SUPPLEMENT

Verotoxin-Producing *Escherichia coli* in Culled Beef Cows Grazing Rangeland Forages

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The objective of this study was to assess prevalence of verotoxin-producing *Escherichia coli* (VTEC) in culled beef cows at the time of shipping to slaughter. Feces were collected from 82 cows on eight Nevada ranches during fall and winter (from September to January) after grazing rangeland forages. A random sample (n = 154) of potential VTEC isolates were tested for verotoxicity and were screened for the presence (polymerase chain reaction) and expression (VTEC-reversed passive latex agglutination assay) of the toxin genes (i.e., VT1 and VT2). Seventeen isolates from four ranches were VTEC. Of these, four had the VT1 gene, five had the VT2 gene, seven had both genes, and one did not have either gene despite its toxicity to Vero cells. Except for one isolate (i.e., untypeable that reacted with VT1-latex beads without having VT1 gene), the genotype and phenotype data of the VTEC isolates matched. Another isolate (O8:H$^-$ [nonmotile]) was verotoxic, but neither had nor expressed the toxin genes. Of the 17 isolates, four (from one cow) were O157:H7, 11 (from five cows on three ranches) were non-O157:H7 (two O8:H$^-$, three O105:H$^-$, three O116:H$^-$, and three O141:H$^-$), and two were untypeable. Because some of these VTEC serotypes (i.e., O8:H$^-$, O141:H$^-$, and O157:H7) are known to cause human illnesses, it is beneficial to identify VTEC-positive cows before slaughter. This is a critical step in any pre- or post-harvest strategy to minimize the risk of beef contamination with such pathogens. Exp Biol Med 228:352–357, 2003

Key words: *Escherichia coli*; beef cattle; culled cows; verotoxins; food-borne pathogens

Outbreaks of human illnesses due to consumption of contaminated foods such as ground beef are on the rise due to changes in food production and its processing methods, globalization of food supply, new packaging technologies, and changing eating habits (1, 2). In the past two decades, it has become evident that verotoxin-producing *Escherichia coli* (VTEC) such as O157:H7 can contaminate beef and its products and cause a variety of human illnesses (2, 3). The illnesses (4, 5) range from mild diarrhea to severe hemorrhagic colitis with some patients developing a life-threatening disease known as hemolytic uremic syndrome (HUS). These illnesses result from verotoxins (VT1 and VT2) that resemble those produced by *Shigella dysentariae* (6) and are known for their cytotoxic effects on African-Green Monkey kidney (Vero) cells in cultures (7). These toxins are different proteins, encoded by different genes, and have similar toxic effects (8). VTEC can produce VT1, VT2, or both toxins (9), and either toxin can cause human illnesses (10).

VTEC such as O157:H7 (11–14) or non-O157:H7 (15, 16) have been more prevalent in cattle than in other animals. This may explain why many outbreaks of food-borne illnesses have been traced to consumption of undercooked ground beef in the United States (17–19) and in other countries (20, 21). Because initial VTEC outbreaks in the United States were attributed to contamination of ground beef with *E. coli* O157:H7, most studies have focused only on the prevalence of this VTEC serotype in beef and dairy cattle (22). However, in the past decade several human illness outbreaks have been attributed to non-O157:H7 VTEC such as O104:H21 in Montana (23) and members of the serogroup O6 and O26 in Minnesota (8). These outbreaks prompted Tarr *et al.* (24) and Acheson *et al.* (25) to describe non-O157:H7 VTEC infections and to express concerns that

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such pathogens may pose an underestimated threat to public health in the United States.

Members of the O6 serogroup were isolated from ground beef samples in Washington State (12). These VTEC strains have been associated with HUS cases in France (26). Screening of beef (27) and dairy (28) herds in Nevada also revealed the presence of O157:H7 and non-O157:H7 (i.e., members of the serogroups O6, O26, O39, and O113) VTEC in cattle feces. Earlier U.S. estimates indicated that non-O157:H7 VTEC have been responsible for 5%–15% of the HUS cases (29). In the past decade, non-O157:H7 VTEC (e.g., O26, O103, O111, O118, O145, and O166) have caused as many as 10%–30% of the sporadic cases of HUS in Germany (30–32), Italy (33), the United Kingdom (34), France (35), and Japan (36). Because of this, it is critical to screen cattle, especially those that enter the food chain as ground beef (i.e., culled cows) for VTEC in general. Therefore, the objective of this study was to assess VTEC prevalence in culled beef cows that were grazing Nevada rangeland forages.

