Vero Cytotoxigenic Escherichia coli Infection in Dairy Farm Families

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Fecal samples from 335 dairy farm residents and 1458 cattle on 80 farms were tested for Vero cytotoxin (VT)-producing Escherichia coli (VTEC). Residents were also tested for antibodies to VT1 and O157 lipopolysaccharide (LPS). Residents and cattle on farms with VTEC-positive persons or E. coli O157:H7-positive cattle were retested. Twenty-one persons (6.3%) on 16 farms (20.8%) and 46% of cattle on 100% of the farms had VTEC in fecal samples. Human VTEC isolates included E. coli O157:H7 and 8 other serotypes, 4 of which were present in cattle on the same farms. More persons had antibodies to VT1 (41%) than to O157 LPS (12.5%). Seropositivity to O157 LPS was associated with isolation of E. coli O157:H7 on the farm (P = .022). Human VTEC infection was negatively associated with age (P < .05) and was not associated with clinical illness. Many dairy farm residents experience subclinical immunizing VTEC infections at a young age, which frequently involve non-O157 VTEC found in cattle.

Escherichia coli O157:H7 is responsible for most cases of hemolytic uremic syndrome in children [1, 2] and is a common cause of bloody and nonbloody diarrhea in Canada and the United States [3–5]. Vero cytotoxin (VT) production has been proposed as the mechanism by which E. coli O157:H7 causes severe disease [6]. This property is not restricted to E. coli O157:H7; VT-producing E. coli (VTEC) belonging to >160 other serotypes have been isolated from humans [7]. While not all of these serotypes have been shown to cause disease, >50 have been associated with outbreaks or sporadic cases of bloody diarrhea and hemolytic uremic syndrome [1, 8–12]. The burden of disease attributable to VTEC serotypes other than E. coli O157:H7 remains unclear because there are no simple tests to detect and identify these organisms.

Consumption of improperly cooked ground beef has been identified most frequently as the risk factor for E. coli O157:H7 infection in outbreaks and among sporadic cases [4, 13, 14]. Infection may also occur following consumption of other contaminated foods, including unpasteurized milk [4, 15], and by person-to-person transfer [16, 17]. E. coli O157:H7 and other VTEC have been isolated from ground beef [18, 19] and bovine feces [20–22], confirming that cattle are an important reservoir of these organisms. However, in both ground beef [19] and bovine feces [20], E. coli O157:H7 is far exceeded in prevalence by non–O157:H7 VTEC serotypes, many of which have been isolated from humans and associated with human illness [7]. Non–O157 VTEC of bovine origin are thus of potentially broad public health significance.

Because dairy farm families are exposed to high levels of bovine VTEC through direct contact with cattle manure and through consumption of unpasteurized milk [15, 23], they constitute a model of naturally occurring transmission of these organisms from cattle to people. Therefore, to assess the role of VTEC serotypes of bovine origin in human disease, we studied a cohort of dairy farm families.

Materials and Methods

Farm Visits. A formal random sample of 80 dairy farms (0.93%) from among the 8562 dairy producers in 12 southern Ontario counties was visited between July 1992 and February 1993. A standardized questionnaire was used to obtain information on potential risk factors for VTEC infection, including patterns of consumption of food, water, and unpasteurized milk; food handling practices; exposure to farm animals and their manure; off-farm employment; and antibiotic use. Families were also questioned regarding episodes of diarrhea (defined as one or more loose stools on 2 consecutive days) in the 1-month periods preceding and following the farm visit and regarding their lifetime histories of bloody diarrhea or renal disease.

Each participant was asked to provide a fecal sample and a blood sample. Fecal samples were collected in an equal volume...
of transport medium [24] and refrigerated for up to 3 days until processed. Blood samples were obtained from participating family members within 3 months of the original farm visit. Sera were harvested and stored at −20°C. On each farm, a rectal swab sample was obtained from all calves <3 months of age and from a random sample of the milking herd, consisting of 25% of the herd or a minimum of 10 cows.

On farms having ≥1 VTEC-positive family member(s) or ≥1 cattle positive for E. coli O157:H7, permission was sought to resample and test fecal samples from all family members and previously tested cattle ~1 and 3 months after the initial visit. Family members were asked to record episodes of diarrheal illness of ≥2 days duration until 1 month after the date of the third visit.

