Isolation and Characterization of Verocytotoxin-Producing
Escherichia coli O157 Strains from Dutch Cattle and Sheep


Departments of Medical Microbiology1 and Pediatrics,2 University Hospital Nijmegen, 6500 HB Nijmegen, Inspectorate for Health Protection, Food Inspection Service, 7200 GN Zaandam,3 Veterinary Public Health Inspectorate, 6800 DR Arnhem,4 and Department of the Science of Food of Animal Origin, Utrecht University, 3580 TD Utrecht,5 The Netherlands

Received 29 August 1997/Accepted 4 December 1997

In the periods from July to November 1995 and 1996, fecal samples from Dutch cattle and sheep were collected at the main slaughterhouses of The Netherlands, located at different geographic sites. The samples were examined for the presence of verocytotoxin (VT)-producing Escherichia coli (VTEC) of serogroup O157. E. coli O157 strains could be isolated from 57 (10.6%) of 540 adult cattle, 2 (0.5%) of 397 veal calves, 2 (3.8%) of 52 ewes, and 2 (4.1%) of 49 lambs. Immunomagnetic separation with O157-specific-antibody-coated beads appeared to be significantly more sensitive than conventional plating for detection of the organism in feces. With the exception of two isolates from adult cattle which appeared to be negative for VT genes, all animal isolates were positive for both VT (VT1 and/or VT2) and E. coli attaching-and-effacing gene sequences, and therefore, they were regarded as potential human pathogens. Although genotypic typing by pulsed-field gel electrophoresis revealed a wide variety of distinct restriction patterns, comparison of the 63 animal isolates with 33 fecal O157 VTEC strains previously isolated from humans with the diarrhea-associated form of the hemolytic-uremic syndrome showed a marked similarity between animal and human isolates: 30 (90.9%) of the 33 human isolates appeared to be of E. coli O157 strain types also isolated from cattle and sheep. It was concluded that Dutch cattle and sheep are an important reservoir of E. coli O157 strains that are potentially pathogenic for humans.

Verocytotoxin (VT)-producing Escherichia coli (VTEC) can be associated with a variety of human diseases, including mild diarrhea, hemorrhagic colitis, and the diarrhea-associated (D+) form of the hemolytic-uremic syndrome (HUS) (10). Of the numerous VTEC serotypes identified, O157:H7 and O157:H− (nonmotile) continue to be the dominant causes of illness in humans (1, 6). VTEC produces either or both of two phage-encoded toxins, VT1 and VT2. VTs are thought to cause the vascular endothelial damage observed in hemorrhagic colitis and HUS patients (26). An additional virulence factor contributing to the pathogenicity of VTEC is the formation of attaching-and-effacing lesions in the intestine of the host. The formation of attaching-and-effacing lesions is mediated by multiple genes encoded on a 35-kb chromosomal region called the locus of enterocyte effacement (21). Humans can become infected with VTEC following the consumption of contaminated foods or by direct transmission of VTEC from infected humans or animals (1, 6, 10). Undercooked ground beef and raw milk have most often been implicated in foodborne infections. Since healthy domestic animals, in particular, ruminants like cattle, sheep, and goats, can harbor VTEC in their feces, they are regarded as natural reservoirs of these pathogens (4).

In The Netherlands, similar to the situation in the rest of the world, an infection with O157 VTEC is the predominant cause of D+ HUS (27). The sources of the infectious agents in these cases of D+ HUS have not been defined. Upon examination of raw meats obtained from retail outlets in The Netherlands, O157 VTEC strains were isolated from 2 (0.3%) of 770 samples of raw minced mixed beef and pork (14). The organisms were not detected in samples of raw minced beef (n = 1,000), minced pork (n = 260), or poultry products (n = 300) (14). An epidemiological survey on the occurrence of O157 VTEC strains in Dutch domestic animals had never been undertaken. As a contribution to the understanding of the epidemiology of human VTEC infections, the present study describes the isolation and characterization of O157 VTEC strains from Dutch cattle and sheep. Fecal samples from adult cattle, veal calves, and sheep were collected at several slaughterhouses located at different geographic sites in the country. The samples were examined for the presence of O157 VTEC by performing both conventional plating and immunomagnetic separation (IMS). To determine the isolates’ potential as human pathogens, they were tested for the presence of three main virulence-associated genes (the VT1, VT2, and E. coli attaching-and-effacing [eae; encoded on the locus of enterocyte effacement] gene loci) and for toxin production. In addition, isolates were further characterized by phage typing, PCR-based fingerprinting, and pulsed-field gel electrophoresis (PFGE). The characteristics of the animal isolates were compared with those obtained previously for fecal O157 VTEC isolates from patients with D+ HUS (13).