Materials and Methods

Animals and Sample Collection. One fecal grab sample was collected from the rectum of each of 82 culled beef cows (8 to 12 years old) at the time of shipping to slaughter during fall and winter (from September to January). The cows were Angus, Hereford, or their crosses, and represented eight Nevada ranches (i.e., A, B, C, D, E, F, G, and H) with an average herd size of 600 cows, of which 10%–20% are culled annually because of production, reproduction, or health problems. Only healthy culled cows were selected for this study. The cows grazed meadow regrowth of rangeland forages (i.e., crested wheatgrass [*Agropyron desertorum*], bromegrass [*Bromus inermis*], and tall fescue [*Festuca arundinacea*]) with supplementation of alfalfa hay (*Medicago sativa*) only during winter. Each fecal sample was placed in a sterile Whirl-pak bag (Nasco, Modesto, CA), and the bags were shipped on ice to our laboratory for analysis. Sample processing began between 24 and 36 hr after collection.

Bacteriological Analysis. Initial selection of *E. coli* isolates to be screened for VTEC was conducted by adding 1 g of fresh feces to 25 ml of enrichment medium (i.e., tryptic soy broth [TSB]; [Hardy Diagnostics, Santa Maria, CA] containing 20 μg/ml novobiocin [Sigma, St. Louis, MO] and 40 μg/ml vancomycin [Sigma]). The feces and medium were mixed vigorously, and the suspension then was incubated at 25°C for 20 min to allow for antibiotic selection. The optimal dilutions for plating were established by preliminary experiments. Fecal suspensions were serially diluted to 10^{-2}, plated in duplicate onto sorbitol-MacConkey (SMAC) agar (Hardy Diagnostics) containing novobiocin (20 μg/ml) and vancomycin (40 μg/ml), and incubated at 37°C for 18 hr. Fecal suspensions were then incubated at 37°C for 18 hr with continuous shaking. The enriched fecal suspensions were serially diluted to 10^{-7}, and the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated in duplicate onto SMAC agar containing novobiocin (20 μg/ml) and vancomycin (40 μg/ml). The plates were incubated at 37°C for 18 hr. All colonies were enumerated by using a Bantex Model 900A illuminated colony counter (American Bantex, Burlingame, CA).

Nonsorbitol-fermenting bacteria (i.e., white colonies) on SMAC plates were subcultured to 4-methylumbelliferyl-β-D-glucuronide (MUG) MacConkey (MMUG; Hardy Diagnostics) agar grid plates. If a SMAC plate contained 10 or less sorbitol-negative (white) colonies, all were transferred to MMUG plates. However, if the SMAC plate had greater than 10 white colonies, a subset (11) of them (i.e., 10%) was randomly selected and transferred to MMUG plates. MMUG plates were incubated at 37°C for 18 hr and were observed on a UV light box (Fotodyne, New Berlin, WI). Results were recorded for MUG positive or MUG negative (i.e., blue fluorescence or no fluorescence under UV light, respectively) and lactose fermentation (11, 37, 38).

Verotoxin Production. The isolates (i.e., sorbitol negative/MUG negative or sorbitol negative/MUG positive) collected (n = 154) from the cows were tested for verotoxicity. The isolates were grown in 5 ml of TSB at 37°C for 18 hr. The cultures were centrifuged (3000*g* for 10 min) and the supernatants were filtered through 0.2-μm sterile syringe filters (ISC BioExpress, Kaysville, UT). The sterile supernatant was used for determination of verotoxicity (7, 38). Negative controls consisting of Eagle's minimal essential medium (Mediatech, Inc., Herndon, VA), TSB medium, and non-VTEC O157:H7 (ATCC 43888) were analyzed with each set of plates. In addition, each set of plates contained a positive control panel of supernatants from three *E. coli* O157:H7 isolates known to produce only VT1 (ATCC 43890), only VT2 (ATCC 43889), or both toxins (ATCC 43895). VTEC-positive and VTEC-negative isolates were determined by absence or presence of a confluent monolayer, respectively. Results were recorded after 24 and 48 hr of incubation.

