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Prevalence and Molecular Characterization of *Escherichia coli* O157:H7 in Bulk Tank Milk and Fecal Samples from Cull Cows: A 12-Month Survey of Dairy Farms in East Tennessee

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ABSTRACT

A study on the prevalence of *Escherichia coli* O157:H7 was conducted on 30 dairy farms in east Tennessee between May 2000 and April 2001. This pathogen was isolated from 8 of 30 (26.7%) dairy farms at various sampling times. A total of 415 fecal samples from cull dairy cows and 268 bulk tank milk samples were analyzed. Overall, 10 of 683 (1.46%) samples (2 of 268 [0.75%] milk samples and 8 of 415 [1.93%] fecal samples) tested positive for *E. coli* O157:H7. *Food and Drug Administration Bacteriological Analytical Manual* protocols were used for the conventional isolation and confirmation of *E. coli* O157:H7. Samples were shake cultured (150 rpm) at 42°C for 24 h in tryptic soy broth containing 2 mg of novobiocin per liter. White colonies isolated on cefixime-tellurite sorbitol MacConkey agar plates were evaluated for fluorescence on sorbitol MacConkey agar supplemented with 0.025 g of methylumbelliferyl-β-D-glucuronide per liter. Nonfluorescing white colonies were biochemically typed and serologically confirmed. Multiplex polymerase chain reaction profiles of *E. coli* O157:H7 isolates indicated the presence of common virulence factors (Shiga toxin, enterohemolysin, and intimin) of Shiga toxin–producing *E. coli,* suggesting the potential human pathogenicity of bacterial isolates. Pulsed-field gel electrophoresis profiles of *Spe*I and *Xba*I restriction enzyme–digested genomic DNA were used to establish relatedness among bacterial isolates. Data from this study indicate that both cull dairy cows and bulk tank milk pose a potential hazard with regard to human foodborne illness. It is therefore imperative to develop on-farm and preharvest pathogen reduction programs to control the carriage of *E. coli* O157:H7 pathogens.

Foodborne diseases are estimated to cause 76,000,000 illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year *(23).* They appear to cause more illnesses but fewer deaths than previously estimated *(6).* Illnesses due to *Escherichia coli* O157:H7 infections result in about 62,458 cases, 1,843 hospitalizations, and 52 deaths, costing $700,000,000, each year *(10, 23).*

The consumption of unpasteurized milk products *(8, 21)* and ground beef, including beef from cull dairy cows *(22, 28),* has been associated with enterohemorrhagic *E. coli* (EHEC) O157:H7 outbreaks in the United States. Significant amounts of the beef consumed in the United States come from dairy cows, with cull dairy cows contributing about 17% of the ground beef produced *(28).* An array of food products, including raw seeds, apple cider, salad, and fruits, as well as contaminated well water, have been implicated in foodborne EHEC O157:H7 disease outbreaks *(12, 25, 27).* Nonfood vehicles such as petting zoo animals and person-to-person transmission have also been implicated *(1).*

EHEC O157:H7 is the most commonly identified member of the Shiga toxin–producing *E. coli* (STEC) family. It is the most notorious emerging pathogen and is considered prototypal for the current paradigm of foodborne diseases in the United States *(2).* The spectrum of diseases caused by EHEC O157:H7 includes asymptomatic infection (carrier status), mild uncomplicated diarrhea, bloody diarrhea and hemolytic colitis, diarrhea-associated hemolytic uremic syndrome, and diarrhea-associated thrombotic thrombocytopenic purpura *(13, 20, 26).* In the worst cases, death may result, usually from kidney failure. Shiga toxins are the main virulence factors associated with hemolytic colitis and hemolytic uremic syndrome, presumably because they interact with endothelial cells at the site of infection and in the glomeruli of kidneys *(13).* hemolytic uremic syndrome has a case fatality rate of 3 to 5%. Apart from Shiga toxins, members of the STEC family produce other accessory virulence factors, including intimin and enterohemolysin A. Intimin is responsible for the characteristic histopathologic feature known as the attaching-effacing lesion *(13).*

The objective of this study was to establish the prevalence of *E. coli* O157:H7 on dairy farms in east Tennessee. The specific aims of the study were to define the population of cull dairy cows carrying *E. coli* O157:H7 by analyzing fecal samples and to determine if pooled samples, i.e., bulk tank milk, could be used to establish the *E. coli* O157:H7 carriage status of a dairy herd. In addition, we used a multiplex polymerase chain reaction (PCR) targeted at genes encoding H7 flagellar and STEC virulence factors (Shiga toxins 1 and 2, intimin, and enterohemolysin) and pulsed-field gel electrophoresis (PFGE) of genomic DNA to subtype *E. coli* O157:H7 isolates. The long-term goal of this

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project is to introduce intensive sampling at more diverse locations on selected farms to enable the identification of reservoirs of *E. coli* O157:H7 pathogens. Data on the prevalence of *E. coli* O157:H7 in cull dairy cows and bulk tank milk is critical for the design of risk-based, on-farm preharvest pathogen reduction programs *(29)* to control these pathogens at the primary production unit, the dairy farm.

