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Prevalence and Level of Enterohemorrhagic *Escherichia coli* in Culled Dairy Cows at Harvest

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ABSTRACT

The primary objective of this study was to determine the prevalence and level of enterohemorrhagic *Escherichia coli* (EHEC) O26, O45, O103, O111, O121, and O145 (collectively EHEC-6) plus EHEC O157 in fecal, hide, and preintervention carcass surface samples from culled dairy cows. Matched samples ($n = 300$) were collected from 100 cows at harvest and tested by a culture-based method and two molecular methods: NeoSEEK STEC (NS) and Atlas STEC EG2 Combo. Both the culture and NS methods can be used to discriminate among the seven EHEC types (EHEC-7), from which the cumulative prevalence was inferred, whereas the Atlas method can discriminate only between EHEC O157 and non-O157 EHEC, without discrimination of the serogroup. The EHEC-7 prevalence in feces, hides, and carcass surfaces was 6.5, 15.6, and 1.0%, respectively, with the culture method and 25.9, 64.9, and 7.0%, respectively, with the NS method. With the Atlas method, the prevalence of non-O157 EHEC was 29.1, 38.3, and 28.0% and that of EHEC O157 was 29.1, 57.0, and 3.0% for feces, hides, and carcasses, respectively. Only two samples (a hide sample and a fecal sample) originating from different cows contained quantifiable EHEC. In both samples, the isolates were identified as EHEC O157, with 4.7 CFU/1,000 cm^2 in the hide sample and 3.9 log CFU/g in the fecal sample. Moderate agreement was found between culture and NS results for detection of EHEC O26 ($\kappa = 0.58$, $P < 0.001$), EHEC O121 ($\kappa = 0.50$, $P < 0.001$), and EHEC O157 ($\kappa = 0.40$, $P < 0.001$). No significant agreement was observed between NS and Atlas results or between culture and Atlas results. Detection of an EHEC serogroup in fecal samples was significantly associated with detection of the same EHEC serogroup in hide samples for EHEC O26 ($P = 0.001$), EHEC O111 ($P = 0.002$), EHEC O121 ($P < 0.001$), and EHEC-6 ($P = 0.029$) based on NS detection and for EHEC O121 ($P < 0.001$) based on detection by culture. This study provides evidence that non-O157 EHEC are ubiquitous on hides of culled dairy cattle and that feces are an important source of non-O157 EHEC hide contamination.

Key words: Dairy cows; Enterohemorrhagic *Escherichia coli*; *Escherichia coli*; Shiga toxin–producing *Escherichia coli*

Shiga toxin–producing *Escherichia coli* (STEC) causes severe illness in humans, including hemorrhagic colitis and hemolytic uremic syndrome *(22)*. STEC strains are naturally found in the intestines of ruminants, shed in the feces, and transmitted to humans through the ingestion of contaminated food or water or by direct contact *(18, 30, 44)*. Enterohemorrhagic *E. coli* (EHEC) strains are a subset of STEC and generally defined as *E. coli* strains that contain genes for Shiga toxin (*stx*) and locus of enterocyte effacement (LEE) proteins, e.g., intimin (*eae*), but may also include LEE-negative *stx*-positive *E. coli* strains that cause hemorrhagic colitis and hemolytic uremic syndrome in human patients *(22)*. EHEC of the serogroups O26, O45, O103, O111, O121, and O145 (collectively EHEC-6) caused 71% of the human STEC infection cases in the United States from 1983 to 2002 *(14)*. Cumulatively, EHEC-6 plus EHEC O157:H7 (collectively EHEC-7) caused >90% of the human STEC infection cases in the United States from 2000 to 2010 *(29)*. Thus, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) *(68)* declared EHEC-7 adulterants in raw, nonintact beef.

The clonality and unique biochemical features of EHEC O157:H7 has allowed for the development of sensitive and specific culture methods for this organism *(8, 51)*. In contrast, culture methods for non-O157 EHEC adulterants have lacked sensitivity and specificity because of the multiplicity of organisms needing to be targeted and the lack of clonality of these organisms, and other than having Shiga toxin and intimin, these organisms lack characteristics that distinguish them from other *E. coli (28, 38, 63, 70)*. FSIS methods for detection and isolation of EHEC from meat products, which involve PCR screening, immunomagnetic separation (IMS), cultural isolation on a chromogenic

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agar, and confirmatory PCR and agglutination, have been improved through a number of modifications but still are not optimal *(67, 70)*. Molecular methods for detecting nucleic acids from EHEC strains have been used in place of culture-based methods or as an initial screening test before culturing of EHEC in foods *(26),* but the approaches also lack specificity because gene targets can be contributed by background microorganisms, leading to false-positive results *(70)*. To increase sensitivity and specificity, the NeoSEEK STEC Detection and Identification test (NS; Neogen, Lansing, MI) and the Atlas STEC EG2 Combo Detection Assay (Roka Bioscience, Warren, NJ) have been used. The NS test includes a proprietary set of genetic markers, has been approved by the FSIS as a confirmation test for EHEC adulterants in beef trim, and has been used to detect EHEC in veal calf hide samples *(72)* and feedlot cattle hide and carcass samples *(63)*. The Atlas test has been used on cattle fecal samples *(12)*.

EHEC strains pose a threat to food safety because they contaminate carcass surfaces during the removal of hides *(1, 2, 5, 13, 25, 39, 40, 60)*, and the predictive virulence of EHEC isolates is a question of major importance. In one study, the prevalence of EHEC-7 in beef feedlot cattle based on NS test results was 80.7% on hides and 6.0% on preintervention carcass surfaces *(63)*. Culled dairy cows also are a significant source of beef; in 2014, 9.5% of cattle slaughtered were classified as dairy cows *(69)*. Whereas the meat from feedlot cattle is primarily manufactured into whole muscle cuts such as steaks and roasts, meat from dairy cattle is primarily used for ground beef. According to a literature review *(34),* fecal samples from dairy cows tested by culture-based methods yielded prevalence results of 0.2 to 48.8% for EHEC O157:H7 and 0.4 to 74.0% for non-O157 STEC. Coombes et al. *(19)* developed a molecular risk assessment (MRA) protocol for assessing the predictive virulence or public health risk of non-O157 STEC strains based on the detection of specific virulence genes. The identification of more highly virulent strains is important because these strains are most likely to cause disease outbreaks and case progression to hemolytic uremic syndrome *(19, 37)*.

Many factors, including management practices, have been implicated in the intestinal colonization of cattle by EHEC *(58, 59, 61, 62)*. Production systems for beef feedlot cattle and dairy cattle differ, which may result in differences in EHEC prevalence. Culled dairy cows (6 to 8 years old) are typically older than feedlot steers and heifers (<2 years old). Cray and Moon *(21)* reported that preweaned (3- to 14-week-old) calves were more susceptible to infection with STEC O157:H7 after experimental inoculation than were adult (1- to 3-year-old) cattle; however, Mir et al. *(48)* reported that cows (≥2 years old) had a higher natural prevalence of STEC than did heifers (1 to 2 years old).

In addition to prevalence, population level is an important contributor to risk of EHEC infection *(24)*. *E. coli* O157:H7 levels $>10^4$ CFU/g are associated with hide contamination *(3),* and even higher levels in the environment may lead to more human exposure *(16)*. The objectives of this cross-sectional study were to (i) determine the prevalence and levels of EHEC-7 in fecal, hide, and preintervention carcass surface samples from culled dairy cows at a commercial processing plant, (ii) conduct an MRA on recovered EHEC isolates, (iii) compare the applicability of a culture-based method, the NS test, and the Atlas assay for detection of EHEC, and (iv) determine the association between detection of an EHEC serogroup in fecal samples and detection of that same serogroup on hides.

MATERIALS AND METHODS

Study design and sample collection. Matched fecal, hide, and carcass surface samples ($n = 300$) were collected from 100 dairy cows (10 to 30 cows per week for 5 weeks) at a small (60 animals per h) western U.S. commercial processing plant from June to July 2014 using a modified protocol for *E. coli* O157:H7 sampling *(66)*. Samples of rectal lumen contents (as a surrogate for feces) were obtained at the viscera table after the cattle were slaughtered and eviscerated. Hides were rinsed with water by plant employees as part of the routine process before the research team collected samples. Hide and carcass surface samples were collected using wet (35 and 20 ml of buffered peptone water, respectively) Speci-sponges (Nasco, Ft. Atkinson, WI) according to methods previously described *(63)*. Hide samples were collected by swabbing an area of approximately 1,000 cm^2 (32 by 32 cm), 15 cm from the ventral midline near the diaphragm. Two carcass surface samples per animal were collected: the first was obtained from an area of approximately 1,000 cm^2 in the brisket–short plate region, and the second was obtained from an area of approximately 3,000 cm^2 in the lateral hock and round-rump regions. The two carcass sponges and their buffer volumes, representing a total of 4,000 cm^2 of swabbed area per carcass, were combined into a single Whirl-Pak bag and shipped overnight on ice to the laboratory.