Detection and isolation of VTEC. Fecal samples and rectal swabs were cultured and screened for the presence of VTEC by detection of VT genes and VT production. Cultures with evidence of VT production were then processed to obtain VTEC isolates. For screening, rectal swabs or ~1 mL of feces in transport medium were grown overnight at 37°C in 9 mL of MacConkey broth (Difco, Detroit) and then subcultured at a 1:10 dilution in 5 mL of brain-heart infusion broth (BHI; Difco). The remaining MacConkey broth culture was stored at 4°C. After overnight incubation at 37°C, filtrates from 1 mL of the BHI cultures were tested at 5-fold dilutions (1:5 to 1:625) by a Vero cell cytotoxicity assay (VCA) [15]. Bacteria pelleted by centrifugation of 1 mL of the same BHI cultures were tested for the presence of VT genes by polymerase chain reaction (PCR) as described below. A portion of the remaining BHI culture was stored in 15% glycerol at −70°C. Results of the VCA were recorded as negative (no cytotoxicity) or as the highest sample dilution with >50% destruction of the Vero cell monolayer. Samples with Vero cytotoxicity affecting <50% of the monolayer at a 1:5 dilution were scored as weak positives.

To obtain VTEC isolates, MacConkey broth cultures corresponding to BHI filtrates with VCA titers of ≥1:5 were streaked onto MacConkey agar and grown overnight at 37°C. Five colonies and a mixture of colonies (sweep) from the initial streak area of each plate were inoculated separately into 1 mL of BHI, cultivated overnight at 37°C, and prepared and tested by VCA as described above. If a sample failed to yield a VCA-positive individual colony but had a sweep VCA titer of ≥1:25, additional groups of 5 individual colonies from the MacConkey agar plate were tested similarly. The maximum number of colonies tested from any 1 bovine or human fecal sample was 20 and 40, respectively. VCA-positive colonies obtained in this way were plated onto sorbitol-MacConkey agar, tested for the presence of the O157 antigen by slide agglutination tests with O157 antiserum (Difco), and held for serotyping. A portion of the BHI culture of these colonies was preserved in 50% glycerol for testing by PCR to confirm the presence of VT genes, to identify toxin type, and for detection of the eaeA gene (see below). Serotyping of all confirmed VTEC isolates and phage typing of E. coli O157:H7 isolates were done by the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control.

The PCR procedure for testing BHI cultures of feces and VCA-positive individual colonies used synthetic oligonucleotide primers designed to target conserved sequences in VT1, VT2, and VTE genes (generic VT primers) [25]. Toxin typing (VT1, VT2, and VTE) and detection of the eaeA gene for confirmed VTEC isolates were done using established primers and protocols [26–28]. Samples were prepared for PCR as described previously [25], except that bacteria were washed with PBS (FA buffer, Difco) rather than normal saline. The amplified product from all PCR protocols was visualized by standard submarine gel electrophoresis.

Serologic testing. Sera were titrated for VT1 neutralizing antibodies as described previously [29]. We did not attempt to detect VT2 neutralizing antibodies since tests for these antibodies are unreliable [30, 31]. Antibodies to the O157 lipopolysaccharide (LPS) were detected by testing sera for a 1:320 dilution of serum with an ELISA having sensitivity, specificity, and positive and negative predictive values of 95%, 94%, 80%, and 98%, respectively for E. coli O157:H7 infection [32].

Classification of VTEC infection status. Cattle and humans were classified as VTEC-positive if their initial fecal cultures were positive for VT genes by PCR with generic VT primers or if a VTEC isolate was obtained from their feces. Individual E. coli isolates were classified as VTEC if they were positive in the VCA and contained VT genes when tested by PCR with generic VT primers. Participants were classified as positive for VT1 neutralizing antibodies if their titers were ≥1:4 and as seropositive for O157 LPS antibodies if their net O157 LPS ELISA values were greater than a cut-off value established by testing sera from a control population of 256 urban residents [32].

Statistical analysis. The relationship between VTEC carriage in humans and potential risk factors and health outcomes at the individual level was tested using logistic regression analysis appropriately weighted to account for the possibility of clustering of individual person effects within families [33]. The relationship between the presence of antibodies to VT1 or O157 antigen and potential risk factors for seropositivity was tested in an analogous manner. The relationship between VTEC carriage or serologic status of families and farm-level risk factors for infection was tested using contingency tables and least-squares regression analysis.