MATERIALS AND METHODS

Sample collection and submission. Thirty dairy herds from east Tennessee were investigated in this study. The sizes of the dairy herds studied differed substantially ($n = 175 \pm 75$ cows [mean ± standard deviation]). Farms were placed into four regional groups for convenience of sample collection and analysis. Members of each group submitted samples to the laboratory once per month on the same sampling date. Samples were transported in ice-packed cooler boxes. Initially, printed and laminated sampling instructions and a video demonstrating aseptic sampling procedures were provided to farmers. Fecal samples were collected from cull dairy cows by digital recovery per rectum with sterile single-service plastic gloves that were individually sterilized in pouches with nitrous oxide gas. Fecal samples collected on dates not coinciding with the once-per-month bulk tank milk sample collection date were frozen (−20°C) and shipped with the next milk samples. Milk was agitated for 5 min before a sample was collected from the top of the bulk tank with a sterilized ladle. Three 45-ml samples were collected in sterile self-standing 50-ml screw-cap centrifuge tubes (Corning Inc., Corning, N.Y.). Two of these tubes were used for the isolation of mastitis and foodborne pathogens, and the third tube was used for counting somatic cells.

Bacteriological methods. General bacteriological methods used for the isolation of *E. coli* O157:H7 were compendial *(14)*. Milk (11 ml) was mixed with 99 ml of modified tryptic soy broth (mTSB; TSB plus 20 mg of novobiocin per liter) enrichment medium in 99-ml dilution bottles. For fecal sample analysis, 11 g was hand-mixed in a filter-stomacher bag. The mixture was strained into a 99-ml dilution bottle and adjusted to a pH of 7.0. For the most-probable-number procedure *(9)*, four aliquots of 10, 1, 0.1, and 0.01 ml were transferred to sterile tubes and adjusted to a total volume of 10 ml with mTSB.

Samples were shake cultured at 150 rpm for 24 h at 42°C and streaked onto tellurite-cefixime sorbitol MacConkey agar for isolation. Sorbitol-negative colonies (white) were streaked onto methylumbelliferyl-β-D-glucuronide sorbitol MacConkey agar for fluorescence testing. All other incubations were conducted at 37°C for 24 h. Suspect colonies were typed biochemically with triple sugar iron agar slants, lactose fermentation, and indole–methyl red–Voges-Proskauer–citrate tests. Isolates demonstrating typical *E. coli* reactions were streaked for isolation on tryptic soy agar (TSA) supplemented with 0.6% (wt/vol) yeast extract (TSAYE) before they were evaluated serologically. Isolates were screened for the presence of O157 antigens with the Reveal *E. coli* O157:H7 test system (Neogen Corp,. Lansing, Mich.) and O157 antiserum (Difco Laboratories, Detroit, Mich.). Latex agglutination with RIM *E. coli* O157:H7 latex (Remel, Lenexa, Kans.) was used to determine the presence of O157 and H7 antigens by following the protocol provided by the manufacturer. Motility enhancement was carried out with soft TSB agar (0.4% agar, wt/vol) tubes before H7 serology was carried out. Actively growing cultures from TSB were stab inoculated into the soft TSB agar and incubated for 24 h. Growth was streaked on TSB blood agar (5% [vol/vol] sheep's blood) to isolate colonies that were tested for the presence of H7-flagellar antigens. In addition, isolates were evaluated for tellurite resistance on antibiotic gradient plates *(11)*.

Maintenance of bacterial cultures. Generic *E. coli*, *E. coli* O157:H7, and O157:H− strains (Table 1) were used for quality control. Generic *E. coli* was obtained from the American Type Culture Collection (ATCC), Manassas, Va., and *E. coli* O157 was obtained from the ATCC and from Dr. D. Golden (Department of Food Science and Technology, University of Tennessee, Knoxville, Tenn.). Working stock cultures were maintained as slants on TSAYE at 5°C for 1 month. For long-term storage, cultures grown in TSB for 24 h at 37°C were mixed with 25% glycerol (1:1, vol/vol) and stored at −80°C.

Multiplex PCR for virulence genes. The multiplex PCR technique described by Fratamico et al. *(15)* was used to subtype isolates by targeting common *E. coli* O157:H7 virulence genes encoding Shiga toxin 1 and 2 (stx_1 and stx_2), enterohemolysin (hly_{933}), intimin (*eaeA*), and flagellar H7 ($flicC_{h7}$) gene sequences.

Bacterial cultures grown in TSB at 37°C for 24 h were streaked onto TSAYE and incubated for 24 h. For each test bacterium, an isolated colony was picked and washed with 1 ml of sterile distilled water in a microfuge tube centrifuged at 13,500 × *g* for 3 min. The supernatant was aspirated to leave 20 μl and pellet. A volume of 200 μl of well-mixed InstaGene Matrix (Bio-Rad, Hercules, Calif.) was added to the microfuge tube, and DNA was isolated using the InstaGene protocol (Bio-Rad).

Oligonucleotide primers of virulence gene sequences were obtained from a commercial source (IDT, Coralville, Iowa). DNA amplification was performed with the protocol described by Fratamico et al. *(15)*. Amplified products were electrophoresed in 1.5% agarose with TBE buffer (0.9 M Tris base, 0.09 M boric acid, 2.5 mM EDTA [pH 8.3]) as described by Gillespie et al. *(17)*. Gels were run at 150 V for 2 h and stained with 1.0 μg/ml ethidium bromide (Sigma Chemical Co., St. Louis, Mo.). Multiplex PCR products were visualized by UV transillumination (Fotodyne Inc., Heartland, Wis.) and photographed *(17)* using type 55 Polaroid film (Polaroid Corp., Cambridge, Mass.).