Isolation and confirmation of EHEC using culture and PCR. The 300 fecal, hide, and carcass samples were processed within 24 h after collection (Fig. 1). One gram of feces was suspended in 9 ml of *E. coli* broth (Oxoid, Basingstoke, UK) and vortexed for 1 min. Ninety milliliters of *E. coli* broth at room temperature was added to each hide sponge sample, and 80 ml of *E. coli* broth at room temperature was added to each carcass sponge sample. All samples were incubated at 40°C for 6 h and then used to inoculate a CHROMagar STEC plate (DRG Int., Springfield, NJ) for isolation, which was incubated overnight at 37°C. Four or fewer pink to mauve colonies (two colonies with and two without UV-fluorescing halos when present) were picked and inoculated into 500 μl of Trypticase soy broth and incubated overnight at 37°C. Genomic DNA was prepared from overnight cultures by centrifuging a 200-μl aliquot at 2,000 × *g* for 20 min, replacing the medium with 200 μl of PBS, vortexing to resuspend the cells, incubating at 95°C for 20 min, centrifuging again at 2,000 × *g* for 20 min, and collecting the supernatant. One microliter of the supernatant was used in a 20-μl PCR containing a 1.3× final concentration of Bullseye HS *Taq* Buffer II (with balanced ammonium and potassium), 29.5 mM $MgCl_2$, 10 mM concentrations of each deoxynucleoside triphosphate, 1.5 U of Bullseye HS *Taq* (all from MidSci, St. Louis, MO), and primers shown in Table 1. PCR amplification was performed as previously described *(56)*.

Primers for the amplification of the type III secreted effector EspK (*espK*) were generated from the *E. coli* O157:H7 EDL933 genome (GenBank accession no. AE005174). The *espK* (Z1829) sequence was loaded into Geneious version 7.1.8 (Biomatters, Auckland, New Zealand), and the Primer 3 software module was used to design PCR primers with compatible melting temperatures

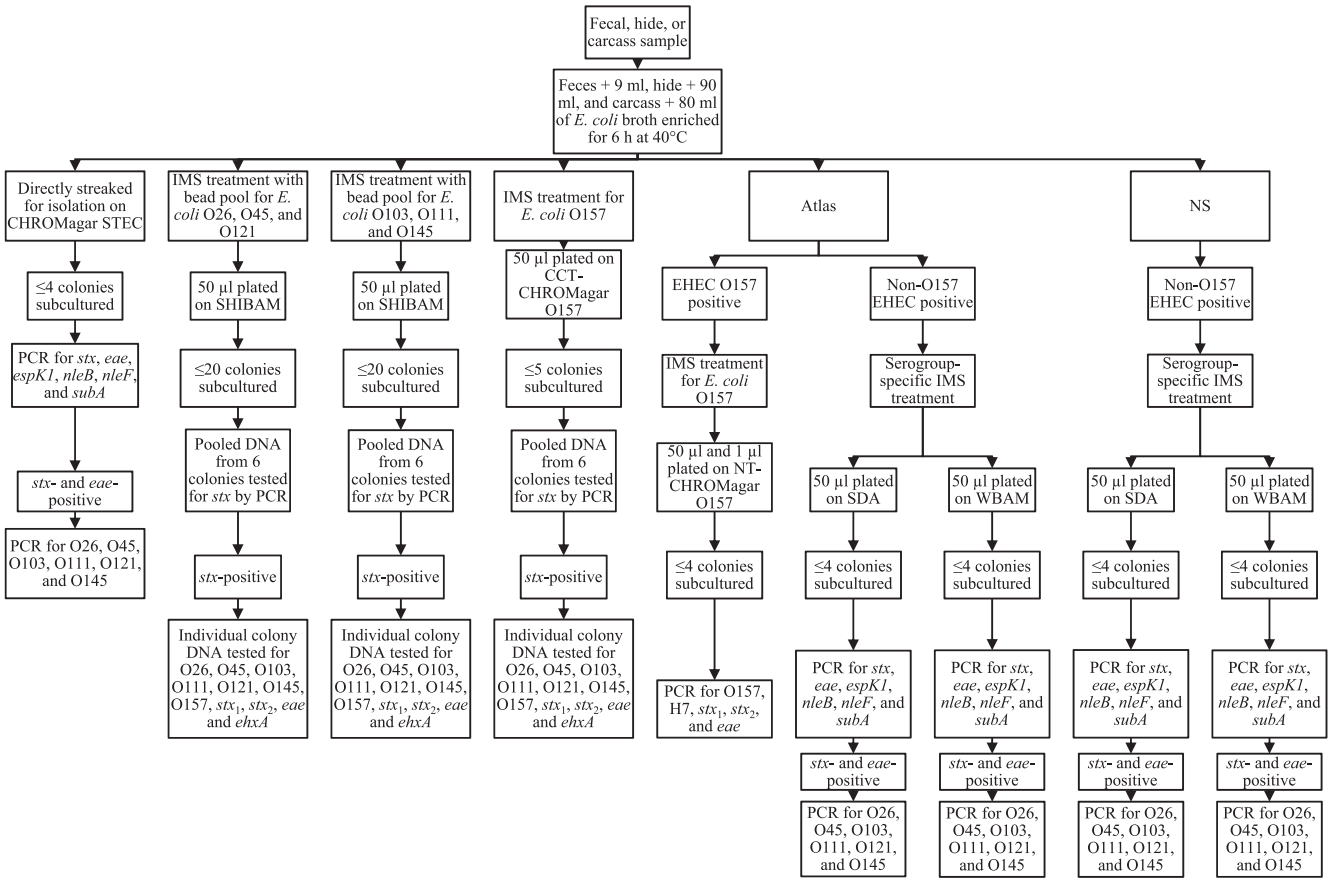

FIGURE 1. *Flowchart of culture-based and molecular methods used for detection of enterohemorrhagic* E. coli. *Atlas, Atlas STEC EG2 Combo Detection Assay; CCT-CHROMagar O157, culture on CHROMagar O157 containing cefixime, cefsulodin, and potassium tellurite; IMS, immunomagnetic separation; NS, NeoSEEK STEC Detection and Identification; NT-CHROMagar O157, culture on CHROMagar O157 containing novobiocin and potassium tellurite; SHIBAM, STEC heart infusion washed blood agar with mitomycin C; SDA, culture on STEC differentiation agar; WBAM, washed blood agar with mitomycin C.*

that generated a 200-bp product. Primers EspK_F1 and EspK_R1 were validated against 86 *espK*-positive STEC strains: the U.S. Meat Animal Research Center (Clay Center, NE) *E. coli* O157:H7 molecular diversity panel (50 unique *E. coli* O157:H7 strains isolated from across North America, each with an individual pulsed-field gel electrophoresis restriction digest pattern) and six STEC strains each of the O26, O45, O103, O111, O121, and O145 serogroups. Negative controls for *espK* included the non-EHEC strains within the 72 *E. coli* strains that make up the *E. coli* reference collection *(54)*.