MATERIALS AND METHODS

Collection and storage of fecal samples. In the periods from 24 July to 30 October 1995 and from 12 July to 22 October 1996, fecal samples from adult cattle and veal calves were collected weekly at the major slaughterhouses of The Netherlands. Five adult cattle slaughterhouses and four veal calves slaughterhouses were visited; these were located in different regions of the country. Immediately after slaughter, samples of the rectal contents were collected aseptically and were kept at 4 to 8°C. The adult cattle sampled were randomly selected. To trace the locations of the farm of origin of the animals, the ear tag...
numbers were recorded at the time that the fecal samples were taken. In The Netherlands, veal calves are fattened in an all-in-all-out system. Per group of veal calves derived from one herd, a random selection of 10% of the total number of calves was sampled, with a maximum of 10 calves being sampled. Flocking ewes and lambs were collected on two occasions (21 October and 25 November 1996) at the only major sheep slaughterhouse in the country. The samples were taken by rectal palpation, just before slaughter. Animals were randomly selected, and no information on the farms of origin was recorded.

The collected fecal samples were transported immediately to the laboratory, where the microbiological examination was started within 20 h.

**Isolation of O157 VTEC by selective plating.** A 20-g portion of each fecal sample was added to 180 ml of modified E. coli broth containing novobiocin (20 mg/ml; Sigma Chemical Co., St. Louis, Mo.); (Dynal) was performed with 1 ml of the filtrates, according to the instructions of 50-μl volumes of the 10−3 and 10−4 dilutions were spread plated onto sorbitol MacConkey agar (SMAC; Oxoid Ltd., Basingstoke, England), and 100-μl volumes of the 10−5 and 10−6 dilutions were spread plated onto SMAC supplemented with ceftaxime (0.05 mg/ml) and potassium tellurite (2.5 mg/liter) (Dynal, Oslo, Norway) (CT-SMAC) (28). The plates were incubated at 42°C for 18 to 20 h. Sorbitol-nonfermenting colonies (up to 12 per sample) were selected for confirmation. The isolates were inoculated onto SMAC supplemented with 4-methylumbelliferyl-β-D-glucuronic acid (MUG; 0.1 g/liter; Sigma) and on Levine’s eosin methylene blue (EMB) (Oxoid). Presumptive O157 VTEC isolates (by typical E. coli metallic sheen on L-EMB; the isolates were both sorbitol nonfermenting and β-glucuronidase negative on SMAC-MUG) were tested for agglutination with an E. coli O157 latex test kit (Oxoid). Isolates that gave a positive latex agglutination result were confirmed to be E. coli by using an API 20E biochemical test strip (bioMérieux, Lyon, France) and were confirmed to be of serotype O157:H7 or serotype O157:H− by serotyping at the National Institute of Public Health and the Environment, Bilthoven, The Netherlands (W. J. van Leeuwen). The sensitivity of the plating method was found to be about one organism g−1 of feces by performing inoculation experiments as described previously (15).

**Isolation of O157 VTEC by IMS.** After 6 to 8 h of incubation, about 5 ml of each mEC− broth culture was filtered through a piece of paper towel to remove particulate matter. IMS with magnetic beads coated with antibody to O157 (Dynal) was performed with 1 ml of the filtrates, according to the instructions of the manufacturer. The concentrates were inoculated onto CT-SMAC, and the plates were incubated at 37°C for 18 to 20 h. For each sample, up to 12 sorbitol-nonfermenting colonies were selected and confirmed as described above. Recovery experiments demonstrated that O157 VTEC could be detected at inoculum levels of about one organism g−1 of feces (15).