Detection of Shiga toxins and enterohemolysin. Shiga toxin production was detected with the Verotoxin-Producing *E. coli* Reversed Phase Latex Agglutination kit and protocol (Oxoid Ltd., Napean, Ontario, Canada) without preincubation at 37°C for 30 min. (The preincubation step was found to be unnecessary.) Serial twofold dilutions (1:2 to 1:128) of supernatant fluid (25 μl) were tested for Shiga toxins 1 and 2 using 96-well U-bottom cell culture plates (Corning). Agglutination was scored visually against a dark background after 24 h of incubation at room temperature. Hemolysin production was performed on Columbia agar base EH (Difco) containing washed sheep's blood (5%, vol/vol) as described by Beutin et al. *(4)* and on TSA blood agar (5% sheep's blood, vol/vol). Plates were incubated at 37°C for 24 h prior to scoring for hemolysis. Strains that did not easily demonstrate hemolysin production were evaluated at least six times.

PFGE of genomic DNA. PFGE was used to characterize and determine relatedness between *E. coli* O157:H7 isolates by the procedure described by Gautom *(16)*. For restriction endonuclease digestion, two plugs of a bacteria-agarose mixture were incubated at 37°C for 1 to 1.5 h with 30 U of *Xba*I or *Spe*I (BioWhittaker, Molecular Applications, Rockland, Maine) restriction endonucleases in 100 μl of the appropriate restriction enzyme buffer. Plug slices were loaded in 1% SeaKem gold agarose (BioWhittaker) and electrophoresed in 2 liters of 0.5× standard Tris-borate-EDTA (pH 8.0) running buffer. Electrophoresis was performed with the CHEF-Mapper (Bio-Rad) with an initial switch time of 2.16 min,

TABLE 1. *Multiplex PCR of virulence factor gene sequences and toxin production by* E. coli *O157 (H7 and H− variants) and generic* E. coli *strains*

Bacteria tested	Serotype[a]	Source[b]	Presence or absence of virulence factor gene sequences					Presence or absence of toxin[d]		
			stx_1	stx_2	*eaeA*	hly_{933}	$flicC_{h7}$	Stx1	Stx2	Hly
Quality control										
Non-O157 *E. coli*										
4350	X:Y	ATCC	−	−	−	−	−	−	−	−
12014	X:H−	ATCC	−	−	+	−	+	ND	ND	−
23513	O18:H7	ATCC	−	−	−	−	+	ND	ND	++
23514	O19:H7	ATCC	−	−	−	−	+	ND	ND	−
23545	X:Y	ATCC	−	+	−	−	−	−	−	++
31619	X:Y	ATCC	−	−	+	−	+	−	−	−
E. coli O157										
700375	O157:H−	ATCC	+	+	+	+	+	−	−	+
Ground beef	O157:H7	DG	+	+	+	+	+	−	−	+
E0019	O157:H7	DG	+	+	+	+	+	ND	ND	+
Cider	O157:H7	DG	+	+	+	+	+	+	+	++
H1730	O157:H7	DG	+	+	+	+	+	ND	ND	+
Salami	O157:H7	DG	+	+	+	−	+	ND	ND	++
994	O157:H7	DG	+	+	+	−	+	ND	ND	−
4546	O157:H7	DG	+	+	+	+	+	ND	ND	+
438888	O157:H7	ATCC	−	−	+	+	+	−	−	−
438889	O157:H7	ATCC	+	+	+	+	+	+	+	+
43894	O157:H7	ATCC	+	+	+	+	+	+	+	+
Isolates[e]										
36E1	O157:H7	This study	+	+	+	+	+	ND	ND	−
43E1	O157:H7	This study	−	+	+	+	+	−	+	+
142E1	O157:H7	This study	−	+	+	+	+	−	+	+
161E1	O157:H7	This study	−	+	+	+	+	−	+	+
309E1	O157:H7	This study	−	+	+	+	+	−	+	+
384E1	O157:H7	This study	+	+	+	+	+	+	+	+
386E1	O157:H7	This study	+	+	+	+	+	+	+	+
427E1	O157:H7	This study	+	+	+	+	+	+	+	+
436E1	O157:H7	This study	+	+	+	+	+	+	+	+
583E1	O157:H7	This study	ND	ND	ND	ND	ND	+	+	+

[a] X, unknown (non-O157) somatic serotype; Y, motile unknown (non-H7) serotype; H−, nonmotile.

[b] ATCC, American Type Culture Collection, Manassas, Va.; DG, Dr. David Golden, Department of Food Science and Technology, University of Tennessee, Knoxville, Tenn.

[c] stx_1, Shiga-like toxin 1 gene; stx_2, Shiga-like toxin 2 gene; *eaeA*, attaching-effacing gene; hly_{933}, enterohemolysin gene (plasmid-encoded); $flicC_{h7}$, H7 flagellar gene; −, absence of target gene sequence; +, presence of target gene sequence; ND, not determined.

[d] Stx1, Shiga toxin 1; Stx2, Shiga toxin 2; Hly, hemolysin (may be a combination of enterohemolysin and other hemolysins for some of the strains with large zones); −, absence of toxin; +, presence of toxin (small zone of hemolysis [<2 mm]); ++, large zone of hemolysis; ND, not determined.