After enrichment, three separate 490-µl aliquots of the broth cultures were diluted with 490 µl of PBS with 0.05% Tween 20 (PBS-Tween), and each aliquot was subjected to one of three IMS treatments using a KingFisher Flex magnetic particle processor (Thermo Scientific, Waltham, MA) (Fig. 1): (i) 20 µl of anti-O157 Dynabeads (Invitrogen, Carlsbad, CA), (ii) a pool of 20 µl of IMS

TABLE 1. *List of primers for screening enterohemorrhagic* E. coli

Gene	Primer	Sequence (5′→3′)	Concn (nM)	Amplicon size (bp)	Reference
nleF	nleF F	ATGTTACCAACAAGTGGTTCTTC	250	567	*19*
	nleF R	ATCCACATTGTAAAGATCCTTTGTT	250		
subA	SubHCDF	TATGGCTTCCCTCATTGCC	300	556	*57*
	SubSCDR	TATAGCTGTTGCTTCTGACG	300		
eae	eaeAF	GACCCGGCACAAGCATAAGC	150	384	*56*
	eaeAR	CCACCTGCAGCAACAAGAGG	150		
nleB	nleB F	GGAAGTTTGTTTACAGAGACG	500	297	*19*
	nleB R	AAAATGCCGCTTGATACC	500		
espK1	EspK_F1	ATCAAAAGCGAAATCACACC	500	200	This report
	EspK_R1	TGTAATTTTTCACAGTTAATGACG	500		
stx[a]	Stx1/2-F	TTTGTYACTGTSACAGCWGAAGCYTTACG	1,000	132	*73*
	Stx1/2-R	CCCCAGTTCARWGTRAGRTCMACDTC	1,000		

[a] Degenerate nucleotide codes are Y (C, T), W (A, T), R (A, G), M (A, C), D (A, G, T), and S (C, G).

beads (6.3 μl each) for *E. coli* O26, O45, and O121 (Abraxis LLC, Warminster, PA), and (iii) a pool of 20 μl of IMS beads (6.3 μl each) for *E. coli* O103, O111, and O145 (Abraxis). Fifty microliters of recovered beads from the *E. coli* O157 IMS treatment was spread onto CHROMagar O157 (DRG Int.) with cefixime trihydrate (0.025 mg/liter), cefsulodin (5.0 mg/liter), and potassium tellurite (2.5 mg/liter) (CCT-CHROMagar O157) and incubated for 18 h at 37°C. Fifty microliters of recovered beads from the remaining two IMS treatments was spread onto STEC heart infusion washed blood agar with mitomycin C (0.5 μg/ml) (SHIBAM) and incubated for 18 h at 37°C. SHIBAM was prepared with 4% defibrinated sheep blood (Quad Five, Ryegate, MT) according to the methods of Feng et al. *(27)*. Five or fewer mauve colonies per CCT-CHROMagar O157 plate and ≤20 enterohemolytic phenotype colonies per SHIBAM plate were picked, streaked for isolation on 5% sheep blood agar (Remel, Lenexa, KS), and incubated for 15 h at 37°C. Isolated colonies were picked from the blood agar plates, suspended in 50 μl of ultrapure water, and heated at 95°C for 10 min for use as DNA template in the PCR assays. Individual DNA preparations from isolated colonies ($n = 6$) were pooled, and the pooled DNA was tested by single-plex PCR for *stx (50)*. When a pool was positive for *stx,* the DNA preparation from each isolate in the pool was individually tested with an 11-plex PCR assay. This 11-plex PCR assay included genes representing each of the EHEC-7 serogroups (*wzx, wbq,* or *rfbE*) plus stx_1, stx_2, and EHEC-hemolysin (*ehxA*) *(4),* which was modified by the use of primers for *eae* as described by Blanco et al. *(9)* and primers for the *wzx* gene of O111 as described by Noll et al. *(53)*.

Additional aliquots of broth enrichment culture of each fecal and hide and carcass surface sample were obtained and held at 4°C (24 to 96 h) until molecular screening assays (Atlas and NS) were completed (Fig. 1). Broth cultures identified as positive for *E. coli* O157:H7 with the Atlas test were subjected to O157 IMS by adding 20 μl of beads (Pickpen IMS, BioControl, Bellevue, WA) to 1 ml of broth culture, shaking at 900 rpm on a bench-top plate shaker for 10 min, and then separating the beads with the KingFisher processor. The IMS protocol included two wash steps in PBS-Tween, and the final captured beads from the IMS-culture treatment were suspended in 200 μl of PBS-Tween. The concentrated beads (50 and 1 μl) were spread plated onto two CHROMagar O157 plates supplemented with novobiocin (5.0 mg/liter) and potassium tellurite (1.0 mg/liter). Plates were incubated overnight at 37°C, and mauve colonies were picked and tested for *E. coli* O157:H7 by multiplex PCR as described by Hu et al. *(32)*.

A broth culture was suspected of containing an EHEC-6 strain when it was positive for non-O157 EHEC with the Atlas assay and/or NS test. Based on those results, IMS for each particular suspect-positive serogroup was conducted; hence, an enrichment broth culture was subjected to one to six IMS treatments depending on the screening results. One milliliter of each EHEC-6 suspect broth culture was subjected to IMS using individual specific O group IMS beads (Romer Laboratories, Union, MO). Captured beads from an IMS-culture treatment were diluted 1:10 and 1:100 and spiral plated with an Autoplate 4000 (Advanced Instruments, Norwood, MA) onto STEC differentiation agar (SDA) *(36)* and washed blood agar with 0.5 μg/ml mitomycin C (WBAM) *(64),* respectively. Plates were incubated overnight at 37°C. Suspect colonies on SDA plates were O-group specific shades of green and blue, and colonies on WBAM had an enterohemolytic phenotype. Four or fewer colonies per plate were picked and screened by multiplex PCR for *stx, eae, espK,* non-LEE genes (*nleB* and *nleF*), and subtilase (*subA*) (Table 1). All *E. coli* isolates from SDA and WBAM that were positive for both *stx* and *eae* were serogrouped using multiplex PCR to identify all EHEC-6 serogroups *(11)* (Fig. 1).

Detection of EHEC by NS. Enriched sample aliquots were tested for EHEC-7 using the NS test, which includes a PCR coupled with mass spectrometry. Based on NS data, a positive result for EHEC was defined as the concurrent detection of *stx,* targeted O-group single nucleotide polymorphisms, and a specific *eae* subtype in conjunction with the O group marker. Identifying combinations of *eae* subtypes and EHEC-7 O group markers were *eae*-β with O26; *eae*-ε with O45, O103, or O121; *eae*-γ2 with O111; and *eae*-γ1 with O145 or O157.

Detection of EHEC by Atlas. Enriched sample aliquots were tested for EHEC O157 and non-O157 EHEC using the Atlas assay. After enrichment, 1.2 ml of culture was transferred to a G2 (Roka Bioscience) room temperature lysis tube and placed in the Atlas system instrument for analysis using the STEC EG2 combo detection assay (Roka Bioscience). Samples were identified as negative, positive for EHEC O157, or positive for non-O157 EHEC.

Quantification of EHEC-7. Preenriched 1-ml sample aliquots were frozen in 500 μl of brain heart infusion broth with 50% glycerol at −80°C. Samples to be quantified were identified based on positive NS or culture results from postenriched samples. Preenriched samples were removed from −80°C and allowed to recover at room temperature for 2 h. Using an Eddy Jet 2 spiral plater (IUL Instruments, Königswinter, Germany), 50 μl of the recovered culture was spiral plated on Possé agar that had been modified by reducing the novobiocin (5.0 mg/liter) and potassium tellurite (0.5 mg/liter) (mPossé) as previously described *(63),* and plates were incubated at 37°C for 18 h. Based on the NS results, blue-purple and red-purple colonies were counted for samples positive for EHEC O26, O45, O103, O111, or O157, and blue-purple, red-purple, and green colonies were counted for samples positive for EHEC O121 or O145. EHEC O157 culture-positive samples were spiral plated on CCT-CHROMagar O157, and mauve colonies were enumerated. Up to 10 target colonies were picked per plate and heated at 95°C in 50 μl of water for use as DNA template. Colonies were tested with the 11-plex PCR assay *(4, 9, 53)*. Colonies positive for one of the EHEC-7 serogroup genes plus *stx* and *eae* were confirmed as an EHEC-7 strain, and the sample was counted as positive. The pathogen level in the sample was determined based on the proportion of target colonies on an individual plate that were confirmed EHEC-7 positive by the 11-plex PCR assay.

