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Non-O157 Shiga toxin-producing *Escherichia coli* in retail ground beef and pork in the Washington D.C. area

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ABSTRACT

The prevalence and characteristics of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in retail ground meat from the Washington D.C. area were investigated in this study. STEC from 480 ground beef and pork samples were identified using PCR screening followed by colony hybridization. The STEC isolates were serogrouped and examined for the presence of virulence genes (*stx*1, *stx*2, *eae* and *hly*A), and antimicrobial susceptibility. PFGE was used to identify the clonal relationships of STEC isolates, and PCR-RFLP was employed to determine *stx* subtypes. In addition, the cytotoxicity of STEC isolates was determined using a Vero cell assay. STEC were identified in 12 (5.2%) of 231 ground pork and 13 (5.2%) of 249 ground beef samples. Among 32 STEC isolates recovered from the 25 samples, 12 (37.5%) carried *stx2dact* and 7 (21.9%) carried *hly*A, but none carried *eae*. Nine isolates were identified as O91, and 17 (53.1%) isolates were resistant to two or more antimicrobials. Verotoxicity was detected in 26 (81.3%) of the STEC isolates. Thus, the retail ground meat was contaminated with a heterogeneous population of non-O157 STEC, some of which were potential human pathogens.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne pathogens worldwide (Islam et al., 2008). Most STEC infections cause self-limiting diarrhea, but some can progress to life-threatening diseases such as hemolytic uremic syndrome (HUS). To date, more than 470 STEC serotypes have been reported to be associated with human illness (Blanco et al., 2004). *E. coli* O157:H7 is the predominant serotype associated with outbreaks and sporadic cases of STEC infections in the United States (Manning et al., 2008). However, a growing number of non-O157 serotypes have also been linked to human illnesses, including HUS (Bettelheim, 2007). In the United States, non-O157 STEC causes an estimated 112,752 cases of illness each year, whereas *E. coli* O157:H7 causes 63,153 cases per year (Scallan et al., 2011). Depending on geographic location, a variety of non-O157 serotypes were isolated from patients with STEC infections (Brooks et al., 2005; Stephan et al., 2008). As only a limited number of laboratories test for non-O157 STEC, the public health risk associated with non-O157 STEC is likely underestimated.

Shiga toxin-producing *E. coli* are defined by their production of one or more Shiga toxins (Stxs). The Stx family consists of two groups: Stx1 and Stx2. Stx1 is highly homogenous and consists of Stx1a, Stx1c and Stx1d (Burk et al., 2003; Zhang et al., 2002), whereas, Stx2 contains several variants including Stx2a, Stx2c, Stx2d, Stx2dact, Stx2e, Stx2f and Stx2g (Feng et al., 2011). Certain Shiga toxin subtypes are highly associated with clinical syndromes (Jelacic et al., 2003). STEC strains carrying certain *stx2* genes were frequently associated with severe diseases such as hemorrhagic colitis (HC) and HUS (Miliwebsky et al., 2007). Additionally, intimin, an outer membrane protein encoded by *eae* that resides in the locus of enterocyte effacement (LEE), is highly associated with STEC infections (Karmali et al., 2010). However, STEC strains lacking *eae* have also been isolated from patients with severe disease (Bonnet et al., 1998). Additional adhesion factors, such as Saa (STEC autoagglutinating adhesion), Iha (IrgA homologues adhesion), and LPF (long polar fimbriae), may contribute to the adhesion process of *eae*-negative STEC (Xia et al., 2010).

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Shiga toxin-producing *E. coli* are transmitted to humans mainly through consumption of contaminated food and water (Erickson and Doyle, 2007). Ground meat presents a greater risk than intact muscle because it can be contaminated with STEC during processing, and the pathogens present inside the ground product are more likely to survive during cooking of meat (Barlow et al., 2006). However, limited information is available about non-O157 STEC contamination in retail ground meat in the United States. The objectives of this study were to determine the prevalence of non-O157 STEC in ground meat in the Washington D.C. area, and to characterize STEC isolates to determine their virulence potential.

2. Materials and methods

2.1. Sampling, culture enrichment and PCR assay

From March 2009 to March 2010, 480 samples (249 ground beef and 231 ground pork) were collected weekly from three grocery chain stores in the Washington D.C. area, USA. The USDA-FSIS enrichment method was used in this study with modification (USDA-FSIS, 2008). Briefly, a 25 g portion of each sample was placed in a plastic filter bag with 225 ml of modified tryptic soy broth (mTSB: 30 g TSB base, 1.5 g bile salts No. 3 and 1.5 g dipotassium phosphate per liter of distilled water) (Becton Dickinson, Sparks, MD). After homogenizing in a stomacher (Seward, Bohemia, NY), each sample was incubated for 15–22 h at 42 °C in a water bath with shaking at 100 rpm. One milliliter of the broth culture was taken for DNA extraction using the InstaGene DNA extraction kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. A PCR assay described by Lin et al was used to identify *stx*-positive samples (Lin et al., 1993). It amplified *stx1a, stx1c, stx1d, stx2a, stx2c, stx2d, stx2dactive*, *stx2e, stx2f* (Bastian et al., 1998; Kruger et al., 2011; Lin et al., 1993; Schmidt et al., 2000). The PCR was performed in a 25 μl reaction mixture containing 3 μl of DNA template, 2.5 μl of 10× PCR buffer, 2 μl of 25 mmol l^{-1} $MgCl_2$, 2 μl 1.25 mmol l^{-1} dNTP mix, 0.125 μl of 5 U μl^{-1} AmpliTaq Gold DNA polymerase mix (Applied Biosystems, Branchburg, NJ) and 0.2 μl of 50 pmol μl^{-1} of each primer. Thermal cycle condition was used as previously described (Lin et al., 1993). *E. coli* O157:H7 EDL933 and *E. coli* K-12 were used as positive and negative controls, respectively, in all PCR assays.