[e] Several isolates were obtained from each sample, and they did not differ phenotypically or genetically.

a final switch time of 35.07 min, an angle of 120°, a gradient of 6.0 V/cm, a temperature of 14°C, linear ramping, and a run time of 14 h. After electrophoresis, gels were stained in 500 ml of distilled water containing 50 μl of ethidium bromide (10 mg/ml; Sigma) for 20 min, and then two 30-min washes were carried out with distilled water. Gels were analyzed with the aid of the Molecular Analyst software (Bio-Rad).

Data analysis. The sensitivity and specificity of multiplex PCR tests were determined by using quality control *E. coli* strains (generic and O157) with known virulence factor profiles (Table 1). TIFF (tagged image file format) images of photographed (Polaroid) gel bands were analyzed with the Molecular Analyst software, version 6 (Bio-Rad). This software was used to compare, correlate, and generate similarity indices (cluster analysis) among *E. coli* O157:H7 isolates.

RESULTS

Bacterial isolation and enumeration. Isolation and enumeration of *E. coli* O157:H7 was conducted for the first 90 samples submitted. One milk sample and one fecal sample tested positive for *E. coli* O157:H7. The most-probable-number values established *(9)* were 240/100 ml (95% confidence interval: lower limit = 36 and upper limit = 1,300) for milk and 9/100 g (95% confidence interval: lower limit = 1 and upper limit = 36) for the fecal sample. Nine of 10 isolates were obtained by the 11:99 dilution method; however, this technique provided only qualitative data. The seasonal distribution of *E. coli* O157:H7 isolates from dairy farms is summarized in Figure 1. All 10 *E. coli* O157:H7 isolates were obtained between June 2000 and January

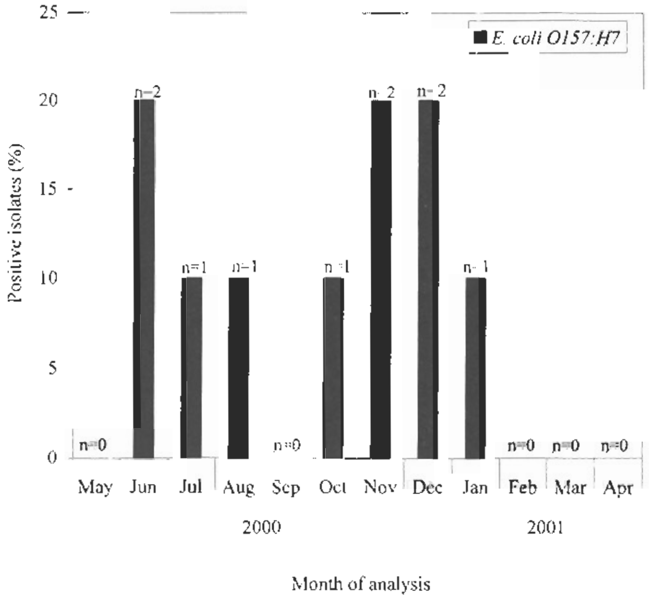

FIGURE 1. *Seasonal distribution of* E. coli *O157:H7 isolates from dairy farms in east Tennessee.*

2001. There were no isolates obtained in May and September of 2000 or in February, March, and April of 2001.

Escherichia coli O157:H7 isolates demonstrated greater tellurite resistance than generic *E. coli* strains (data not shown).

Multiplex PCR. The multiplex PCR protocol permitted the identification of *E. coli* strains, including non-O157:H7 bacteria possessing H7 flagellar genes, and determined the presence of gene sequences coding for enterohemolysin, intimin, and Shiga toxin 1 and 2 (Table 1). The experiment was repeated with freshly grown cultures with identical results. Multiplex PCR correctly typed ATCC quality control *E. coli* O157 (H7 and H− variants) with known virulence factor profiles (*E. coli* O157:H7 strain ATCC 43888 [Fig. 2A, lane 1] and *E. coli* O157:H− strain ATCC 700375 [Fig. 2A, lane 10]), which lack Stx, and *E. coli* O157:H7 strain ATCC 43894 (Fig. 2A, lane 5), which has both Stx1 and Stx2. Interestingly, although *E. coli* O157:H7 strain ATCC 43889 (Fig. 2A, lane 7) was represented as an Stx2 producer when purchased (ATCC), we demonstrated that it harbors an stx_1 gene sequence. Original stock cultures made from the strain were tested for the presence of the two Shiga toxin gene sequences, and results agreed. Other *E. coli* O157:H7 strains tested positive for all five virulence factors evaluated. The *E. coli* O157:H7 strains Salami and 994 lacked hly_{933} but possessed the other four virulence genes. The quality control generic *E. coli* strains possessing H7 flagellar genes typed correctly for $flicC_{h7}$. *E. coli* strain 31619 was nonmotile according to motility tests, although it occasionally tested positive for H7; it possessed the $flicC_{h7}$ flagellar gene sequence.