Characterization of EHEC isolates. H-antigen (*fliC*) typing for H2, H7, H8, H11, and H28 was done as previously described *(46)*. All other H-antigen typing was done at the Pennsylvania State *E. coli* Reference Center *(45)* or using the methods of Wang et al. *(71)*. Serogroups for EHEC-7–positive isolates were confirmed by latex agglutination tests (Abraxis). When EHEC isolates were not identified as an EHEC-7 serogroup, they were O:H serotyped at the Pennsylvania State University *E. coli* Reference Center using the methods of Ørskov et al. *(55)* and Machado et al. *(45)* or O serogrouped by agglutination testing with pooled and specific Statens Serum Institute antisera (Cedarlane Labs, Burlington, NC) according to the manufacturer's instructions. Isolates were tested by MRA PCR for genes associated with hemolytic uremic syndrome and foodborne outbreaks: *nleA, nleB, nleB2, nleC, nleD, nleE, nleF, nleG, nleG2-1, nleG2-3, nleG5-2, nleG6-2, nleG9, nleH1-1, nleH1-2,* and *ent*. Primers and conditions

TABLE 2. *Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic* E.coli *(EHEC) in fecal samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and Atlas STEC EG2 Combo Detection Assay*[a]

	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI
EHEC O26	1	1.01	0.09–9.90	1	1.01	0.09–9.90	NA[b]	NA	NA
EHEC O45	0			11	11.00	6.20–18.78	NA	NA	NA
EHEC O103	0			7	7.00	3.37–13.96	NA	NA	NA
EHEC O111	1	1.00	0.12–7.94	7	4.90	1.20–17.96	NA	NA	NA
EHEC O121	0			0			NA	NA	NA
EHEC O145	0			7	6.74	2.47–17.05	NA	NA	NA
EHEC O157	6	6.13	2.22–15.84	4	4.00	1.51–10.18	29	29.10	20.23–39.92
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	29	29.11	18.27–43.00
EHEC-6	2	2.10	0.45–9.26	24	23.59	13.86–37.21	NA	NA	NA
EHEC-7	7	6.54	2.40–16.63	26	25.90	16.08–38.94	NA	NA	NA
stx	20	20.42	9.50–38.54	90	90.00	82.40–94.53	NA	NA	NA
eae	31	31.40	22.06–42.53	83	83.00	74.34–89.16	NA	NA	NA

[a] Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date. $n = 100$ for each method.
[b] NA, not applicable.

were as previously described *(19)*. Isolates also were tested for putative virulence factor genes (*pagC, sen,* and *efa1*) and *eae* subtyped as previously described *(9, 37)*. The U.S. Food and Drug Administration *E. coli* identification array (ECID) *(35)* was used to determine whether isolates from different sample types of the same cattle type were the same strain.

Statistical analysis. The overall agreement on detection of EHEC groups, beyond that due to chance, between the culture-based method, the NS test, and the Atlas assay independent of sample type was determined by computing the Cohen κ coefficient and by the McNemar chi-square test *(23)*. The κ coefficient was interpreted on the scale of <0, 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8, and 0.81 to 1.0 as poor, slight, fair, moderate, substantial, or almost perfect agreement, respectively *(41)*.

Associations between the detection of an EHEC serogroup or virulence gene (presence or absence of a gene) in fecal samples (explanatory variables) with the presence of an EHEC serogroup or virulence gene on hide samples (dependent variables) were evaluated using generalized linear mixed models (GLMMs) in SAS version 9.3 (SAS Institute, Cary, NC). Similar models were fitted to estimate associations between EHEC serogroup or virulence gene detected in hide samples and their presence in carcass surface samples. An independent model was fitted for each EHEC serogroup and detection method. Mixed models were fitted using a residual pseudo-likelihood estimation, binary distribution, logit link, Kenward-Roger degrees of freedom approximation, and a random intercept for sampling date to account for the clustering effect of samples nested within date of sample collection. Mean probability estimates and 95% confidence intervals (CIs) were computed. P values of <0.05 were deemed significant.

Model-adjusted sample-level prevalence estimates and their 95% CIs were computed for all EHEC serogroups and virulence genes, based on each detection protocol, using GLMMs. Prevalence estimates were calculated from model intercepts using the formula $e^{\beta^\circ}/(1 + e^{\beta^\circ})$, where β° is the coefficient of the model intercept.

RESULTS

Detection of EHEC strains in fecal, hide, and carcass samples by culture-based methods, the NS test, and the Atlas assay. One hundred matched fecal, hide, and carcass samples were tested for EHEC by culture, NS, and Atlas. For the fecal samples, culture results revealed *stx*-positive isolates in 20%, *eae*-positive isolates in 31%, and EHEC-7 in 7% of samples. EHEC-7 strains were detected by NS in 26% of the fecal samples. The percentages of fecal samples testing positive with the NS test for each EHEC serogroup were 11% for O45, 7% for O103, 7% for O111, 7% for O145, 4% for O157, 1% for O26, and 0% for O121. With the Atlas assay, 29% of fecal samples tested positive for non-O157 EHEC and 29% tested positive for EHEC O157 (Table 2).

For hide samples, culture results revealed *stx*-positive isolates in 24%, *eae*-positive isolates in 38%, and EHEC-7 in 16% of samples. EHEC-7 strains were detected by NS in 65% of the hide samples. The percentages of hide samples testing positive with the NS test for each EHEC serogroup were 36% for O45, 23% for O145, 15% for O111, 10% for O103, 7% for O26, 7% for O157, and 3% for O121. With the Atlas assay, 46% of the hide samples tested positive for non-O157 EHEC and 51% tested positive for EHEC O157 (Table 3).

For carcass samples, culture results revealed *stx*-positive isolates in 3%, *eae*-positive isolates in 12%, and EHEC-7 in 1% of the samples. EHEC-7 strains were detected by NS in 7% of the carcass samples. The percentages of carcass samples testing positive with the NS test for each EHEC serogroup were 4% for O103, 3% for O26, 2% for O145, 1% for O157, and 0% for O45, O111, and O121. With the Atlas assay, 28% of the carcass samples tested positive for

TABLE 3. *Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic* E. coli *(EHEC) on hide samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and Atlas STEC EG2 Combo Detection Assay*[a]

	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI
EHEC O26	5	3.19	0.50–17.59	7	1.53	0.06–28.70	NA[b]	NA	NA
EHEC O45	0			36	27.10	10.77–53.36	NA	NA	NA
EHEC O103	0			10	10.00	5.47–17.60	NA	NA	NA
EHEC O111	1	1.00	0.14–6.75	15	12.03	4.76–27.26	NA	NA	NA
EHEC O121	1	1.01	0.09–9.90	3	1.71	0.14–17.99	NA	NA	NA
EHEC O145	0			23	18.59	7.71–38.45	NA	NA	NA
EHEC O157	9	6.65	1.85–21.22	7	6.04	2.05–16.51	51	57.03	32.43–78.59
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	46	38.29	17.27–64.83
EHEC-6	7	6.68	2.39–17.31	62	61.37	46.84–74.13	NA	NA	NA
EHEC-7	16	15.56	8.35–27.16	65	64.94	49.44–77.83	NA	NA	NA
stx	24	24.11	16.08–34.52	98	98.00	92.36–99.50	NA	NA	NA
eae	38	39.34	21.39–60.72	98	98.00	92.36–99.50	NA	NA	NA

[a] Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date. $n = 100$ for each method.
[b] NA, not applicable.

non-O157 EHEC and 3% tested positive for EHEC O157 (Table 4).

Model-adjusted prevalence estimates of EHEC in fecal, hide, and carcass samples. The model-adjusted prevalence estimates for EHEC serogroups and virulence genes as detected by culture, NS, and Atlas are shown in Tables 2 through 4.

Quantification of EHEC-7. Preenriched aliquots from 26 fecal, 67 hide, and 7 carcass samples that were NS or culture positive were spiral plated on mPossé to quantify EHEC-7, and 6 fecal, 9 hide, and 1 carcass sample were spiral plated on CCT-CHROMagar O157 to quantify EHEC O157. No EHEC-7 were quantifiable from the carcass samples. One hide sample was positive for EHEC O26, and one fecal sample and one hide sample were positive for EHEC O157 as detected by spiral plating, and of these, the EHEC O157 samples were quantifiable. EHEC O157 level was estimated as 3.9 log CFU/g in one fecal sample and 3.7 log CFU/100 cm^2 in one hide sample. EHEC isolates that were of a serogroup other than the EHEC-7 were quantified

TABLE 4. *Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic* E. coli *(EHEC) on preintervention carcass surface samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and Atlas STEC EG2 Combo Detection Assay*[a]

	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI	No. of positive samples	% mean prevalence	Prevalence 95% CI
EHEC O26	0			3	1.39	0.09–17.35	NA[b]	NA	NA
EHEC O45	0			0			NA	NA	NA
EHEC O103	0			4	4.00	1.51–10.18	NA	NA	NA
EHEC O111	0			0			NA	NA	NA
EHEC O121	0			0			NA	NA	NA
EHEC O145	0			2	2.00	0.50–7.64	NA	NA	NA
EHEC O157	1	1.00	0.12–7.93	1	1.00	0.14–6.75	3	3.00	0.97–8.89
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	28	28.01	19.48–38.50
EHEC-6	0			7	7.00	3.37–13.96	NA	NA	NA
EHEC-7	1	1.00	0.12–7.94	7	7.00	3.37–13.96	NA	NA	NA
stx	3	2.80	0.61–11.90	45	41.61	24.78–60.65	NA	NA	NA
eae	12	12.00	6.94–19.95	50	50.06	32.25–67.86	NA	NA	NA

[a] Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date. $n = 100$ for each method.
[b] NA, not applicable.