Characterization of Isolates. The isolates that were toxic to Vero cells (n = 17) were confirmed as *E. coli* by biochemical tests using the API 20E Identification System (bioMerieux, Inc., Hazelwood, MO).

Detection of the Verotoxin Genes. The verotoxin genotype of the 154 isolates collected was confirmed by polymerase chain reaction (PCR). In a single tube, a 50-μl PCR reaction mixture was prepared using MasterTaq kit (Eppendorf Scientific, Westbury, NY) following the manufacturer's instructions (5× TaqMaster PCR enhancer, 10× Taq buffer with 0.15 m*M* $MgCl_2$, 0.1 μl each of 100 m*M* nucleotide, and 1.0 unit Taq polymerase). One hundred nanograms of both forward and reverse primers were used for each reaction. Whole cells from each bacterial culture (1 μl of liquid culture) were added to the PCR mixture instead of purified nucleic acids. Oligonucleotide primers were commercially prepared (Life Technologies/GIBCO-BRL, Rockville, MD). The sequence of the primers and the length

of their predicted products are listed in Table I. The PCR was performed using a Mastercycler Gradient thermocycler (Eppendorf Scientific) following the temperature program previously reported (39). Amplified DNA fragments were visualized by gel electrophoresis on 1% agarose-containing ethidium bromide (10 mg/ml).

Sequencing of PCR Products. PCR products of the correct size were excised and purified using the GeneClean kit (Bio101 Inc., La Jolla, CA) according to the manufacturer's instructions. DNA was dried in a SpeedVac (Savant, Farmingdale, NY) and was resuspended in sterile deionized water as needed for sequencing. For automatic sequencing, an Applied Biosystems DNA sequencer (ABI Prism 310 Genetic Analyzer; Perkin-Elmer, Foster City, CA) and a dideoxy terminator cycle sequencing kit (Perkin-Elmer) were used following the manufacturer's instructions. A 10-μl PCR reaction was set up according to the manufacturer's instructions. Briefly, terminator reaction mix, purified PCR product (10–30 ng), and sequencing primer (15 ng) were used for the PCR reaction. The PCR mixture was placed in a Mastercycler Gradient thermocycler (Eppendorf Scientific) programmed to 94°C/10 s, 50°C/5 s, and 60°C/4 min for 25 cycles. Each sample was ethanol precipitated (1 μl of 3 *M* sodium acetate [pH 5.2] and 25 μl of 100% ethanol) followed by drying. The pellet was resuspended in 15 μl of template suppression reagent (Perkin-Elmer). The samples were denatured at 95°C for 2 min, quick cooled on ice, transferred to sequencer tubes, and stored at −20°C until sequencing.

Expression of the Verotoxin Genes. The VTEC-Reversed Passive Latex Agglutination (VTEC-RPLA) assay (Denka Seiken Co., Tokyo, Japan) was performed according to the manufacturer's instructions. Isolates were grown in 5 ml of TSB at 37°C for 18 hr without shaking. From this culture, 1 ml was placed in a 1.5-ml Eppendorf tube and was centrifuged at 3000*g* for 20 min. In 96-well V-bottom microtiter plates (Costar, Corning, NY), culture supernatants were mixed 1:1 (25 μl) with the supplied diluent. An equal volume of latex particles sensitized with rabbit polyclonal anti-VT1 or anti-VT2 immunoglobulin G antibody was mixed in appropriate wells. Plates were covered, incubated at room temperature, and examined for latex agglutination after 18 hr. The positive and negative control toxins supplied with the kit were run with each assay. A positive result was recorded when agglutination in the sample well was two levels above the control well.

Serotyping of VTEC Isolates. The VTEC isolates were serotyped at the *Escherichia coli* Reference Center (Pennsylvania State University, University Park, PA).

