Research Note

Detection of Verocytotoxin-Producing *Escherichia coli* Serogroups O157 and O26 in the Cecal Content and Lymphatic Tissue of Cattle at Slaughter in Italy

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ABSTRACT

Verocytotoxin-producing *Escherichia coli* (VTEC) has emerged as a foodborne pathogen that can cause severe and potentially fatal illnesses, such as hemorrhagic colitis or the hemolytic uremic syndrome. In this study, 182 cattle at slaughter (119 dairy cows and 63 feedlot cattle) were randomly selected and tested for the presence of VTEC serogroups O26, O103, O111, O145, and O157 in their cecal content and lymphatic tissue (tonsils or mesenteric lymph nodes). A total of 364 samples were evaluated with an immunomagnetic separation technique followed by slide agglutination. Presumptive VTEC O26, O103, O111, O145, and O157 isolates were tested by Vero cell assay for verocytotoxin production and by multiplex PCR assay for the detection of vtx_1, vtx_2, *eae,* and *E-hlyA* genes. VTEC O157 was detected in 6 (3.3%) of 182 animals, and VTEC O26 was detected in 1 (0.5%) of 182 animals. No VTEC O103, VTEC O111, or VTEC O145 isolates were found in cattle feces, but one VTEC O91:H⁻ vtx_2^+, *eae⁻*, *E-hlyA⁺* strain nonspecifically cross-reacted with the VTEC O103 type. The prevalence of VTEC O157 in the lymphatic tissue of cattle was 1.1% in both tonsils (1 of 93 samples) and mesenteric lymph nodes (1 of 89 samples). Lymphatic tissue contamination was observed only in VTEC O157 intestinal carriers; two (33.3%) of six fecal carriers were simultaneously VTEC O157 lymphatic carriers. This finding suggests that VTEC O157 contamination of meat does not necessarily come from feces or the environment. No other VTEC serogroups were detected in the lymphatic tissue of slaughtered cattle.

Verocytotoxin-producing *Escherichia coli* (VTEC), also called Shiga toxin–producing *E. coli,* has emerged as a pathogen that can cause food poisoning and severe and potentially fatal illnesses in humans. VTEC strains produce two phage-encoded cytotoxins called verocytotoxins (VT1 and VT2) or Shiga-like toxins (Stx1 and Stx2) as their key virulence factors. VT consists of five identical B subunits responsible for binding the holotoxin to the glycolipid receptor Gb3 on the cell surface and of a single A subunit that cleaves ribosomal RNA, causing protein synthesis to cease *(22).* VT is produced in the colon of infected humans and travels through the bloodstream to the endothelial cells of the kidney and the large bowel, which are rich in the Gb3 receptor, causing damage to the vascular region of those organs *(1).* VT also induces apoptosis in intestinal epithelial cells *(18).*

Another virulence-associated factor expressed by VTEC is a 94-kDa outer membrane protein called intimin, which is responsible for the intimate attachment of VTEC to intestinal epithelial cells, causing attaching-and-effacing lesions in the intestinal mucosa. Intimin is encoded by the chromosomal gene *eae,* which is part of a pathogenicity island termed the locus for enterocyte effacement *(19).* Se-

vere diarrhea, especially hemorrhagic colitis and hemolytic uremic syndrome, are closely associated with VTEC types carrying the *eae* gene for intimin. A factor that may also affect the virulence of VTEC strains is enterohemolysin, also called enterohemorrhagic *E. coli* hemolysin, which is encoded by the *E-hlyA* gene *(20).*

VTEC strains are part of the normal intestinal flora of animals. VTEC transmission to humans occurs through consumption of undercooked meat, raw milk, unpasteurized dairy products, and vegetables or water contaminated by the feces of animal carriers. Person-to-person transmission also has been documented *(21).*