TABLE 5. *Distribution of virulence genes in enterohemorrhagic* E. coli *isolates from postenrichment cultures*

Serotype	Virulence genes	No. of detected MRA[a] genes	*pagC*	*sen*	*efa1*	No. of isolates			
						Feces	Hide	Carcass	Total
O26:[H11]	*stx*$_1$, *eae*-β1, *ehxA*	13	−	+	+	1	4	0	5
O26:[H11]	*stx*$_1$, *eae*-β1, *ehxA*	6	−	+	+	0	1	0	1
O84:[H2]	*stx*$_1$, *eae*-ζ, *ehxA*	6	+	+	−	0	0	1	1
O98:NM	*stx*$_1$, *eae*-ζ, *ehxA*	8	+	+	−	6	0	1	7
O109:[H10]	*stx*$_2$, *eae*-ι, *ehxA*	6	−	−	−	1	0	0	1
O111:[H8]	*stx*$_1$, *eae*-γ2, *ehxA*	9	+	+	+	1	1	0	2
O121:[H19]	*stx*$_2$, *eae*-ε, *ehxA*	7	+	+	+	0	1	0	1
O157:[H7]	*stx*$_2$, *eae*-γ1, *ehxA*	16	+	+	+	5	2	0	7
O157:[H7]	*stx*$_1$, *stx*$_2$, *eae*-γ1, *ehxA*	16	+	+	+	5	23	5	33
O177:[H25]	*stx*$_2$, *eae*-β1, *ehxA*	12	−	+	−	3	8	0	11

[a] MRA, molecular risk assessment.

in one fecal and three hide samples. O-nontypeable EHEC levels were estimated as 4.2 log CFU/g in one fecal sample. In hide samples, two EHEC O177 isolates were quantified at 3.7 and 2.8 log CFU/100 cm^2, and one EHEC O101 isolate was quantified at 3.6 log CFU/100 cm^2.

Characterization of EHEC-7 isolates. The results of molecular serotyping and testing for virulence genes in EHEC isolates are shown in Table 5. EHEC O157 isolates tested positive for all 16 genes in the MRA, and the non-O157 EHEC isolates tested positive for 6 to 13 of the 16 genes. Only EHEC-7 isolates tested positive for the EHEC factor for adherence (*efa1*).

Comparison of methods for the detection of EHEC-7. The McNemar chi-square test results comparing the culture-based method and NS results, independent of sample type, were significant ($P < 0.05$) or not applicable (at least one of the tests did not detect any positive samples). For EHEC O45, O103, O111, and O145, the McNemar chi-square test was not applicable because at least one of the tests did not detect any positive samples. The κ coefficient was calculated for the comparison of the culture-based method and NS for EHEC O26 ($\kappa = 0.58$, $P < 0.001$), EHEC O121 ($\kappa = 0.50$, $P < 0.001$), and EHEC O157 ($\kappa = 0.40$, $P < 0.001$), indicating fair to moderate agreement (Table 6). The McNemar chi-square test indicated significant disagreement ($P < 0.05$) between NS and Atlas and between culture and Atlas and no significant agreements.

TABLE 6. *Cohen κ coefficient and McNemar χ^2 analysis for agreement between a culture-based method and NeoSEEK STEC Detection and Identification test, independent of sample type*

Serogroup	κ		χ^2 (*P* value)
	Value	*P* value	
EHEC O26	0.58	<0.0001	0.059
EHEC O45	NA[a]	NA	NA
EHEC O103	NA	NA	NA
EHEC O111	0.16	<0.0001	<0.0001
EHEC O121	0.50	<0.0001	0.157
EHEC O145	NA	NA	NA
EHEC O157	0.40	<0.0001	0.317

[a] NA, not applicable (at least one of the tests did not detect any positive samples).

Associations of EHEC detection. Two EHEC O98 isolates from one fecal sample and one carcass sample from the same animal were inferred to be the same strain based on the ECID information. Similarly, two EHEC O157 isolates from one fecal sample and one carcass sample from the same animal were inferred to be the same strain. Detection of an EHEC serogroup in fecal samples was significantly associated with the detection of the corresponding EHEC serogroup in hide samples for EHEC O26 ($P < 0.001$), EHEC O111 ($P = 0.002$), EHEC O121 ($P < 0.001$), and EHEC-6 ($P = 0.029$) based on NS detection and for EHEC O121 ($P < 0.001$) based on detection by culture (Table 7).

TABLE 7. P *values for associations between the detection of an enterohemorrhagic* E. coli *(EHEC) serogroup or virulence gene in fecal samples and the presence of the same EHEC serogroup or virulence gene on hide samples using generalized linear mixed models*

Serogroup or gene in fecal samples	*P* values for comparisons of results for fecal and hide samples		
	Culture	NS[a]	Atlas[b]
EHEC O26	0.989	<0.001	NA[c]
EHEC O45	NA	0.973	NA
EHEC O103	NA	0.764	NA
EHEC O111	0.989	0.002	NA
EHEC O121	<0.001	<0.001	NA
EHEC O145	NA	0.139	NA
EHEC O157	0.418	0.323	0.262
Non-O157 EHEC	NA	NA	0.519
EHEC-6	0.985	0.029	NA
EHEC-7	0.952	0.251	NA
stx	0.625	0.998	NA
eae	0.373	0.242	NA

[a] NS, NeoSEEK Detection and Identification test.
[b] Atlas, Atlas STEC EG2 Combo Detection Assay.
[c] NA, not applicable.

No significant associations were found between detection of an EHEC serogroup in hide samples with the detection of an EHEC serogroup in carcass samples.

DISCUSSION

Management practices and cattle type may play a role in the prevalence of STEC in animals and their production environments *(6, 15, 17, 31, 61, 62, 68)*. In one study, a lower prevalence of STEC was detected in feedlot cattle than in dairy and range cattle *(17)*. In the present study, 65% of culled dairy cattle hides were positive for EHEC-7 by the NS test, whereas in a previous study >80% of feedlot cattle hides were positive by the same method *(63)*. Interventions such as high pressure water washes containing sanitizing agents (e.g., 1% cetylpyridinium chloride) that reduce bacterial levels on hides have been effective for reducing transfer of *E. coli* O157 onto carcasses during processing *(1, 10, 52)*. EHEC-7 prevalence on surfaces of preintervention carcasses of culled dairy cows was 7% by NS, which was similar to the findings for feedlot beef cattle *(63)*. However, these studies were done in different locations and different years, making it difficult to make reliable comparisons.

Other than our recent publication in beef feedlot cattle at harvest *(63)*, we are aware of only four publications that reported EHEC-6 data for cattle hides and carcasses based on culture-based protocols. In those four studies, prevalence estimates were based on proportion of samples positive instead of model-adjusted estimates. In two studies conducted at a beef export abattoir in Ireland, the source of the cattle (i.e., beef or dairy) was not reported, but 100 cm^2 of hides and carcasses were sampled, and very low EHEC-6 prevalence was detected. In one of the studies, which did not include IMS as part of the protocol, only EHEC O26 was detected, and this serogroup was found in only 2 (0.4%) of 450 carcasses and 0 of 450 hides *(49)*. In the other study from Ireland, IMS was used for EHEC O26, O103, O111, and O145, and of 402 hides and carcasses cultured, 1 hide was positive for O26 (0.2%) but no other EHEC-6 serogroups were found *(65)*. In one study that was conducted on cattle at an abattoir in Poland but did not specify whether the animals were of beef or dairy origin, 400 cm^2 of hide and carcass surface area were sampled, but the protocol did not include non-O157 IMS and no EHEC-6 isolates were obtained *(74)*. However, Arthur et al. *(1),* who cultured samples from 8,000 cm^2 of carcass surface from beef feedlot cattle in the Midwest United States and used a protocol that included enrichment followed by a colony blot screen for *stx* on agar plates, detected EHEC-6 in 4 (1.2%) of 334 carcass samples. These isolates included EHEC of serogroups O103, O121, and O145. These results suggest that based on culture, EHEC-6 is relatively low in prevalence, but prevalence estimates are higher in studies with larger sampling areas and protocols that include serogroup-specific IMS.