Results

Characterization of the VTEC Isolates. Based on the microbiological methods (i.e., sorbitol fermentation and β-glucuronidase activity), 154 initial isolates representing samples from each of the eight ranches were selected and screened for verotoxicity. Seventeen isolates (i.e., 11% of the total tested) were confirmed to be verotoxic (Table II). These isolates were from fecal samples collected from culled cows representing four of the eight ranches. Based on the API identification system (bioMerieux, Inc.), the 17 verotoxic isolates were confirmed as *E. coli*. Due to the large numbers of bacteria present in bovine feces, we limited initial selection and screening of potential VTEC isolates to sorbitol-negative colonies because classic O157:H7 demonstrate these characteristics. To increase the probability of detecting sorbitol-negative non-O157:H7 VTEC, we did not limit our selection to MUG-negative isolates (a second unique characteristic of O157:H7). Because the identification of sorbitol negative is subjective (based on the color of colonies on SMAC agar), 10 of our isolates (Table II) were confirmed biochemically as sorbitol positive by using the API identification system. The VTEC isolates (Table II) illustrated various combinations of sorbitol fermentation and β-glucuronidase activity.

Detection of the Verotoxin Genes. Determination of the genotype of the 17 VTEC isolates by PCR (Table II) revealed that four isolates (from Ranch A) had only the VT1 gene, five isolates (three from Ranch B and two from Ranch C) had only the VT2 gene, and seven isolates (two, one, three, and one from Ranches A, B, C, and D, respectively) had both VT1 and VT2 genes. One isolate from Ranch C was toxic to Vero cells but did not have either VT1 or VT2 genes. The sequences of the PCR products from the 16 VTEC isolates (i.e., having VT1 [$n = 4$], VT2 [$n = 5$], or both toxin genes [$n = 7$]) matched the published ones (40, 41).

Expression of the Verotoxin Genes. The VTEC-RPLA kit (Denka Seiken Co.) was used to examine expression of the toxin genes in the 17 VTEC isolates (Table II). Except for one isolate, the PCR results (i.e., presence of the toxin genes) corresponded with the RPLA data (i.e., expression of the toxin genes). Two isolates from Ranch C (i.e., untypeable ones) reacted with the VT1-latex beads but did not have the VT1 gene based on PCR. Interestingly, another

Table I. Primer Sequences and Predicted Amplification Length of PCR Products

Primer	Direction	Sequence (5′–3′)	Product size (base pairs)	Reference
VT1	Forward	CAGTTAATGTGGTGGCGAA	475	56
	Reverse	CACAGACTGCGTCAGTGAGG		
VT2	Forward	TTAACCACACCCCACCGGCAGTTA	346	39
	Reverse	GCTCTGGATGCATCTCTGGT		

Table II. Characteristics of VTEC Isolates Detected in Feces of Culled Beef Cows

Ranch	Number of isolates	Characteristics								
		Biochemical		Toxicology	Genotype			Phenotype[a]		Serotype
		Sorb[b]	MUG[c]	Vero[d]	VT1	VT2	Sequence[e]	VT1	VT2	
A	4	−	−	+	+	−	+	+	−	O157:H7
B	2	−	+	+	+	+	+	+	+	O116:H^{-f}
	3	+	+	+	−	+	+	−	+	O141:H$^-$
C	1	+	−	+	+	+	+	+	+	O8:H$^-$
	1	+	+	+	−	−	−	−	−	O8:H$^-$
	3	+	+	+	+	+	+	+	+	O105:H$^-$
	2	+	+	+	−	+	+	+	+	Untypeable
D	1	−	+	+	+	+	+	+	+	O116:H$^-$

[a] Expression of the toxin genes determined by the RPLA-VTEC kit (Denka Seiken Co.).
[b] Sorbitol fermentation.
[c] 4-Methylumbelliferyl-β-D-glucuronide.
[d] Toxicity to vero cells (7).
[e] Sequence of PCR product matched published sequence (40, 41).
[f] Nonmotile.

isolate from Ranch C (i.e., O8:H$^-$ [nonmotile]) was toxic to Vero cells, but neither had nor expressed the toxin genes (Table II).

Serotyping of VTEC Isolates. Results of serotyping the VTEC isolates (Table II) revealed that four (from one cow) were O157:H7, 11 (from five cows) were non-O157:H7, and two (from one cow) could not be serotyped (i.e., unreactive with the "O" and "H" monovalent antisera available). One-half of the ranches tested had cows harboring VTEC. *E. coli* O157:H7 was detected only on one ranch, whereas non-O157:H7 VTEC (i.e., O8:H$^-$, O105:H$^-$, O116:H$^-$, and O141:H$^-$) were detected on three ranches.