VTEC serogroup O157 is a major cause of gastroenteritis; this infection may be complicated by hemorrhagic colitis or the hemolytic uremic syndrome, which has been responsible for acute renal failure in children *(21).* Since its identification as a pathogen in 1982, enterohemorrhagic VTEC O157:H7 has been associated with several outbreaks, especially in Canada, Japan, the United Kingdom, and the United States *(24).* VTEC strains that cause human infections belong to a large number of O:H serotypes, and ruminants (particularly cattle, sheep, and goats) are the most important reservoirs of VTEC strains *(4, 11).* Infections with some non-O157 VTEC types (e.g., O26:H11 or O26:H⁻, O91:H21 or O91:H⁻, O103:H2, O111:H⁻, O113:

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H21, O117:H7, O118:H16, O121:H19, O128:H2 or O128: H−, O145:H28 or O145:H−, and O146:H21) frequently have been associated with severe illness in humans *(2, 5)*.

Although cattle are regarded as a major reservoir of VTEC O157 *(11),* in many non-O157 VTEC outbreaks the source of human infection is unknown *(17)*. The underestimation of non-O157 VTEC prevalence in domestic animals is largely due to the absence of effective differential media available to discriminate between VTEC serogroups and between VTEC and non-VTEC strains, which share common phenotypic features. However, the development of molecular biological techniques and immunologically based methods and verotoxin assays have allowed identification of more than 400 VTEC serotypes that have been responsible for human illness and are detectable in samples of human, animal, food, and environmental origin *(3, 5)*.

The goal of this study was the examination of the cecal content and lymphatic tissue (tonsils or mesenteric lymph nodes) of cattle at slaughter to determine the carriage rate of VTEC O26, O103, O111, O145, and O157 and the related risk of contamination of foodstuffs.

MATERIALS AND METHODS

Sample collection. From October 2003 to October 2005, 182 samples of cecal content and lymphatic tissue were collected from 182 cattle slaughtered in three abattoirs in northern Italy. This group of animals included 119 dairy cull cows and 63 feedlot cattle (cattle reared intensively, not on pasture; live weight of 550 to 650 kg). The sampling protocol had two parts. In part 1, 93 samples of cecal material and tonsils were collected from 93 cattle (67 dairy cows and 26 feedlot cattle). In part 2, 89 samples of cecal material and mesenteric lymph nodes were collected from 89 animals (52 dairy cows and 37 feedlot cattle). The animals came from 165 different farms located in nine regions of the country.

All samples were aseptically collected immediately after slaughter, placed in separate sterile containers, transported at 4°C to the laboratory, and examined on the day of collection.

Part 1. Immunomagnetic separation and slide agglutination tests for VTEC O26, O103, O111, O145, and O157: cecal material and tonsils. From October 2003 to September 2004, 93 fecal and 93 tonsil samples were collected from 93 slaughtered cattle and examined using an immunomagnetic separation (IMS) technique followed by slide agglutination (SA) with specific antisera. Aliquots of 10 g of cecal material and 20 g of lymphatic tissue were suspended in 90 and 180 ml, respectively, of modified tryptone soya broth (Oxoid, Basingstoke, UK) supplemented with 20 mg/liter novobiocin (Sigma-Aldrich, Steinheim, Germany). Following static incubation at 37°C overnight, 1 ml of the broth culture was added to 20 μl of five different sets of serogroup-specific IMS beads (Dynabeads anti–*E. coli* O157, Dynabeads EPEC/VTEC O26, Dynabeads EPEC/VTEC O103, Dynabeads EPEC/VTEC O111, and Dynabeads EPEC/VTEC O145, Dynal, Oslo, Norway) in five separate sterile microcentrifuge tubes. IMS was performed following the manufacturer's instructions. Following all steps, beads were suspended in 100 μl of phosphate-buffered saline (Oxoid) with 0.05% Tween 20.

