle, and humans, and none of them contained the *eae* gene (55). Gobius et al. investigated 311 STEC strains possessing *stx*<sub>2</sub> from food and livestock and found 12 STEC isolates carrying activatable *stx*<sub>2d</sub>, all of which did not have *eae* either (21). It has been suggested that the expression of an activatable toxin may compensate for the lack of intimin (34). Due to their strong association with severe clinical outcomes, more surveillance of STEC strains expressing activatable *stx*<sub>2d</sub> in food and human illness is warranted.

Another interesting finding of this study was the identification of several atypical EPEC isolates. EPEC is a leading cause of infant diarrhea in developing countries and is also an im-

portant cause of diarrhea in developed countries (53). Typical EPEC strains contain both *eae* and *bfp*, while atypical EPEC strains contain only *eae*. In industrialized countries, typical EPEC infections have decreased and atypical *E. coli* infections seem to have increased in recent years (25, 53). Unlike typical EPEC strains, which are found only in humans, atypical EPEC strains have been isolated from a variety of animal species, such as cattle, goats, sheep, chickens, pigeons, and gulls (13). In addition to chicken and beef, we also identified atypical EPEC isolates among *E. coli* isolates from pork, indicating that pigs may also be potential reservoirs for the pathogen. Atypical EPEC strains found in this study belonged to a variety of serogroups, most of which were not found in atypical EPEC strains involved in human infections. However, isolate N11573 belonged to O26, a serogroup that is frequently found in classic human EPEC strains (8). Further studies are needed to determine whether atypical EPEC strains of animal origin can actually cause human infections when ingested.

In conclusion, retail meats, especially ground beef, were contaminated with STEC, although at a very low frequency, and some of the strains contained Shiga toxins associated with high potential to cause severe human disease. Moreover, the identification of atypical EPEC strains in retail meats is noteworthy, and the potential role of animal-derived atypical EPEC strains in causing human infections requires further investigation.

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