**VT and E. coli attaching-and-effacing genes.** The presence of VT1, VT2, and eae gene sequences was determined by a multiplex PCR assay as described previously (13). The IMS technique proved to be significantly more sensitive for the detection of E. coli O157 in fecal samples from naturally colonized cattle than appropriately sized pieces that were washed five times for 1 h at room temperature in 100 mM Tris (pH 8)−5 mM MgCl2, preincubated for 1 h at 4°C in complete restriction enzyme buffer (10 mM Tris-acetate [pH 7], 10 mM magnesium acetate, 50 mM potassium acetate, 0.1 mg of RNase A per ml) without restriction enzyme, and preincubated for 1 h at 4°C in complete restriction enzyme buffer containing the enzyme. Digestion was carried out with XbaI (10 U), Boehringer Mannheim, Mannheim, Germany) at 37°C for 15 h. The resulting DNA fragments were resolved in 1.2% agarose gels (Pulsed Field Certified Agarose; Bio-Rad) in 0.5% Tris-borate-EDTA buffer at 13°C in a contour-clamped homogeneous electric field (CHEF) DR II apparatus (Bio-Rad). The run time was 24 h, with a constant voltage of 200 V and a linearly ramped pulse time of 3 to 50 s. Lambda concatemers (Bio-Rad) were used as DNA size markers. After staining with ethidium bromide (1 μg/ml), the gels were photographed under UV transillumination. The genomic patterns were analyzed with a Biogene program (vilber Lourmat, marne la Vallee, France), which uses the algorithm Nei and Li (2%) confidence. To compare the PFGE patterns of isolates of animal and human origin, chromosomal DNAAs from O157 VTEC strains isolated from patients with D+ HUS between 1989 and 1996 were analyzed by PFGE as well.

**RESULTS**

**Isolation of E. coli O157.** In 1995, E. coli O157 strains were isolated from 30 (11.1%) of 270 fecal samples from adult cattle and from 1 (0.5%) of 183 samples from veal calves (Table 1). Similar results were obtained in 1996: E. coli O157 strains were isolated from 27 (10.0%) of 270 fecal samples from adult cattle and from 1 (0.5%) of 214 samples from veal calves. For both adult cattle and veal calves, the location of slaughter was not linked to the location of origin of the animals. The majority of adult cattle sampled comprised dairy cows originating from different dairy farms located throughout the country (Fig. 1). No marked geographic variation in the prevalence of E. coli O157 was demonstrated. Cattle originating from farms located in the western part of the country seemed to be less often infected than cattle originating from farms located in other parts of the country, but this may be the result of the smaller number of animals originating from the west delivered for slaughter (Fig. 1). The farms of origin of 39 animals, including 6 animals positive for E. coli O157, could not be traced. The farms of origin of the 397 veal calves sampled (45 herds) were located predominantly in the central part of the country, where calf-fattening herds are most concentrated.

| Year of isolation | Animal source of isolates | No. (%) of animals E. coli O157 positive | No. of isolates obtained by the following methoda |
|------------------|--------------------------|----------------------------------------|-------------------------------------------------
| 1995             | Adult cattle             | 30/270 (11.1)                          | SMAC 7/9 (100) CT-SMAC 4/9 (23) IMS 2/9 (11) |
|                  | Veal calves              | 1/183 (0.5)                            | NDb                                                  |
| 1996             | Adult cattle             | 27/270 (10.0)                          | SMAC ND b CT-SMAC ND b IMS 0/9 (0)                   |
|                  | Veal calves              | 1/214 (0.5)                            | NDb                                                  |

a SMAC, isolation on SMAC following enrichment in mEC+n for 18 to 20 h; CT-SMAC, isolation on CT-SMAC following enrichment in mEC+n for 18 to 20 h; IMS, isolation on CT-SMAC following enrichment in mEC+n for 6 to 8 h and IMS of E. coli O157.

b ND, not done.
the conventional plating methods (sign test; \( P < 0.0001 \)) (Table 1), although in 1995 one false-negative result was obtained by IMS. Therefore, only the IMS procedure was performed for the isolation of fecal O157 VTEC from sheep.