For VTEC O157 detection, two 50-μl aliquots of the bacteria-bead complexes were streaked onto sorbitol MacConkey agar with 0.05 mg/liter cefixime and 2.5 mg/liter potassium tellurite (CT-SMAC; Oxoid) *(29)* and onto Chromocult Coliform Agar ES

(Merck, Darmstadt, Germany). Plates were incubated at 37°C for 18 to 24 h. Up to 10 sorbitol-nonfermenting colorless colonies grown on CT-SMAC or red β-glucuronidase–negative colonies grown on Chromocult Coliform Agar ES were selected and seeded into tryptone soya broth (TSB; LAB M, Bury, UK). The indole test was performed after 24 h of incubation at 37°C by using the Kovacs reagent. Indole-positive cultures were tested with an *E. coli* O157 latex agglutination test kit (Oxoid), and agglutinating cultures were confirmed biochemically as *E. coli* (API 20E, bioMérieux, Marcy l'Etoile, France).

For VTEC O26 detection, two 50-μl aliquots of the O26 Dynabeads–bacteria suspensions were plated onto MacConkey agar base (Difco, Becton Dickinson, Sparks, Md.) with 1 g/liter L(+)-rhamnose-monohydrate (Merck) and 0.05 mg/liter cefixime and 2.5 mg/liter potassium tellurite (CT-RMAC; Oxoid) and onto Chromocult Coliform Agar ES (Merck). Plates were incubated at 37°C for 18 to 24 h. Up to 10 rhamnose-nonfermenting colorless colonies grown on CT-RMAC and dark blue to violet β-glucuronidase–positive colonies grown on Chromocult Coliform Agar ES were selected and seeded into TSB for the indole test. Indole-positive cultures were tested with O26 antiserum (Denka Seiken, Tokyo, Japan), and agglutinating strains were confirmed biochemically (API 20E) as *E. coli.*

For detection of VTEC O103, O111, and O145, two 50-μl aliquots of the O103, O111, and O145 Dynabeads-bacteria complexes were seeded onto plates of Enterohemolysin Agar with blood (EHLY agar; Oxoid) and Chromocult Coliform Agar ES (Merck). After 18 to 24 h of incubation at 37°C, up to 10 colonies on EHLY agar that were surrounded by a narrow zone of hemolysis and resembling *E. coli* and dark blue to violet colonies grown on Chromocult Coliform Agar ES were seeded into TSB for the indole test. Indole-positive cultures were tested with specific antisera (Denka Seiken), and agglutinating cultures were subjected to biochemical identification.

Putative VTEC O157, O26, O103, O111, and O145 strains were tested for toxic activity on Vero cell monolayers as described by Caprioli et al. *(10).* Verocytotoxin-producing cultures were subjected to multiplex PCR (m-PCR) for the detection of the genes coding for cytotoxins (vtx_1 and vtx_2), intimin (*eae*), and enterohemolysin (*E-hlyA*). Crude DNA extracts were prepared from characterized VTEC strains by boiling one colony for 5 min in 50 μl of distilled water. Samples (2 μl) of each extract were amplified in 25-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl$_2$, 0.2 mM concentrations of each dNTP, 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif.), and 0.5 μM concentrations of each primer (pairs stx1F-stx1R, stx2F-stx2R, eaeAF-eaeAR, and hlyAF-hlyAR) *(25)*. Samples were amplified with a touchdown protocol. The initial step was incubation at 95°C for 10 min. This step was followed by 35 amplification cycles of denaturation at 95°C for 60 s, primer annealing at 65°C for 60 s decreasing 0.5°C every cycle for the first 10 cycles and then at 60°C for 60 s for the remaining 25 cycles, and extension at 72°C for 90 s. The final step was incubation at 72°C for 7 min. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

VTEC strains were sent to the reference national laboratory (Istituto Superiore di Sanità, Rome, Italy) for serogroup confirmation.