In addition to adequate sampling area and IMS, many other factors, such as variability in the carbohydrate source for fermentation and resistance to antimicrobials, affect the detection of non-O157 STEC or EHEC. Based on the variability of these organisms, the use of multiple agar media for isolation (as in our study) is recommended *(28, 33)*. In the present study, EHEC O157 was the most common serogroup isolated by culture because either it is truly the most prevalent or it is more easily detected because of the greater specificity of the media. Approximately half of the samples that tested positive for EHEC O157:H7 by NS were confirmed by culture, and significant agreement ($P < 0.001$) was observed between NS and culture for detection of EHEC O157:H7. However, most samples that tested positive for non-O157 EHEC by NS or Atlas did not test positive by culture. This finding is consistent with our previous observations *(63),* and improved agar media are needed for detection of non-O157 EHEC. The molecular screening methods may produce false-positive results for some samples, in which case improved specificity is needed. Discrepancies between molecular and culture-based methods for detection of EHEC-7 have been previously reported. Wang et al. *(72)* reported that 93.9% of veal calf hide samples were positive for EHEC-7 by NS but only 53.0% were positive by culture. Wasilenko et al. *(73)* used another molecular screen (BAX) and culture to test retail ground beef for EHEC-7. With the BAX method, 14 of 308 samples were positive for *stx, eae,* and at least one EHEC-7 serogroup, but culture confirmed the result for only 4 of these samples *(73)*. Because the NS test sample consists of DNA extracted from an aliquot of an enrichment broth culture, it is able to concurrently test thousands or even millions of bacterial cells in contrast to the culture-based method, which would have tested approximately 50 bacterial colonies from the same sample (Fig. 1). This difference in the size of the tested population could be one reason why the NS test might be more sensitive than culture. An advantage of culture, however, is the recovery of isolates, which may include other EHEC serogroups that could be useful in subsequent studies. For example, in the present study, EHEC O84, O98, O109, and O177 were isolated by culture. These EHEC serogroups have previously been isolated from cattle *(47, 58)* and have caused disease in humans *(7)*.

Only two samples had EHEC-7 level high enough to be quantified by the methods used. In both samples, the serogroup was EHEC O157 at high levels; the fecal sample contained 7,900 CFU/g, and the hide sample contained 100,000 CFU/100 cm^2. EHEC-7 were not detected in most preenrichment samples, suggesting they were at low levels in most samples and indicating the importance of enrichment for their detection. Current methods to quantify EHEC have relied upon real-time PCR assays for *eae* or *stx* and may overestimate the level of EHEC because these genes can be amplified from various microorganisms *(20)*. Recently, *E. coli* attaching and effacing gene–positive conserved fragment 1 (*ecf1*) has been used as a gene target for detection of STEC *(42)*, and *ecf1* and *eae* have been used as targets to enumerate EHEC directly in cattle feces *(43)*. However, this assay allows for enumeration of the total EHEC load and not specifically EHEC-7.

Previously, for most EHEC-7 serogroups no significant agreement was observed between the results of a culture-based method and the NS test *(63)*. In the present study, significant agreement in results was observed between the culture-based method and the NS test for three of the seven EHEC-7 serogroups; however, there was no significant

agreement in results beyond that due to chance between culture and the Atlas assay or the NS test and the Atlas assay. Atlas detects all EHEC, whereas NS detects only EHEC-7 and the culture-based method used in this study targeted EHEC-7 by using IMS beads directed against those seven serogroups. Therefore, Atlas results for only EHEC O157 could be compared with those obtained with NS and culture.

Hides can be contaminated by multiple sources, e.g., feces from one or multiple animals in lairage *(2)*. Significant association between the detection of an EHEC in fecal samples and detection of the same EHEC on hide samples was determined for EHEC O26, O111, O121, and EHEC-6 and supports the hypothesis that feces are a major source of hide contamination. Effective preharvest interventions that reduce carriage of EHEC in the intestines, e.g., vaccines *(6, 61, 62),* may reduce EHEC prevalence on hides.

In summary, these data were collected to determine the prevalence of EHEC-7 in culled dairy cattle by using multiple detection methods. Molecular screening assays detected more EHEC types than did the culture-based method. Although there was significant agreement for some EHEC-7 serogroups between the NS and culture-based method, continued improvement is needed for accurate detection and isolation of EHEC-7 in the matrices tested. Significant associations between EHEC detected in fecal samples and EHEC detected in hide samples supports the need to identify effective preharvest interventions to reduce EHEC contaminated hides. EHEC prevalence and estimated levels can be used to populate a quantitative microbial risk assessment model.

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REFERENCES

1. Arthur, T. M., G. A. Barkocy-Gallagher, M. Rivera-Betancourt, and M. Koohmaraie. 2002. Prevalence and characterization of non-O157 Shiga toxin–producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *Appl. Environ. Microbiol*. 68:4847–4852.
2. Arthur, T. M., J. M. Bosilevac, D. M. Brichta-Harhay, M. N. Guerini, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2007. Transportation and lairage environment effects on prevalence, numbers, and diversity of *Escherichia coli* O157:H7 on hides and carcasses of beef cattle at processing. *J. Food Prot*. 70:280–286.
3. Arthur, T. M., J. E. Keen, J. M. Bosilevac, D. M. Brichta-Harhay, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, X. Nou, and M. Koohmaraie. 2009. Longitudinal study of *Escherichia coli* O157:H7 in a beef cattle feedlot and role of high-level shedders in hide contamination. *Appl. Environ. Microbiol*. 75:6515–6523.
4. Bai, J., Z. D. Paddock, X. Shi, S. Li, B. An, and T. G. Nagaraja. 2012. Applicability of a multiplex PCR to detect the seven major Shiga toxin–producing *Escherichia coli* based on genes that code for serogroup-specific O-antigens and major virulence factors in cattle feces. *Foodborne Pathog. Dis*. 9:541–548.
5. Barkocy-Gallagher, G. A., T. M. Arthur, M. Rivera-Betancourt, X. Nou, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2003. Seasonal prevalence of Shiga toxin–producing *Escherichia coli,* including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J. Food Prot*. 66:1978–1986.
6. Besser, T. E., C. E. Schmidt, D. H. Shah, and S. Shringi. 2014. "Preharvest" food safety for *Escherichia coli* O157 and other pathogenic Shiga toxin–producing strains. *Microbiol. Spectr*. doi:10.1128/microbiolspec.EHEC-0021-2013.
7. Bettelheim, K. A. 2007. The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. *Crit. Rev. Microbiol*. 33:67–87.
8. Beutin, L., and P. Fach. 2014. Detection of Shiga toxin–producing *Escherichia coli* from nonhuman sources and strain typing. *Microbiol. Spectr*. doi:10.1128/microbiolspec.EHEC-0001-2013.
9. Blanco, M., J. E. Blanco, A. Mora, G. Dahbi, M. P. Alonso, E. A. González, M. I. Bernárdez, and J. Blanco. 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)–producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae-*ξ). *J. Clin. Microbiol*. 42:645–651.
10. Bosilevac, J. M., T. M. Arthur, T. L. Wheeler, S. D. Shackelford, M. Rossman, J. O. Reagan, and M. Koohmaraie. 2004. Prevalence of *Escherichia coli* O157 and levels of aerobic bacteria and *Enterobacteriaceae* are reduced when hides are washed and treated with cetylpyridinium chloride at a commercial beef processing plant. *J. Food Prot*. 67:646–650.
11. Bosilevac, J. M., and M. Koohmaraie. 2011. Prevalence and characterization of non-O157 Shiga toxin–producing *Escherichia coli* isolates from commercial ground beef in the United States. *Appl. Environ. Microbiol*. 77:2103–2112.
12. Bosilevac, J. M., B. E. Luedtke, T. M. Arthur, N. Kalchayanand, J. W. Schmidt, S.D. Shackelford, and T. L. Wheeler. 2013. Identification of bovine reservoirs of human pathogenic non-O157 Shiga toxin–producing *E. coli*. Report to the North American Meat Institute. Available at: http://www.namif.org/research/11-129/. Accessed 14 August 2015.
13. Brichta-Harhay, D. M., M. N. Guerini, T. M. Arthur, J. M. Bosilevac, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2008. *Salmonella* and *Escherichia coli* O157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States: an evaluation of prevalence and bacterial loads by immunomagnetic separation and direct plating methods. *Appl. Environ. Microbiol*. 74:6289–6297.
14. Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M Griffin, R. M. Hoekstra, and N. A. Strockbine. 2005. Non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis*. 92:1422–1429.
15. Callaway, T. R. 2010. Pre-harvest management controls and intervention options for reducing *Escherichia coli* O157:H7 shedding in cattle. Beef safety. Cattlemen's Beef Board and National Cattlemen's Beef Association. Available at: http://www.beefresearch.org/CMDocs/BeefResearch/Pre-harvest%20Control%20of%20E.%20coli%20Literature%20Review.pdf. Accessed 10 August 2015.
16. Chase-Topping, M., D. Gally, C. Low, L. Matthews, and M. Woolhouse. 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Natl. Rev. Microbiol*. 6:904–912.
17. Cobbold, R. N., D. H. Rice, M. Szymanski, D. R. Call, and D. D. Hancock. 2004. Comparison of Shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in Washington State. *Appl. Environ. Microbiol*. 70:4375–4380.
18. Cole, D., P. M. Griffin, K. E. Fullerton, T. Ayers, K. Smith, L. A. Ingram, B. Kissler, and R. M. Hoekstra. 2014. Attributing sporadic and outbreak-associated infections to sources: blending epidemiological data. *Epidemiol. Infect*. 142:295–302.
19. Coombes, B. K., M. E. Wickham, M. Mascarenhas, S. Gruenheid, B. B. Finlay, and M. A. Karmali. 2008. Molecular analysis as an aid to