Results

Compliance. We contacted residents on 138 farms to obtain 80 farm families that would agree to respond to questionnaires and to allow testing of their cattle (farm compliance, 58%). Stool samples were obtained from 335 (89.6%) of 374 family members on 77 (96.3%) of 80 farms on the first visit. Blood samples were provided by 235 persons (62.8%) in 68 families (85%). Diarrheal histories for the 30 days following the first visit were returned by 51 (63.8%) of 80 families. Eighteen farms were retested ∼30 and 90 days later, and one additional farm was revisited only at 90 days. Of 115 family members on these farms, 61 (55%) provided stool samples on all visits, and at least 1 person in 15 of the 19 families provided a stool sample on all visits. Diarrheal histories for the 30 days following stool collection were returned by 9 of 18 families after the second sampling, and 5 (26.3%) of 19 families after the third visit. Compliance for cattle testing was 100% at each visit. On retested farms, 76% of the cattle sampled on the first visit were tested at subsequent visits.
Table 1. Summary rates of VTEC infection in Ontario dairy farm families and family members over a 3-month period.

<table>
<thead>
<tr>
<th></th>
<th>No. of samples VTEC-positive*</th>
<th>no. of samples submitted (%)</th>
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<tbody>
<tr>
<td></td>
<td>First visit (month 0)</td>
<td>Second visit (month 1)</td>
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<tr>
<td>Families</td>
<td>16/77 (21)</td>
<td>4/18 (22)</td>
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<tr>
<td>Persons</td>
<td>21/335 (6)</td>
<td>4/76 (5)</td>
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* Stool cultures were positive for verocytotoxin genes by polymerase chain reaction or yielded VTEC isolate.

Human stool culture. Evidence of VTEC was found in stool samples from 21 (6.3%) of 335 family members on 16 (20.8%) of 77 farms on the first visit. Twenty-four persons on these 16 farms were VTEC-positive on at least one of the three visits (tables 1, 2). VTEC were isolated from 9 (37.5%) of the 24 VTEC-positive persons, and the remaining 15 were VTEC-positive by PCR only (table 2). The VTEC isolates belonged to 9 serotypes, including E. coli O157:H7, which was isolated from a healthy 6-month-old child on one farm. Four of these serotypes were also isolated from cattle on the same farm. Among families having VTEC-positive persons, 6 (37.5%) had a VTEC-positive person on more than one visit, whereas among VTEC-positive persons, 3 (12.5%) were positive on more than one visit (table 2). Human VTEC carriage had a negative linear relationship with age by logistic regression analysis ($P = .045$) (figure 1).

VTEC carriage in cattle. All 80 herds and 679 (45.9%) of 1478 cattle were VTEC-positive on the first visit. Twelve cattle (0.8%) on 7 (8.8%) of 80 farms were positive for E. coli O157:H7 on at least one visit. On the 16 farms having a VTEC-positive family member, 50% of 303 cattle were VTEC-positive on the first visit. Among isolates from these cattle, 61% produced VT1 either alone (42%) or in combination with VT2 (19%), and 39% produced only VT2. The eaeA gene was present in 37% of these isolates, and at least 1 of the VTEC serotypes was isolated from cattle on 15 of the 16 farms over all visits (figure 2). On the initial visit, 679 cattle (44.9%) were positive for VTEC on the 64 farms having no VTEC-positive family members.

Table 2. Microbiologic findings from 3 fecal cultures of 24 VTEC-positive residents on 16 Ontario dairy farms over a 3-month period.

<table>
<thead>
<tr>
<th>Farm (no. of persons in family)</th>
<th>Person code</th>
<th>Age (years)</th>
<th>PCR</th>
<th>Serotype isolated*</th>
<th>Toxin type</th>
<th>Sample 1 (month 0)</th>
<th>Sample 2 (month 1)</th>
<th>Sample 3 (month 3)</th>
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NOTE. VTEC-positive residents had stool cultures positive for verocytotoxin (VT) genes by polymerase chain reaction (PCR) or yielded VTEC isolate. NS, not sampled; NM, non-motile.

* [+] and [−], presence or absence, respectively, of eaeA gene in VTEC isolate.

† Also isolated from cattle on same farm.
**Human serologic status.** Among 68 families submitting blood samples, 46 (67.7%) had at least 1 person seropositive for VT1, and 20 (29.4%) had at least 1 person seropositive for O157 LPS. Overall, 97 (41.3%) of 235 family members were seropositive for VT1, and 30 (12.5%) were seropositive for O157 LPS. Seropositivity rates to each antigen showed markedly different age trends (figure 1). The proportion of persons with antibodies to O157 LPS increased gradually from birth, with a peak prevalence (26%) in the 40- to 50-year-old age group that was significantly greater than that of other age groups ($P < .05$). In contrast, the prevalence of VT1 antibodies was greatest (78%) in children $<5$ years of age, and antibodies declined significantly with increasing age ($P < .001$). The proportion of family members seropositive for VT1 was positively associated with family size ($P = .05$).