Shiga toxin and enterohemolysin production. The production of Shiga toxins and enterohemolysin was used to verify data from multiplex PCR (Table 1). The tests confirmed that strains with toxin genes identified using multiplex PCR were expressing these gene products and that *E. coli* O157:H7 ATCC 43889 produced both Stx1 and Stx2, and not Stx2 alone. However, the generic *E. coli* quality

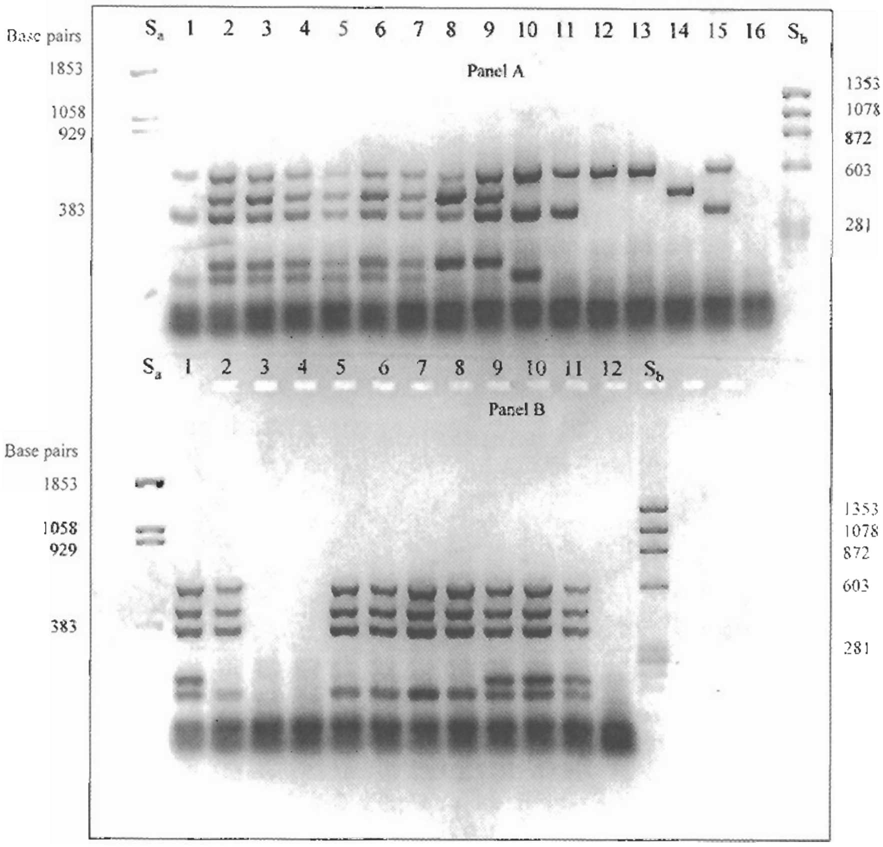

FIGURE 2. *Representative multiplex PCR fragments for the identification of* E. coli *O157:H7 virulence factor gene sequences. Lanes* S_a, *pBR322 DNA* BstNI *digest; Lanes* S_b, *ϕX174 DNA* HaeIII *digest. (A) Lanes 1 to 9 show* E. coli *O157:H7 strains: lane 1, 43888; lane 2, ground beef; lane 3, E0019; lane 4, H1730; lane 5, 43894; lane 6, 4546; lane 7, 43889; lane 8, salami; lane 9, 994. Lane 10,* E. coli *O157:H− strain 700375. Lanes 11 to 16 show non-O157 generic* E. coli *strains: lane 11, 12014 (nonmotile); lane 12, 23513 (H7 flagellar phenotype); lane 13, 23514 (H7 flagellar phenotype); lane 14, 23545; lane 15, 31619; lane 16, 4350. (B) Lanes 1, 3, and 5 through 11 show* E. coli *isolates from this study. Lanes 3 and 4 show strains (isolates 75E1 and 75E2 from the same sample) that were serologically O157:H7-positive but failed the Reveal O157:H7 test. Lane 12 shows a reagent control.*

TABLE 2. *Sensitivity and specificity of virulence factor tests*[a]

	Result for toxin/virulence gene				
Test	Shiga toxin 1/*stx_1*	Shiga toxin 2/*stx_2*	Enterohemolysin/*hlyA*	H7 flagellar/*$fliC_{h7}$*	Intimin/*eaeA*
Sensitivity (%)	100	100	88.9	100	ND
Specificity (%)	100	87.5	62.5	40	ND

[a] Specificities and sensitivities were determined by using *E. coli* O157 (H7 and H− variants) and generic *E. coli* quality control strains. A positive test was one that was verifiable by both genetic and toxin production phenotypic tests. A negative test was one with two different test outcomes. The best tests had both high sensitivity and high specificity. Sensitivity (%) = true positive/(true positive + false negative); specificity (%) = true negative/(true negative + false positive). ND, not determined because phenotypic expression of the intimin-encoding gene was not evaluated.

control strain ATCC 23545, which harbors *stx* gene sequences, did not produce Shiga toxin. When the two toxins were coproduced, Stx2 titers were generally higher than Stx1 titers. A positive enterohemolysin test was taken to indicate the presence of hemolysis zones in at least three of six tests (Table 1). Small zones were interpreted as an indication of enterohemolysin production *(3)*. No major differences in the detection of hemolysis zones were observed with Columbia agar base EH and TSA blood agar.

Sensitivity and specificity of virulence factor tests. The values obtained with both sensitivity and specificity tests for Shiga toxins were high (87.5 to 100%; Table 2). Values obtained with sensitivity tests for enterohemolysins and the flagellar phenotype were high (88.9 and 100%, respectively; Table 2). However, the values for the corresponding specificity tests were low (62.5 and 40%, respectively).