Characterization of E. coli O157 isolates. The results of the PCR assay, the Vero cell assay, and phage typing are presented in Table 2. RAPD-PCR of the E. coli O157 isolates from cattle and sheep showed highly monomorphic patterns which mutually differed only by minor differences in the intensities of single bands. The banding profile produced by the animal isolates could not be distinguished from the DNA fingerprint exhibited by the human isolate that represented the major group of human isolates (79% of the strains) characterized previously (13). Digestion of genomic DNA from the animal and human isolates with XbaI and analysis by CHEF-PFGE yielded between 17 and 25 discernible fragments that ranged from approximately 20 to 590 kb in length. The results are summarized in the last column of Table 2. Forty-six distinct genomic patterns were generated from the 59 cattle isolates. The four isolates from sheep exhibited four distinct patterns. Among the collection of 33 human isolates, 28 distinct restriction patterns could be discriminated. Figure 2 shows the fragment patterns generated from the cattle and human isolates of phage types 2, 4, and 8.

DISCUSSION

Cattle are regarded as a major source of human pathogenic O157 VTEC strains. Reported estimates of the prevalence of

<table>
<thead>
<tr>
<th>Phage type</th>
<th>Vero cell assay for VT</th>
<th>PCR result for the following gene:</th>
<th>Cattle (n = 59)</th>
<th>Sheep (n = 4)</th>
<th>Human (n = 33)</th>
<th>No. of distinct XbaI patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VT1</td>
<td>VT2</td>
<td>eae</td>
<td>No. (%) of isolates</td>
<td>No. of distinct XbaI patterns</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>15 (45.5)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>3 (5.1)</td>
<td>0</td>
<td>10 (30.3)</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4 (6.8)</td>
<td>0</td>
<td>1 (3.0)</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6 (10.2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2 (3.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>7 (11.8)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2 (3.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2 (3.4)</td>
<td>1 (25.0)</td>
<td>0</td>
</tr>
<tr>
<td>49</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>3 (5.1)</td>
<td>0</td>
<td>2 (6.1)</td>
</tr>
<tr>
<td>54</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rdnc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8 (13.5)</td>
<td>1 (25.0)</td>
<td>0</td>
</tr>
<tr>
<td>rdnc</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

---

a Including a group of four and a group of three identical patterns, all of which were generated from human isolates.

b Isolate from a lamb.

c The three cattle isolates of phage type 4 and the three cattle isolates of phage type 10 generated identical patterns.

d Including a group of three identical patterns generated from two cattle and one human isolate.

e Two cattle isolates shared the same pattern.

f One isolate from a veal calf.

g Three isolates generated identical patterns.

h Including two groups of two identical patterns.

i One of the cattle isolates showed a pattern identical to that of the sheep isolate.

j rdnc, reacted with phage set but did not correspond to recognized phage type.
O157 VTEC in North American and European cattle range from 0 to almost 10% (1). Sheep, the second most commonly reared species of ruminant food animals, appears to have a role similar to that of cattle as a natural reservoir of O157 VTEC. Kudva et al. (19) studied 35 free-ranging healthy ewes of a single flock in Idaho and reported that the incidence of fecal shedding varied from 31% of sheep in June to none in November. In the United Kingdom, O157 VTEC strains were found in the feces from 18 (2.6%) of 700 sheep sampled at a slaughterhouse (8). In the present study, O157 VTEC strains could be isolated from about 10% of Dutch adult cattle, from 0.5% of Dutch veal calves, and from 4.0% of Dutch sheep. The ability to compare published prevalence data is limited because of the use of a large variety of screening methods. Several recent studies have demonstrated that selective enrichment significantly improved the sensitivity of direct plating of fecal samples from cattle and sheep (e.g., dairy herd versus slaughterhouse). Furthermore, geographic and seasonal variations in prevalence may occur. It has been observed that shedding of O157 VTEC by cattle and sheep peaks during the summer (11, 19, 20), parallel to the seasonal variation in the reported cases of O157 VTEC infections in humans (6, 10). The noted difference in the rate of isolation of O157 VTEC from mature cattle and veal calves may be due to differences in the composition of the gastrointestinal flora resulting from differences in diet. Orally administered antibiotics can also interfere with the gastrointestinal flora. Besides differences in the composition of the intestinal flora, differences in the handling of the animals may explain the differences in the rate of isolation of O157 VTEC. Whereas veal calves are directly sold and transported from fattening herds to a slaughterhouse, adult cattle (as well as sheep) can pass several stations and therefore are at risk of coming into contact with infected animals.