Part 2. Immunomagnetic separation and slide agglutination tests for VTEC O26, O103, O111, O145, and O157: cecal material and mesenteric lymph nodes. From January 2005 to October 2005, 89 fecal and 89 mesenteric lymph node samples

TABLE 1. *Number and prevalence of* E. coli *isolates positive for verocytotoxin production and other virulence determinants detected in the cecal content, tonsils, and mesenteric lymph nodes of cattle at slaughter[a]*

Sample type	Total no. of samples	VTEC O157		VTEC O26		Putative O157		VTEC O91		Total (%) VTEC-positive samples
		No. (%) of positive samples	Isolate virulence factors	No. (%) of positive samples	Isolate virulence factors	No. (%) of positive samples	Isolate virulence factors	No. (%) of positive samples	Isolate virulence factors	
Cecal content	182	6 (3.3)	vtx_1, vtx_2, eae, E-hlyA vtx_1, vtx_2, eae, E-hlyA vtx_2, eae, E-hlyA vtx_2, eae, E-hlyA vtx_2, eae, E-hlyA vtx_2, eae, E-hlyA	1 (0.5)	vtx_1, eae, E-hlyA	1 (0.5)	vtx_1, eae, E-hlyA	1 (0.5)	vtx_2, eae, E-hlyA	9 (4.9)
Tonsils	93	1 (1.1)	vtx_2, eae, E-hlyA	0		0		0		1 (1.1)
Mesenteric lymph nodes	89	1 (1.1)	vtx_2, eae, E-hlyA	0		0		0		1 (1.1)

[a] Isolates were identified based on results of immunomagnetic separation with slide agglutination, Vero cell assay, and m-PCR assay.

were collected from 89 slaughtered cattle randomly selected from three abattoirs in northern Italy. Samples were examined using the IMS-SA procedure with specific antisera (as described for part 1). Putative VTEC O157, O26, O103, O111, and O145 isolates were tested for toxic activity on Vero cell monolayers *(10),* and positive cultures were subjected to m-PCR for the detection of the vtx_1, vtx_2, eae, and E-hlyA genomic sequences, following the protocol of Paton and Paton *(25)* (as described for part 1). VTEC strains were sent to the reference national laboratory for serogroup confirmation.

RESULTS

A total of 364 samples of bovine origin were examined by IMS-SA for VTEC O26, O103, O111, O145, and O157 followed by the Vero cell assay and m-PCR for the detection of the virulence genes vtx_1, vxt_2, eae, and E-hlyA (Table 1). The prevalence of VTEC intestinal carriers among slaughtered cattle was 4.9% (9 of 182 animals). VTEC O157 was shed by 3.3% (6) of the 182 animals, O26 VTEC was shed by 0.5% (1 of 182), O91:H⁻ VTEC was shed by 0.5% (1 of 182), and a putative O157 strain that was positive for vtx_1, eae, and E-hlyA (whose preliminary SA result was not confirmed by the tube agglutination assay) was shed by 0.5% (1 of 182). The VTEC O91 strain was previously identified as *E. coli* O103 by IMS-SA because it cross-reacted with the O103 Dynabeads and the O103 antiserum and thus was reclassified as VTEC O91:H⁻ by the reference laboratory. Cattle shedding VTEC came from 9 (5.4%) of the 165 farms.

The prevalence of VTEC O157 in the lymphatic tissue was 1.1%, both in the tonsils (1 of 93 samples) and in the mesenteric lymph nodes (1 of 89 samples). The single lymph node in which the VTEC O157 strain was detected was hemorrhagic and enlarged. This pathologic change was uncommon in the other mesenteric lymph nodes examined. Lymphatic tissue contamination by VTEC O157 was observed only in animals that also were VTEC O157 fecal carriers and only during the warm season (June and September). No other VTEC serogroups were detected in the lymphatic tissue of slaughtered cattle.