2.2. Colony hybridization

A colony hybridization procedure targeting *stx* genes was used to identify suspect STEC as previous described (Stephan et al., 2008). A DNA probe targeting both *stx1* and *stx2* was prepared by labeling *stx*-PCR amplicons from *E. coli* EDL933 using the PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The probe was tested with control strains listed in subsection 2.3. In order to isolate STEC, up to six hybridization positive colonies were randomly picked and grown at 37 °C overnight on LB agar plate (Becton Dickinson, Sparks, MD). Isolates were determined as *E. coli* using the Vitek 2.0 system (bioMerieux, Marcyl'Etoile, France).

2.3. Molecular characterization of virulence genes

Two multiplex PCRs were used to determine the presence of *stx*1, *stx*2, *eae* and *hly*A in STEC isolates (Xia et al., 2010). PCRs were performed in a 25 μl reaction system with 0.2 μl of 50 pmol μl^{-1} of each primer. *stx* subtypes were determined using PCR-RFLP as described previously (Beutin et al., 2007) with the following control strains: EDL933 (*stx1a* and *stx2a*), E32511 (*stx2c*), EH250 (*stx2d*), S1191 (*stx2e*), B2F1 (*stx2dactive*) and N15018 (*stx1c*). *stx2dact* was confirmed by PCR according to our previously reported method (Zheng et al., 2008).

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed following the updated protocol for non-O157 from PulseNet (2009). *Salmonella* Braenderup H9812 was used as control for PFGE. PFGE gel pictures were analyzed with Bionumerics software (Applied Maths, Austin, TX) using dice coefficients and unweighted pair group method with a 1.5% band position tolerance.

2.5. Vero cell cytotoxicity assay

Shiga toxin production of STEC isolates was evaluated using a Vero cell cytotoxicity assay as previously described (Xia et al., 2010; Zheng et al., 2008). Briefly, Vero cells were grown in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% fecal calf serum (Phenix Research Product, Candler, NC) under 5% CO_2 at 37 °C. STEC isolates were inoculated in LB broth (Becton Dickinson, Sparks, MD) and incubated at 37 °C overnight with shaking at 100 rpm. After adjusting the cell concentration to 10^9 CFU/ml with LB broth, 2 ml of the culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.45 μm pore-size membrane filter (Fisher HealthCare, Houston, TX). The filtrate was serially diluted (1:5) and 100 μl of each dilution was added to each well that was preseeded with Vero cells. After incubation under 5% CO_2 at 37 °C for 48 h, 200 μl of 2% formalin in 0.067 M phosphate-buffered saline (pH 7.2) was added to fix Vero cells. After 1 h, the fixed cells were stained with 0.13% crystal violet in 5% ethanol for 30 min. The color density of each well was measured using a Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT) at 600 nm. EDL933 and *E. coli* k-12 were used as positive and negative controls, respectively. All assays were conducted in triplicate and repeated independently three times.

2.6. Molecular serogrouping

Serogroups including O8, O26, O28, O45, O91, O103, O111, O121, O145, and O157 were screened using PCR assays with primers based on the *wzy* genes (Table 1). Each PCR was performed in

Table 1
Oligonucleotide primers based on *wzy* for PCR serogrouping.

Serogroup	Primer	Product size	Accession number
O8	5′-ACACCACAAACGATAATCAATGCC-3′ 5′-GACCGATTAACAGCCAACACAGGT-3′	272	AF013583.1
O26	5′-TAATTGGCTTGCTGGGTTTATTCC-3′ 5′-AGAATGAACCTTAATGCCATCAGC-3′	422	AF529080.1
O28	5′-GTCCAAAACGCAAGACGGTTC-3′ 5′-CCATACGCACGAGTGAATGTCC-3′	387	DQ462205.1
O45	5′-GGCTCATCATTTGGTGCTTTGTG-3′ 5′-ATAAGGATTTTCAGCGCCCCTG-3′	404	AY771223.1
O91	5′-CTGGAATGCTTGATGAACCTGGG-3′ 5′-AAGCCCCGACTCACTGTCAGAAAT-3′	284	AY035396.1
O103	5′-TTATACAAATGGCGTGGATTGGAG-3′ 5′-TGCAGACACATGAAAAGTTGATGC-3′	385	AY532664.1
O111	5′-TAGGGGGCAGATTTTATATTCCGT-3′ 5′-AACCAATGCTCCTATCACACCAAT-3′	379	AF078736.1
O121	5′-AGTGGGGAAGGGCGTTACTTATC-3′ 5′-CAATGAGTGCAGGCAAAATGGAG-3′	366	AY208937.1
O145	5′-CCTGTCTGTTGCTTCAGCCCTTT-3′ 5′-CTGTGCGCGAACCACTGCTAAT-3′	392	AY863412.1
O157	5′-TCGTTCTGAATTGGTGTTGCTCA-3′ 5′-CTGGTGTCGGAAAGAAATCGTTC-3′	278	AF061251.1

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