With the exception of two isolates, all E. coli O157 isolates from cattle and sheep contained VT and eae genes and therefore appeared to be potential human pathogens. It might be possible that the two VT-negative E. coli O157 isolates have lost their VT-encoding phages (17). By comparison of the animal and human isolates by their phage types and VT genotypes, 23 types of E. coli O157 were identified among the 96 isolates, of which only 5 were found in both animals and humans. Of the 33 human isolates, 30 (90.9%) were of types also isolated from cattle, whereas only 20 (33.9%) of the 59 cattle isolates were of types also found in humans. A similar degree of shared strain types among cattle and sporadic human isolates has been reported by Chapman and Siddons (7), based on

![Agarose gels showing XbaI digestion patterns (designated A to AI) of fecal E. coli O157 isolates of phage types (PT) 2, 4, and 8 from cattle (A) and from patients with D+ HUS (B).](image-url)
phage type, VT genotype, and plasmid content. The marked similarity between cattle and human isolates further supports the theory that cattle are an important reservoir of O157 VTEC potentially pathogenic for humans. However, it remains unclear why certain types appeared to be more often associated with human disease than others. In our study, VT2-producing strains of phage types 2 and 4 represented approximately 75% of the human isolates, whereas these types accounted for only circa 7% of the cattle isolates. Among the four isolates from sheep, we identified four strain types, of which one was also isolated from one patient with D+ HUS. The RAPD-PCR profiles of the E. coli O157 isolates were highly monomorphic, whereas there was polymorphism among the PFGE profiles. Seventy-six distinct XbaI patterns were identified among the 96 E. coli O157 strains. On the basis of the combined results of phage typing, VT genotyping, and PFGE, 77 distinct E. coli O157 types were identified, and only 1 (3.0%) of the 33 human isolates could not be discriminated from 2 (3.4%) of the 59 isolates from cattle (Fig. 2, pattern K). These two identical cattle isolates possibly originated from the same farm, since their ear tag numbers corresponded to the same postal box number. The small degree of overlap between the animal and human isolates observed by comparing PFGE results does not lead to a different conclusion regarding the potential pathogenicity of E. coli O157 strains harbored by cattle and sheep for humans, but it supports the observations of previous studies demonstrating the value of PFGE as an epidemiologic tool for the differentiation of E. coli O157 strains (2, 3, 12, 16, 22). Although PFGE was clearly more sensitive, in one instance phage typing could distinguish between two strain types that generated identical XbaI patterns.

From the data presented in this survey, it can be concluded that Dutch cattle and sheep are natural hosts of E. coli O157 strains potentially pathogenic for humans. The use of a sensitive isolation technique has proven to be essential for the detection of these pathogens in fecal samples. PFGE has appeared to be a highly sensitive method for distinguishing between apparently unrelated E. coli O157 strains. A further study to determine the prevalence of O157 VTEC in dairy herds in The Netherlands is being performed in our laboratory. By analyzing the relationship between these pathogens and the farm environment, we hope to eventually reduce the risk of O157 VTEC-positive animals going to slaughter and, in turn, the risk of O157 VTEC infections in humans.

ACKNOWLEDGMENTS

We thank the several slaughterhouses for their cooperation with this research. Also, the excellent assistance of Karel Wernars and Paula Leeflang in analyzing the restriction fragment patterns is gratefully acknowledged.

This study was supported by the Prevention Fund (grant 28-2354).

REFERENCES