assess the public health risk of non-O157 Shiga toxin–producing *Escherichia coli* strains. *Appl. Environ. Microbiol.* 74:2153–2160.
20. Conrad, C. C., K. Stanford, T. A. McAllister, J. Thomas, and T. Reuter. 2014. Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces. *J. Microbiol. Methods* 105:22–30.
21. Cray, W. C., Jr., and H. W. Moon. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 61:1586–1590.
22. Croxen, M. A., R. J. Law, R. Scholz, K. M. Keeney, M. Slodarska, and B. B. Finlay. 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiol. Rev.* 26:822–880.
23. Dohoo, I., W. Martin, and H. Stryhn. 2009. Veterinary epidemiologic research, 2nd ed. VER, Inc., Charlottetown, Prince Edward Island, Canada.
24. Ekong, P. S., M. W. Sanderson, and N. Cernicchiaro. 2015. Prevalence and concentration of *Escherichia coli* O157 in different seasons and cattle types processed in North America: a systematic review and meta-analysis of published research. *Prev. Vet. Med.* 121:74–85.
25. Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA* 97:2999–3003.
26. Etcheverría, A. I., N. L. Padola, M. E. Sanz, R. Polifroni, A. Krüger, J. Passucci, E. M. Rodríguez, A. L. Taraborelli, M. Ballerio, and A. E. Parma. 2010. Occurrence of Shiga toxin–producing *E. coli* (STEC) on carcasses and retail beef cuts in the marketing chain of beef in Argentina. *Meat Sci.* 86:418–421.
27. Feng, P., S. D. Weagant, and K. Jinneman. 2011. Diarrheagenic *Escherichia coli*. *In* R. I. Merker (ed.), Bacteriological analytical manual, 8th ed., rev. A. U.S. Food and Drug Administration, College Park, MD. Available at: http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm2006949.htm. Accessed 19 May 2015.
28. Gill, A., G. Huszczynski, M. Guthier, and B. Blais. 2014. Evaluation of eight agar media for the isolation of Shiga toxin–producing *Escherichia coli*. *J. Microbiol. Methods* 96:6–11.
29. Gould, L. H., R. K. Mody, K. L. Ong, P. Clogher, A. B. Cronquist, K. N. Garman, S. Lathrop, C. Medus, N. L. Spina, T. H. Webb, P. L. White, K. Wymore, R. E. Gierke, B. E. Mahon, P. M. Griffin, and Emerging Infections Program FoodNet Working Group. 2013. Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: epidemiological features and comparison with *E. coli* O157 infections. *Foodborne Pathog. Dis.* 10:453–460.
30. Gyles, C. L. 2007. Shiga toxin–producing *Escherichia coli*: an overview. *J. Anim. Sci.* 85:E45–E62.
31. Hancock, D. D., T. E. Besser, M. L. Kinsel, P. I. Tarr, D. H. Rice, and M. G. Paros. 1994. The prevalence of *Escherichia coli* O157.H7 in dairy and beef cattle in Washington State. *Epidemiol. Infect.* 113:199–207.
32. Hu, Y., Q. Zhang, and J. C. Meitzler. 1999. Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. *J. Appl. Microbiol.* 87:867–876.
33. Hussein, H. S., L. M. Bollinger, and M. R. Hall. 2008. Growth and enrichment medium for detection and isolation of Shiga toxin–producing *Escherichia coli* in cattle feces. *J. Food Prot.* 71:927–933.
34. Hussein, H. S., and T. Sakuma. 2005. Prevalence of Shiga toxin–producing *Escherichia coli* in dairy cattle and their products. *J. Dairy Sci.* 88:450–465.
35. Jackson, S. A., I. R. Patel, T. Barnaba, J. E. LeClerc, and T. A. Cebula. 2011. Investigating the global genomic diversity of *Escherichia coli* using a multi-genome DNA microarray platform with novel gene prediction strategies. *BMC Genomics* 12:349. doi:10.1186/1471-2164-12-349.
36. Kalchayanand, N., T. M. Arthur, J. M. Bosilevac, J. E. Wells, and T. L. Wheeler. 2013. Chromogenic agar medium for detection and isolation of *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from fresh beef and cattle feces. *J. Food Prot.* 76:192–199.
37. Karmali, M. A., M. Mascarenhas, S. Shen, K. Ziebell, S. Johnson, R. Reid-Smith, J. Isaac-Renton, C. Clark, K. Rahn, and J. B. Kaper. 2003. Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J. Clin. Microbiol.* 41:4930–4940.
38. Kase, J. A., A. Maounounen-Laasri, S. Insook, A. Lin, and T. S. Hammack. 2015. Comparison of eight different agars for the recovery of clinically relevant non-O157 Shiga toxin–producing *Escherichia coli* from baby spinach, cilantro, alfalfa sprouts and raw milk. *J. Food Prot.* 46:280–287.
39. Keen, J. E., and R. O. Elder. 2002. Isolation of Shiga-toxigenic *Escherichia coli* O157 from hide surfaces and the oral cavity of finished beef feedlot cattle. *J. Am. Vet. Med. Assoc.* 220:756–763.
40. Koohmaraie, M., T. M. Arthur, J. M. Bosilevac, M. Guerini, S. D. Shackelford, and T. M. Wheeler. 2005. Post-harvest interventions to reduce/eliminate pathogens in beef. *Meat Sci.* 71:79–91.
41. Landis, J. R., and G. G. Koch. 1977. The measurement of observed agreement for categorical data. *Biometrics* 33:159–174.
42. Livezey, K. W., B. Groschel, and M. M. Becker. 2015. Use of the *ecf1* gene to detect Shiga toxin–producing *Escherichia coli* in beef samples. *J. Food Prot.* 78:675–684.
43. Luedtke, B. E., J. L. Bono, and J. M. Bosilevac. 2014. Evaluation of real time PCR assays for the detection and enumeration of enterohemorrhagic *Escherichia coli* directly from cattle feces. *J. Microbiol. Methods* 105:72–79.
44. Luna-Gierke, R. E., K. Wymore, J. Sadlowski, P. Clogher, R. W. Gierke, M. Tobin-D'Angelo, A. Palmer, C. Medus, C. Nicholson, S. McGuire, H. Martin, K. Garman, P. M. Griffin, and R. K. Mody. 2014. Multiple-aetiology enteric infections involving non-O157 Shiga toxin–producing *Escherichia coli*—FoodNet, 2001–2010. *Zoonoses Public Health* 61:492–498.
45. Machado, J., F. Grimont, and P. A. D. Grimont. 2000. Identification of *Escherichia coli* flagellar types by restriction of the amplified *fliC* gene. *Res. Microbiol.* 151:535–546.
46. Madic, J., C. Peytavin de Garam, N. Vingadassalon, E. Oswald, P. Fach, E. Jamet, and F. Auvray. 2010. Simplex and multiplex real-time PCR assays for the detection of flagellar (H-antigen) *fliC* alleles and intimin (*eae*) variants associated with enterohaemorrhagic *Escherichia coli* (EHEC) serotypes O26:H11, O103:H2, O111:H8, O145:H28 and O157:H7. *J. Appl. Microbiol.* 109:1696–1705.
47. Mekata, H., A. Iguchi, K. Kawano, Y. Kirino, I. Kobayashi, and N. Misawa. 2014. Identification of O serotypes, genotypes, and virulotypes of Shiga toxin–producing *Escherichia coli* isolates, including non-O157 from beef cattle in Japan. *J. Food Prot.* 77:1269–1274.
48. Mir, R. A., T. A. Weppelmann, M. Kang, T. M. Bliss, N. DiLorenzo, G. C. Lamb, S. Ahn, and K. C. Jeong. 2015. Association between animal age and the prevalence of Shiga toxin–producing *Escherichia coli* in a cohort of beef cattle. *Vet. Microbiol.* 175:325–331.
49. Monaghan, Á., B. Byrne, S. Fanning, T. Sweeny, D. McDowell, and D. J. Bolton. 2012. Serotypes and virulotypes of non-O157 Shiga-toxin producing *Escherichia coli* (STEC) on bovine hides and carcasses. *Food Microbiol.* 32:223–229.
50. Monday, S. R., A. Beisaw, and P. C. Feng. 2007. Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR. *Mol. Cell. Probes* 21:308–311.
51. Moxley, R. A. 2003. Detection and diagnosis of *Escherichia coli* O157:H7 in food-producing animals, p. 143–154. *In* M. E. Torrence and R. E. Isaacson (ed.), Microbial food safety in animal agriculture: current topics. Iowa State University Press, Ames.
52. Moxley, R. A., and G. R. Acuff. 2014. Peri- and postharvest factors in the control of Shiga toxin–producing *Escherichia coli* in beef. *Microbiol. Spectr.* doi:10.1128/microbiolspec.EHEC-0017-2013.
53. Noll, L. W., P. B. Shridhar, D. M. Dewsbury, X. Shi, N. Cernicchiaro, D. G. Renter, and T. G. Nagaraja. 2015. A comparison of culture- and PCR-based methods to detect six major non-O157 serogroups of