PFGE. Nine *E. coli* O157:H7 isolates obtained from bulk tank milk and from fecal samples from cull cows from six dairy farms were digested with *Spe*I and *Xba*I DNA restriction enzymes, and then PFGE of the digests was carried out. PFGE profiles for the respective enzymes are shown in Figures 3A and 3B. A cluster analysis of PFGE patterns from *Spe*I and *Xba*I digests was carried out. A matrix of coefficients was generated, and a correlation analysis was conducted by using the unweighted-pair group method of arithmetic averages and the clustering algorithm to create a dendrogram.

Analysis of the *Spe*I digests generated two distinct

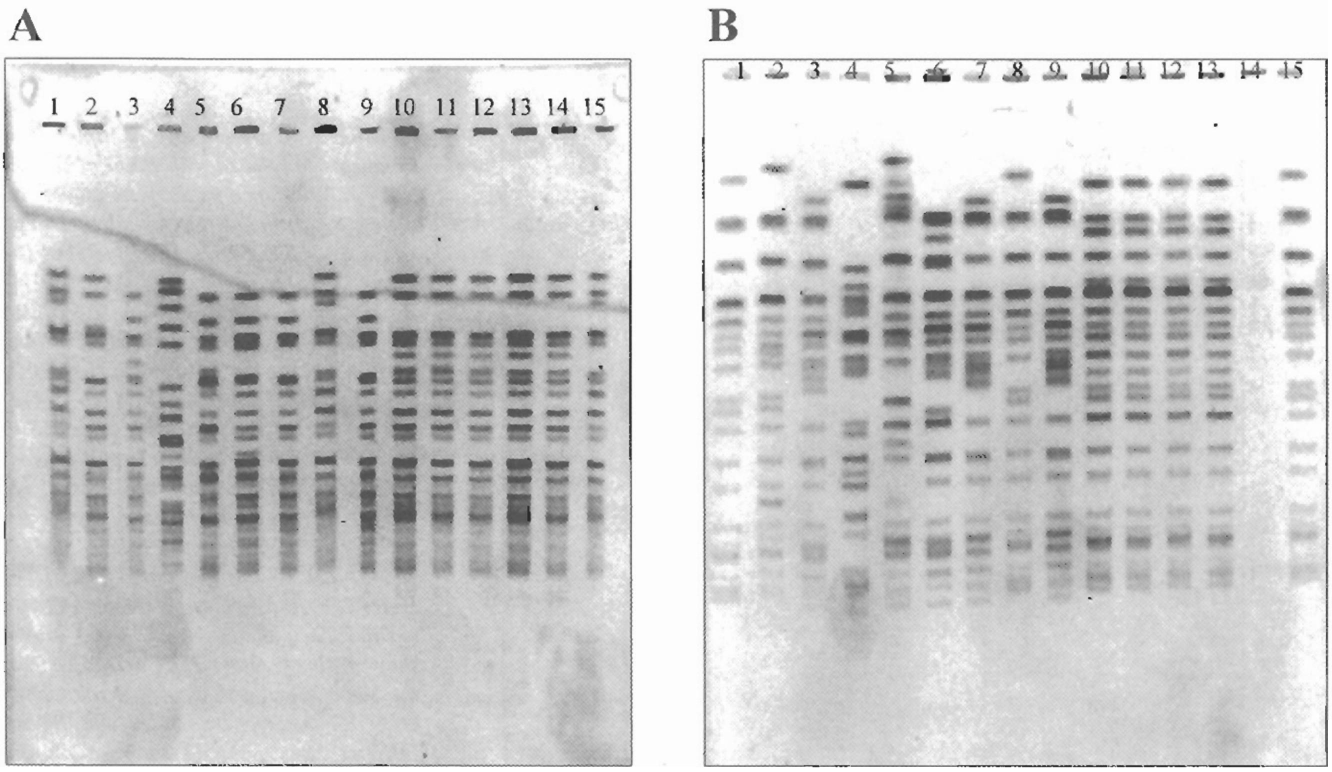

FIGURE 3. *Representative agarose gels showing PFGE patterns of (A)* SpeI*-digested and (B)* XbaI*-digested genomic DNA from* E. coli *isolates. (A) Lanes 1, 8, and 15 show* E. coli *G5244 (reference standard). The remaining lanes show isolates from this study (all except 75E1 are* E. coli *O157:H7): lane 2, 36E1; lane 3, 43E1; lane 4, 75E1; lane 5, 142E1; lane 6, 161E1; lane 7, 309E1; lane 9, 309E2; lane 10, 348E1; lane 11, 384E2; lane 12, 386E1; lane 13, 427E1; lane 14, 436E1. (B) Lanes 1, 8, and 15 show* E. coli *G5244 (reference standard). The remaining lanes show isolates from this study (all except 75E1 are* E. coli *O157:H7): lane 2, 36E1; lane 3, 43E1; lane 4, 75E1; lane 5, 142E1; lane 6, 161E1; lane 7, 309E1; lane 9, 309E2; lane 10, 348E1; lane 11, 384E2; lane 12, 386E1; lane 13, 427E1.*