The presence of a person with antibody to O157 LPS was associated with isolation of *E. coli* O157:H7 from an animal or human on the same farm ($P = .022$). However, antibody to O157 LPS was not associated with VT1 antibodies in individuals ($P = .94$). Seropositivity to VT1 in farm residents was also not associated with the isolation from animals of VTEC serotypes previously implicated in human disease [7] or VTEC carrying the *eaeA* gene.

**Health outcomes and risk factors for human VTEC carriage and seropositivity to VT1 and O157 LPS.** Twenty-nine family members (8.7%) reported at least one episode of diarrhea lasting $\geq 2$ days during the period from 1 month before to 1 month after the initial visit. Diarrhea was not associated with either VTEC carriage or with seropositivity to O157 LPS or VT1 in family members ($\alpha = 0.05$). Renal failure, hemolytic uremic syndrome, or bloody diarrhea were not reported in the lifetime histories of any family members. Among 374 members of 80 families, 269 (71.9%) in 67 families (83.8%) reported routine consumption of raw milk, and 18 (4.8%) in 9 families (11.3%) reported consumption of rare or raw hamburger during the month prior to the initial visit. The probability of human VTEC carriage was negatively associated with consumption of hamburger ($P = .0009$). No additional risk factors for human VTEC carriage or seropositivity to O157 LPS or VT1 were identified.

**Discussion**

A substantial number of dairy farm residents participating in this study had evidence of current or past infection with VTEC on the basis of stool culture (6.3%) or serologic status (41.3%). Most of these infections appeared to be due to VTEC other than serogroup O157 since a much higher proportion of persons had antibodies to VT1 (41.3%) than to O157 LPS (12.5%) and there was no association between seropositivity to VT1 and O157 LPS. In addition, VT1 antibodies were acquired early in life, whereas the frequency of antibodies to O157 LPS peaked in those persons 40–50 years of age. Furthermore, *E. coli* O157:H7 was isolated on only 8.8% of farms and only 1 of the 9 human VTEC isolates was *E. coli* O157:H7. Seven of the 8 other human VTEC isolates belonged to serotypes previously associated with human infection [7].

The most likely source of VTEC infecting family members was cattle on the same farm. Non-O157 VTEC producing VT1 and VT2 either alone or in combination were prevalent in cattle on all farms, and many of the VTEC serotypes isolated from cattle have been associated with human illness [7]. Of the 9 VTEC serotypes found in family members, 4 were also isolated from cattle on the same farm, and the presence of *E. coli* O157:H7 on the farm was associated with antibodies to O157 LPS.
LPS in family members. Transmission of VTEC from cattle to people in this setting is not surprising, since dairy farm families are exposed directly to cattle manure and many consume raw milk, both known risk factors for human VTEC infection [34, 35]. Although exposure to cattle or manure was not identified as a risk factor for VTEC infection in this study, VTEC are probably widespread in the farm environment, making differences in environmental exposure difficult to detect by the study methods used. The apparent clustering of VTEC carriage within certain families (table 2) could have been due to common practices leading to exposure in these families or to person-to-person transmission.

The eaeA gene encodes a colonization factor that has been proposed as an additional virulence factor required for infection by VTEC [36]. We have shown that presence of this gene was highly associated with certain E. coli O serogroups from animals on these farms and that these serogroups more commonly produced VT1 [28]; however, only 5 of the 9 VTEC isolated from farm residents carried the eaeA gene, and there was no correlation between the presence of eaeA-positive VTEC on the dairy farms and seropositivity to VT1 in farm family members. These findings suggest that the role of the eaeA gene in human VTEC infection needs to be better delineated.

VTEC infection occurred at a very young age in subjects in this study, as indicated by both the presence of VTEC in feces and seroprevalence of antibodies to VT1. More than 60% of farm children <10 years old had antibodies to VT1, while in a separate study [37], only 5.0% of urban children in the same age group in Toronto had these antibodies. However, the absence of clinical disease was notable among the 13 children <10 years old who had evidence of current VTEC infection, because this age group in the general population has the highest incidence of VTEC-associated hemolytic uremic syndrome [1] and diarrhea [3]. While this may reflect lesser virulence of the VTEC strains infecting the children, at least 2 isolates from the children, E. coli O157:H7 and E. coli O5:NM, have been associated with bloody diarrhea and hemolytic uremic syndrome [1, 8].