Shiga toxin–producing *Escherichia coli* in cattle feces. *PLoS One* 10:e0135446.

54. Ochman, H., and R. K. Selander. 1984. Standard reference strains of *Escherichia coli* from natural populations. *J. Bacteriol.* 157:690–693.
55. Ørskov, I., F. Ørskov, B. Jann, and K. Jann. 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. *Bacteriol. Rev.* 41:667–710.
56. Paton, A. W., and J. C. Paton. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA,* rfb_{O111}, and rfb_{O157}. *J. Clin. Microbiol.* 36:598–602.
57. Paton, A. W., and J. C. Paton. 2005. Multiplex PCR for direct detection of Shiga toxigenic *Escherichia coli* strains producing the novel subtilase cytotoxin. *J. Clin. Microbiol.* 43:2944–2947.
58. Renter, D. G., J. G. Morris, Jr., J. M. Sargeant, L. L. Hungerford, J. Berezowski, T. Ngo, K. Williams, and D. W. Acheson. 2005. Prevalence, risk factors, O serogroups, and virulence profiles of Shiga toxin–producing bacteria from cattle production environments. *J. Food Prot.* 68:1556–1565.
59. Sargeant, J. M., M. W. Sanderson, R. A. Smith, and D. D. Griffin. 2004. Associations between management, climate, and *Escherichia coli* O157 in the faeces of feedlot cattle in the Midwestern USA. *Prev. Vet. Med.* 66:175–206.
60. Schmidt, J. W., T. M. Arthur, J. M. Bosilevac, N. Kalchayanand, and T. L. Wheeler. 2012. Detection of *Escherichia coli* O157:H7 and *Salmonella enterica* in air and droplets at three U.S. commercial beef processing plants. *J. Food Prot.* 75:2213–2218.
61. Smith, D. R. 2014. Cattle production systems: ecology of existing and emerging *Escherichia coli* types related to foodborne illness. *Annu. Rev. Anim. Biosci.* 2:445–468.
62. Smith, D. R. 2014. Vaccination of cattle against *Escherichia coli* O157:H7. *Microbiol. Spectr.* doi:10.1128/microbiolspec.EHEC-0006-2013.
63. Stromberg, Z. R., N. W. Baumann, G. L. Lewis, N. J. Sevart, N. Cernicchiaro, D. G. Renter, D. B. Marx, R. K. Phebus, and R. A. Moxley. 2015. Prevalence of enterohemorrhagic *Escherichia coli* O26, O45, O103, O111, O121, O145, and O157 on hides and pre-intervention carcass surfaces of feedlot cattle at harvest. *Foodborne Pathog. Dis.* 12:631–638.
64. Sugiyama, K., K. Inoue, and R. Sakazaki. 2001. Mitomycin-supplemented washed blood agar for the isolation of Shiga toxin producing *Escherichia coli* other than O157:H7. *Lett. Appl. Microbiol.* 33:193–195.
65. Thomas, K. M., M. S. McCann, M. M. Collery, A. Logan, P. Whyte, D. A. McDowell, and G. Duffy. 2012. Tracking verocytotoxigenic *Escherichia coli* O157, O26, O111, O103 and O145 in Irish cattle. *Int. J. Food Microbiol.* 153:288–296.
66. U.S. Department of Agriculture, Food Safety and Inspection Service. 2005. Availability of specialized methodologies for incident investigation teams. FSIS notice 50-05. Available at: http://www.fsis.usda.gov/wps/wcm/connect/a5e3b125-ec50-4a28-bd4f-8bf882362069/IIT_Methodology_for_Ecoli.pdf?MOD=AJPERES&CACHEID=78354479-1cc8-4d32-9fee-fb01ba929268. Accessed 12 May 2015.
67. U.S. Department of Agriculture, Food Safety and Inspection Service. 2014. Detection and isolation of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) from meat products and carcass and environmental sponges, sect. 5B.05. *In* Microbiology laboratory guidebook. Available at: http://www.fsis.usda.gov/wps/wcm/connect/7ffc02b5-3d33-4a79-b50c-81f208893204/MLG-5B.pdf?MOD=AJPERES. Accessed 12 May 2015.
68. U.S. Department of Agriculture, Food Safety and Inspection Service. 2014. Pre-harvest management controls and intervention options for reducing Shiga toxin–producing *Escherichia coli* shedding in cattle: an overview of current research. Available at: http://www.fsis.usda.gov/wps/wcm/connect/d5314cc7-1ef7-4586-bca2-f2ed86d9532f/Reducing-Ecoli-Shedding-in-Cattle.pdf?MOD=AJPERES. Accessed 8 August 2015.
69. U.S. Department of Agriculture, National Agricultural Statistics Service. 2015. Livestock slaughter 2014 summary. Available at: http://www.usda.gov/nass/PUBS/TODAYRPT/lsan0415.pdf. Accessed 12 May 2015.
70. Wang, F., Q. Yang, J. A. Kase, J. Meng, L. M. Clotilde, A. Lin, and B. Ge. 2013. Current trends in detecting non-O157 Shiga toxin–producing *Escherichia coli* in food. *Foodborne Pathog. Dis.* 10:665–677.
71. Wang, L., D. Rothemund, H. Curd, and P. R. Reeves. 2003. Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. *J. Bacteriol.* 185:2936–2943.
72. Wang, R., M. Koohmaraie, B. E. Luedtke, T. L. Wheeler, and J. M. Bosilevac. 2014. Effects of in-plant interventions on reduction of enterohemorrhagic *Escherichia coli* and background indicator microorganisms on veal calf hides. *J. Food Prot.* 77:745–751.
73. Wasilenko, J. L., P. M. Fratamico, N. Narang, G. E. Tillman, S. Ladely, M. Simmons, and W. C. Cray, Jr. 2012. Influence of primer sequences and DNA extraction method on detection of non- O157 Shiga toxin–producing *Escherichia coli* in ground beef by real-time PCR targeting the *eae, stx,* and serogroup-specific genes. *J. Food Prot.* 75:1939–1950.
74. Wieczorek, K., L. Beutin, and J. Osek. 2011. Rare VTEC serotypes of potential zoonotic risk isolated from bovine hides and carcasses. *Vet. Rec.* 168:80.