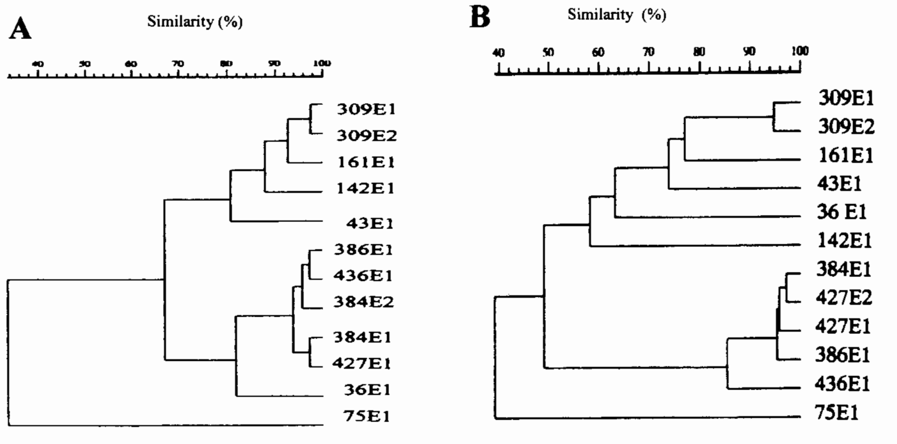

FIGURE 4. *Cluster analysis of PFGE patterns of (A)* SpeI-*digested and (B)* XbaI-*digested genomic DNA from* E. coli *isolates from dairy farms. Isolates 309E1 and 309E2 were obtained from farm E, isolate 161E1 was obtained from farm D, isolate 142E1 was obtained from farm C, isolate 43E1 was obtained from farm B, isolates 386E1, 386E2, 436E1, 384E1, 384E2, 427E1, and 427E2 were obtained from farm F, isolate 36E1 was obtained from farm A, and isolate 75E1 was obtained from farm G. An isolate number followed by E1 or E2 indicates multiple isolates from the same sample; these isolates were not genetically distinguishable.*

clusters (Fig. 4A). Cluster 1 grouped together four *E. coli* O157:H7 isolates (309E1 and 309E2 from the same sample, which were identical, and 161E1, 142E1, and 43E1) with similarity coefficients of 80 to 98%. These isolates were obtained from cull dairy cow fecal samples from four different farms. Cluster 2 contained four *E. coli* O157:H7 isolates (384E2 and 384E1, from the same sample, which were considered the same strain, and 386E1, 436E1, and 427E1) with similarity coefficients of >92% from fecal samples obtained from one farm. The *E. coli* O157:H7–positive fecal samples at this farm were collected over a 2-month period. Cluster 2 also contained the only bulk tank milk *E. coli* O157:H7 isolate 36E1 from a dairy farm. Isolate 75E1 was remotely related to other *E. coli* O157:H7 isolates.

Analysis of *Xba*I digests generated a pattern that was somewhat different (Fig. 4B) from that obtained with *Spe*I digests. Two clusters were formed. Cluster 1 was similar to that established with *Spe*I (Fig. 4A); in addition, it included *E. coli* O157:H7 isolate 36E1, which was in cluster 2 of the *Spe*I digests. Isolates from different samples in cluster 1 (Fig. 4B) had lower coefficients of similarity (58 to 95%) than did those established with *Spe*I (80 to 98%). *Xba*I grouped isolates 427E1 and 427E2 (which were isolated from the same sample), 384E1, and 386E1 into a close cluster with similarity coefficients of 86 to 97%. However, isolate 436E1 was more distantly related to other *E. coli* O157:H7 isolates from the same farm. Like *Spe*I, *Xba*I indicated the distant genetic relatedness of isolate 75E1 to *E. coli* O157:H7 isolates. API 20E profiles indicated that isolate 75E1 identified closely with *Escherichia vulneris* (95%).

DISCUSSION

Escherichia coli O157:H7 was isolated from 8 of 30 (26.7%) dairy farms at different sampling times. Two of 268 (0.75%) bulk tank milk samples and 8 of 415 (1.93%) cull dairy cow fecal samples tested positive for *E. coli* O157:H7. These prevalence rates probably underestimate the true herd carriage rates because of the small number of cull cows available for analysis per farm and the intermittent nature of pathogen shedding by animals. However, there could have been a predominance of pathogens in other classes of dairy animals that were not sampled, that is, calves and heifers, including individual milking cows. Very low isolation rates for bulk tank milk could be due to dilution effects or a low prevalence of the pathogen.

Wells et al. *(30)* found a higher incidence of *E. coli* O157:H7 in bulk tank milk than did other investigators (Table 3). Prevalence rates of the pathogen in cull dairy cow fecal samples in the United States (Table 3) are low *(22)* compared with carriage rates reported in Europe *(5, 7)*. Other dairy animals can be significant carriers of these pathogens (Table 3), which indicates that other animals on dairy farms, i.e., heifers and calves, should be tested. Hancock et al. *(18)* established a prevalence rate of 0.28% for dairy cow fecal samples (n = 3,570). Bornadi et al. *(5)* and McDonough et al. *(22)* reported carriage rates of 16.1 and 1.32%, respectively, for cull dairy cow fecal samples. However, reported isolation rates of *E. coli* O157:H7 in fecal samples and rectal swabs for dairy cattle other than cull cows (Table 3) have ranged from 0 to 16.6% *(5, 18, 24, 30)*. Isolation of *E. coli* O157:H7 from raw milk and bulk tank milk samples has indicated prevalence rates ranging from 0 to 10% *(18, 21, 24, 30)*.