<table>
<thead>
<tr>
<th>Farm No.</th>
<th>VTEC serotypes isolated from cattle over three farm visits</th>
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<tbody>
<tr>
<td>29</td>
<td><strong>O103:H21</strong>[+], O113:H4, <strong>O113:H21</strong>, <strong>O157:H71</strong>[+]</td>
</tr>
<tr>
<td>67</td>
<td><strong>O22:H8</strong>, O163:NM, O?:H2, O?:NM[+]</td>
</tr>
<tr>
<td>74</td>
<td><strong>O22:H8</strong>, <strong>O26:H11</strong>[+], O84:NM[+], <strong>O103:H27</strong>[+], <strong>O111:NM</strong>[+], O115:H8, O119:NM[+], <strong>O145:NM</strong>[+], O156:NM, O?:H8, O?:H16, <strong>O171:NM</strong>, OR:H8, OR:H21, OR:NM</td>
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<tr>
<td>79</td>
<td><strong>O103:H2</strong>[+], O156:NM, <strong>O157:H71</strong>[+], O?:H21</td>
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<tr>
<td>82</td>
<td>O8:H19, O76:H7, O84:NM[+], O98:H25[+], <strong>O111:NM</strong>[+], <strong>O111:NM</strong>[+], O119:NM, O146:H8, O156:NM, <strong>O7:H21</strong>, O?:NM</td>
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<tr>
<td>87</td>
<td><strong>O103:NM</strong>[+], O132:NM, O153:H31, O156:NM, O?:H21</td>
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<tr>
<td>92</td>
<td>O76:H7, O84:NM[+], O98:H25[+], O111:NM[+], O119:NM, O146:H8, O156:NM, O?:H21, O?:NM</td>
</tr>
<tr>
<td>97</td>
<td><strong>O2:H29</strong>, O76:H7, O26:HI11[+], O76:H7, O84:NM[+], O156:NM, <strong>O157:H71</strong>[+], O?:H4, <strong>O2:H21</strong></td>
</tr>
<tr>
<td>115</td>
<td><strong>O111:NM</strong>[+], O113:H21, O?:NM[+]</td>
</tr>
<tr>
<td>121</td>
<td>O8:H19, O69:HI11[+], O136:NM, O?:H25</td>
</tr>
<tr>
<td>128</td>
<td>O69:HI11[+], <strong>O7:H21</strong>, O?:H25, O?:H28, O?:NM</td>
</tr>
<tr>
<td>133</td>
<td><strong>O26:H11</strong>[+], O113:NM, O119:H25[+], O136:H12, OR:NM</td>
</tr>
</tbody>
</table>
The lack of disease due to VT1-producing *E. coli* in farm residents in this study may reflect protection associated with VT1 antibodies induced by previous exposure [37]. However, the 4 persons infected with VT2-producing *E. coli* also were asymptomatic, including an infant infected with *E. coli* O157:H7 producing only VT2. Since *E. coli* producing VT2, either alone or with VT1, were as prevalent (58%) in cattle as those producing VT1 (61%), farm residents may have had sufficient exposure to VT2-producing *E. coli* to develop antibodies to this toxin. We did not attempt to measure VT2 neutralizing antibodies; however, repeated exposure has been suggested to explain the development of VT2 antibodies detectable by ELISA [38].

Continuing or recurrent exposure to VTEC in the farm environment may offer protection against subsequent exposure to *E. coli* O157:H7 or other virulent VTEC serotypes. This hypothesis is supported by the pattern of illness in a family outbreak of VT1-producing *E. coli* O111:NM infection on a dairy farm in the same geographic area as the farm cohort in this study. All VT1-seronegative urban relatives visiting the farm developed diarrhea, including a child who developed hemolytic uremic syndrome, whereas none of the VT1-seropositive relatives living on the farm became ill [37]. As evidenced in the present study, immunizing infections appear to occur early in life, and in view of the absence of bloody diarrhea from family histories, are frequently caused by VTEC other than serotype O157:H7. This is consistent with the predominance of non-O157:H7 serotypes among VTEC isolated from cattle in both the current study and previous studies [20, 21]. Initial immunizing infections of farm residents may cause uncomplicated diarrhea, since many VTEC isolated from cattle belong to serotypes associated with this form of VTEC-related human illness [1, 7]. More serious disease may occur where susceptible farm residents are exposed to *E. coli* O157:H7 present in cattle on the farm [35].

Our findings indicate that many dairy farm residents experience immunizing VTEC infections, which frequently are mild or subclinical and often involve non-O157 VTEC found in cattle. Exposure to the dairy farm environment may have greater health significance for urban residents and specific subgroups within the rural community. Urban residents who visit farms, have direct contact with cattle [35, 37], or consume unpasteurized milk [15, 23] may be expected to have a higher risk of VTEC infection and disease due to less prior exposure to VTEC. In addition, children with declining maternal immunity, the elderly, and other immunocompromised individuals who live on dairy farms may have increased risk of infection and VTEC-associated disease.

Acknowledgments

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References