Discussion

Of the 97.3 million cattle in the United States (42), approximately six million cows are culled annually due to decreased production, health problems, or reproductive inefficiency. Because these cows enter the food chain as ground beef (43), it is important to understand their carriage of VTEC. Identification of VTEC-positive cows before slaughter may be beneficial, especially if pre- and postharvest control measures are adopted to minimize ground beef contamination with such pathogens. Although the pathways for contamination of ground beef are still not well understood, a positive correlation between prevalence of VTEC in culled cows and in their ground beef was established (44). Rice *et al.* (44) examined culled cows on the farm (205 cows from 19 herds) and at slaughter (103 cows from 15 herds) in Idaho, Oregon, and Washington State for *E. coli* O157:H7. Results showed that 3.4% and 3.9% of the cows tested on the farm or at slaughter, respectively, were positive for *E. coli* O157:H7. These data suggest a higher prevalence rate of *E. coli* O157:H7 in culled cows than in younger cattle (1%–2%) as previously reported (11, 45, 46).

Potential microbiological hazards for food-borne illnesses from culled dairy cows are similar to those of culled beef cows because they also enter the food chain as ground beef (43). Prevalence rates of *E. coli* O157:H7 in culled dairy cows, however, have been variable. In a Canadian study, a 0.5% prevalence rate of this pathogen was reported in culled dairy cows (47). A much higher VTEC prevalence rate (16.1%) was found in the United Kingdom (48) when a large number (1666) of culled dairy cows were tested at slaughter over a 1-year period. Further testing of VTEC isolates revealed that 86.3% were O157:H7 and 19.7% were non-O157:H7. In contrast, a U.S. investigation (49) of *E. coli* O157:H7 prevalence in 80 culled cows on the farm (fresh feces) or at slaughter (hide surface sponge samples) revealed its absence. Based on our data (Table II) and those of others (48), isolates of non-O157:H7 VTEC may have been present in the National Cattlemen's Beef Association study (49) but were never tested.

Of our isolates (Table II), four were *E. coli* O157:H7 and three were O105:H$^-$. Between 1982 and 1996, *E. coli* O157:H7 was reported to be the cause of 40 major human illness outbreaks associated with consumption of contaminated ground beef in the United States (50). Two of our non-O157:H7 VTEC isolates (i.e., O8:H$^-$ and O141:H$^-$) were detected in humans affected by outbreaks of food-borne illnesses (51, 52). A member of the VTEC serogroup O105 (i.e., O105:H18) was isolated from healthy cattle and sick (HUS) humans in Spain (53) and Canada (54). Two of our VTEC isolates (Table II) were O116:H$^-$ that have been isolated from cattle in the past but were not associated with any food-borne illness outbreaks (15). Three *E. coli* O141:H$^-$ isolates were also found in our cows (Table II). Interestingly, these VTEC isolates are common in swine feces (55) and did not appear be isolated from cattle previously.

Our results indicated that testing culled beef cows at the time of shipping to slaughter revealed the presence of several VTEC serotypes in the cows' feces. Of these, several serotypes (i.e., O8:H$^-$, O141:H, and O157:H7) are known to cause human illnesses. Because culled beef cows enter the

food chain as ground beef, determination of VTEC-positive cows before shipping to slaughter can provide significant benefits to the beef industry. Isolation of such cows and possibly subjecting them to pre- and/or post-harvest control measures would assure safety of their beef and the beef from other animals slaughtered and processed at the same packing facilities. The preharvest (e.g., water trough cleaning, use of feed additives or probiotics, proper manure handling, changing feeding regime, and feeding specific dietary ingredients) and postharvest (e.g., trimming, spraying with sanitizers, and hot-water washing of the carcass) control methods have been summarized and evaluated previously (22). However, efficacy validation and cost/benefit evaluation of the control methods of high potential, especially those at the farm level, remain to be determined. Enforcing the preharvest control point and improving the postharvest control methods should maintain a competitive beef industry and reduce consumer safety concerns.

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