DISCUSSION

This report is the first description of the simultaneous examination of bovine intestinal content and lymphatic tissue for VTEC O26, O103, O111, O145, and O157 in Italy. Previous surveys carried on in the country more commonly investigated VTEC O157 shedding by slaughtered cattle *(8, 9, 13)* or VTEC O157 fecal carriage by cattle on farms *(12).* In Italy, other studies concerning VTEC O157 and non-O157 carriage by slaughtered cattle have been focused on fecal shedding only *(6, 7).*

In this survey, VTEC strains of the O157 and O26 serogroups were detected, as was one VTEC O91:H⁻ strain previously considered a putative VTEC O103 strain. No VTEC O103, O111, and O145 strains were detected in the intestinal content or lymphatic tissues of these cattle.

The absence of selective media for VTEC O103, O111, and O145 serogroups made it necessary to use the time-consuming SA technique to test a large number of suspect colonies. Cross-reactions with bacteria other than *E. coli* also were observed: colonies of *Salmonella, Citrobacter freundii, Citrobacter diversus,* and *Enterobacter cloacae* were frequently misrecognized as *E. coli* because they had phenotypic characters similar to those of *E. coli* colonies on selective media and cross-reacted with VTEC O157, O26, and O145 antisera. Therefore, biochemical identification of suspect colonies was essential to avoid testing false-positive cultures with the Vero cell assay and m-PCR methods. The performance of the O157 latex agglutination test was satisfactory but not excellent; one (14.3%) of seven putative VTEC O157 isolates was not confirmed by the tube agglutination test. Other putative VTEC O26 and O103 isolates were not confirmed as such by the reference laboratory; therefore, the use of the tube agglutination test is recommended to confirm SA results.

The use of the IMS-SA technique followed by the Vero cell assay reduced the number of *E. coli* isolates processed by molecular assay for detection of virulence determinants. Because only 11 of 182 *E. coli* colonies detected with IMS-SA had toxic activity on Vero cell monolayers, the m-PCR

for vtx_1, vtx_2, *eae,* and *E-hlyA* genes was performed on a small number of isolates.

Compared with the results of previous surveys *(8, 9, 13),* the prevalence of VTEC O157 fecal carriers among cattle at slaughter in Italy was lower. The detection of VTEC O26 in cattle cecal content is of the greatest importance because in Italy this serogroup has surpassed *E. coli* O157 as the major cause of hemorrhagic colitis and hemolytic uremic syndrome in pediatric patients *(28).*

The isolation of VTEC O157 from the lymphatic tissue of cattle was of special interest. Two (33.3%) of six VTEC O157 fecal carriers were simultaneously lymphatic carriers of VTEC O157 strains. Because nine cattle were shedding VTEC, the prevalence of lymphatic tissue VTEC contamination was 22.2%.

One problem associated with lymphatic tissue contamination by *E. coli* O157:H7 in cattle is the well-known cross-reactivity between *E. coli* and *Brucella* spp. *(14).* Thus, cattle infected with VTEC O157:H7 would produce false-positive reactions in assays for diagnosis of bovine brucellosis. Nevertheless, the most important finding related to the detection of VTEC O157 in the lymphatic tissue of cattle at slaughter is that lymph nodes and tonsils could be a source of VTEC O157 meat contamination in addition to the previously identified fecal and environmental sources. Undercooking of minced beef products is a common cause of VTEC O157–associated foodborne outbreaks *(26),* and the Scientific Veterinary Committee of the European Commission *(27)* identified hamburgers and other ground beef products as high-risk foodstuffs for VTEC transmission to humans. Because the infectious VTEC dose can be as low as 10 cells *(15),* the accidental presence of VTEC O157–positive tonsil or lymph node fragments in raw or undercooked ground beef, hamburger patties, or fresh sausages could increase the risk of consumer exposure to VTEC strains that are pathogenic for humans. Therefore, the prevention of VTEC O157 contamination of bovine carcasses at slaughter should not be focused only on the reduction of VTEC fecal shedding by cattle, in which fecal concentrations can range from 10^2 to 10^5 CFU/g *(30)* or even 10^6 CFU/g *(23)* and 10^8 CFU/g *(16),* but should also focus on avoiding the accidental presence of lymphatic tissue fragments in all beef products.

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