One study recovered *E. coli* O157:H7 significantly more often from young animals than from adult animals *(30)*. From the literature, it appears that dairy cattle carry and shed relatively low numbers of *E. coli* O157:H7. However, meat and dairy products are a significant component of the human diet. The higher incidences of the pathogen found at slaughter may be due to stress (e.g., transport, feed withdrawal) undergone by animals awaiting slaughter; these stress factors may exacerbate the shedding of *E. coli* O157:H7 *(28)*. The results of the present study (Fig. 1) concur with those of other studies in that isolation rates are normally higher in warmer months *(7, 18)*.

Our finding of a higher incidence of *E. coli* O157:H7 in feces than in raw and bulk tank milk seems to support reports that *E. coli* O157:H7 outbreaks are associated most often with the consumption of undercooked ground beef (that could have been contaminated from fecal matter) and less often with the consumption of raw milk *(28)*. Thus, there are significant microbiological hazards and public health risks associated with the consumption of products made from unpasteurized milk and meat from cull dairy cows. It is imperative to establish management-associated

TABLE 3. *Prevalence rates*[a] *of* E. coli *O157:H7 on dairy farms*

Bulk tank milk	Cull dairy cows	Other dairy animals[b]	Country	Reference
2/268 (0.75%)	8/415 (1.93%)	ND[c]		This study
1/23 (4.3%)	ND	17/604 (2.8%) Cv + H; 1/662 (0.15%) DCt	USA (Wisconsin and Washington State)	*30*
0/131 (0%)	ND	ND	USA (South Dakota and west Minnesota)	*19*
0/630 (0%)	ND	7/1083 (0.65%) WCv; 0/649 (0%) UWCv; 2/1273 (0.16%) LDCw; 1/477 (0.21%) NLDCw	USA (Washington State)	*18*
ND	22/1668 (1.32%)	ND	USA (New York State)	*22*
0/327 (0%)	ND	153/3593 (4.3%) Cw + H + Cv	UK	*24*
ND	268/1661 (16.1%)	ND	UK	*7*
ND	22/137 (16.1%)	0/90 (0%) VCv; 37/223 (16.6) FA	Italy	*5*

[a] Number of samples positive for *E. coli* O157:H7/number of samples analyzed.

[b] Cv, calves; H, heifers; D, dairy; Ct, cattle; Cw, cow; W, weaned; UW, unweaned; L, lactating; NL, non-lactating; V, veal; FA, feedlot animals. For nonmilkers, fecal samples were analyzed.

[c] ND, not done.

risk factors that result in the emergence and persistence of STEC on dairy farms.

Shiga toxin analysis indicated that *E. coli* O157:H7 quality control strain ATCC 43889 produces Stx1 in addition to Stx2. Stock cultures prepared from the original ATCC cultures had the same toxin profiles as the isolate we evaluated. Importantly, the multiplex PCR assay was able to confirm that isolates from fecal samples and bulk tank milk were O157:H7 STEC. Moreover, isolates that often typed serologically positive for the O157 phenotype with antiserum and negative with the Neogen Reveal O157:H7 test system also produced differing results with H7 antiserum and were confirmed to be non-O157:H7 with the multiplex PCR test; they carried none of the virulence genes tested (data not shown). In addition, it was easier to determine the presence of enterohemolysin gene sequences with multiplex PCR than to test for the actual production of enterohemolysins (small zones), since some strains may produce other types of hemolysin (large zones) in addition to enterohemolysin, thus confounding interpretation of zone data. In summary, *eaeA* and $\mathit{flicC}_{\mathrm{h7}}$ sequences were the most common virulence factor–encoding genes found in this study. The multiplex PCR assay promises to be an effective tool for the rapid detection and subtyping of STEC according to virulence gene sequence profiles and can be developed to differentiate *E. coli* O157 H7 and H− variants using DNA-based flagellar phenotype discrimination without cumbersome serology tests.

Comparison of isolates by genomic DNA fingerprinting of restriction enzyme digests demonstrated that some isolates had different PFGE profiles, indicating distant relationships (Figs. 3 and 4). However, four isolates from cull dairy cow fecal samples from the same farm had identical PFGE profiles, suggesting the persistence of *E. coli* O157:H7 in nonculled animals in the dairy herd.

The use of DNA-based methods for the detection and confirmation of *E. coli* O57:H7 is critical in establishing on-farm preharvest food safety and pathogen reduction programs. Techniques for detecting STEC with multiplex PCR may become increasingly important in the future as tools for studying *E. coli* O157 (H7 and H− variants), O157:H7 strains that ferment sorbitol, and non-O157 STEC. Reports have indicated higher isolation rates for non-O157:H7 STEC than for *E. coli* O157:H7 serotypes in dairy animals *(30, 31)*.

This study established that the prevalence of *E. coli* O157:H7 on dairy farms in east Tennessee was low (i.e., 1.46% of the samples analyzed); 8 of 30 farms were *E. coli* O157:H7–positive. Multiplex PCR and PFGE enabled detailed characterization and subtyping of isolates. The next phase of this study will involve more extensive study of selected farms to establish potential reservoirs of the pathogen.

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