Escherichia coli O157 Prevalence in Different Cattle Farm Types and Identification of Potential Risk Factors

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Abstract

Although the prevalence of Escherichia coli O157 on cattle farms has been examined extensively, the relationship between this pathogen and farm type has been established only rarely. A large-scale study was designed to determine the prevalence of E. coli O157 in the Flemish region of Belgium on farms of dairy cattle, beef cattle, and veal calves. The effect of various factors on the occurrence at the pen level also was evaluated. In 2007, 180 farms were randomly selected based on region, farm size, and number of animals purchased and were examined using the overshoe sampling method. When possible, overshoes used in areas containing animals in three different age categories (<8 months, 8 to 30 months, and >30 months) were sampled on each farm. In total, 820 different pens were sampled and analyzed for the presence of E. coli O157 by enrichment, immunomagnetic separation, and plating on selective agar. Presumptive E. coli O157 colonies were identified using a multiplex PCR assay for the presence of the rfbO157 and flicO157 genes. The statistical analysis was carried out with Stata SE/10.0 using a generalized linear regression model with a logit link function and a binomial error distribution. The overall farm prevalence of E. coli O157 was 37.8% (68 of 180 farms). The highest prevalence was found on dairy cattle farms (61.2%, 30 of 49 farms). The prevalences on beef, mixed dairy and beef, and veal calf farms were 22.7% (17 of 75 farms), 44.4% (20 of 45 farms), and 9.1% (1 of 11 farms), respectively. A significant positive correlation between age category and E. coli O157 prevalence was found only on mixed dairy and beef farms and dairy farms. No influence of farm size or introduction of new animals was demonstrated.

Since the outbreak of hemorrhagic colitis in 1982, which was associated with the consumption of contaminated hamburgers (36), Escherichia coli O157 has become the most important enterohemorrhagic E. coli serotype from a public health perspective and has been associated with human morbidity and mortality in industrialized countries (35). The major virulence factors are the verocytotoxins: VT1, VT2, intimin, and enterohemolysin (30). In Belgium, E. coli O157 is the predominant verocytotoxin-producing E. coli (VTEC) serotype associated with human disease (39). However, strains lacking the vti1 and vti2 genes have occasionally been isolated from patients with hemolytic uremic syndrome (3, 16, 28).

Humans can become infected with VTEC by various transmission routes, including consumption of fecally contaminated vegetables (8), beef products (24), raw milk (22, 26), dairy products (29, 34), and drinking water (1). Other routes include animal to person (12, 42) and person to person (7). Human illness caused by VTEC strains usually has been traced back to cattle (4, 12). Therefore, cattle farming and beef processing are targeted areas for interventions aimed at reducing the contamination of food and environment with pathogenic VTEC. Postslaughter antimicrobial treatments and hazard analysis critical control point plans in slaughter plants may significantly reduce carcass contamination (14). However, these measures applied at the processing level have not resulted in a decrease in the number of human illnesses. Therefore, emphasis has now been placed on intervention strategies targeting the occurrence of E. coli O157 at the farm level (6).

The prevalence of E. coli O157 on cattle farms has been examined extensively, with or without identification of risk factors associated with animals such as age (21) or breed (46) or of factors associated with the management such as group housing (17), introduction of new animals (31, 48), and type of feed (20, 37). However, most of these studies have been restricted to one cattle farm type and/or included only a limited number of farms (17–19, 21, 31, 45). Comparison of the data from these various studies is difficult because of inconsistent experimental approaches, the lack of uniformity of study design, and the differences among laboratory methods.

In this study, a large-scale survey was conducted to determine the prevalence of E. coli O157 on different types of cattle farms located in the Flemish part of Belgium. By exploring possible factors affecting E. coli O157 epidemiology at the farm level, risk factors for E. coli O157 infection of cattle were identified.

Materials and Methods

Selection of the farms. The study group consisted of 180 cattle farms located in 5 of the 10 Belgian provinces: West and East Flanders, Antwerp, Flemish Brabant, and Limburg. Farms
were selected from a list provided by the Belgian cattle registration system (Sanitel) that included all cattle farms in Flanders in 2006. In each province, the farms were initially subdivided into three groups according to farm size: small (<10 animals), medium (10 to 99 animals), and large (≥100 animals). Within each province, 12 farms of each group were visited. A further subdivision was made within each farm size group based on the number of animals purchased. All farms in a group were listed in a random order and contacted for willingness to cooperate. Farmers also were asked whether their animals were all on pasture or not. The design called for taking samples only inside the stables to eliminate any influence of external environmental factors (e.g., sunlight and rain). When a farmer refused to participate or when all animals were on pasture, the next farm on the list within the same group was contacted. The number of farms sampled in each specific category was proportional to the number of farms belonging to a specific group.

Sampling at the smallest farms (<10 animals) was not possible because all animals were already sold at the time of contact or all animals were on pasture. Therefore, the number of small farms that should have been sampled was distributed equally between the group with 10 to 99 animals and no animals purchased and the group with ≥100 animals and the most purchases to have as much dispersal as possible. The final sampling scheme is shown in Figure 1.

**Farm visits and sample collection.** One hundred eighty farms dedicated to veal, beef, dairy cattle, and mixed dairy and beef cattle were visited once between June 2007 and August 2007. Samples were collected inside the cattle stables. On each farm, up to 10 pairs of overshoes were collected as described previously (9). On the medium farms, two pairs of overshoes were collected from each pen, whereas on large farms one pair of overshoes was collected per pen. The number of cattle within a pen ranged from 6 to 80. Because dairy cows are not housed in pens, overshoes used in the stable or the passageway to the milking house were collected. When possible, samples associated with three different animal age categories were collected: <8 months, 8 to 30 months, and >30 months. Because on veal farms the animals are slaughtered before the age of 8 months, only one age category from these farms was used. The samples were transported under cool conditions and stored at 4°C until analysis the next day.

At each visit, additional information was obtained about the exact number of animals purchased within the last year.

**Isolation and identification.** To isolate *E. coli* O157 from the overshoes, 250 ml of modified trypton soya broth (Oxoid, Basingstoke, UK) supplemented with novobiocin (20 mg/liter; Sigma-Aldrich, St. Louis, MO) was added to each pair of overshoes. After incubation in a warm water bath at 42°C for 6 h, immunomagnetic separation (Dynal, Oslo, Norway) was performed according to the manufacturer’s recommendations. One hundred microliters of culture was spread onto sorbitol MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter; Dynal) and incubated overnight at 42°C.

After incubation on the selective medium, up to three colonies per plate that did not ferment sorbitol and that had a typical morphology (gray to white with a brown center) were transferred to plate count agar (PCA; Oxoid), incubated for 18 to 24 h at 37°C, and identified serologically with the O157 antigen latex agglutination assay (Oxoid).

From the isolates positive for agglutination, a maximum of nine isolates per farm were chosen (three per sampled age category) and further examined with a multiplex PCR assay for identification confirmation. The cell lysates were prepared by suspending a loop of a colony from a PCA plate in 100 μl of water and heating the suspension for 15 min at 92.5°C. Before use in the PCR, the lystate was centrifuged for 1 min at 12,500 × g to pellet the cell debris. The somatic **rfp**<sub>3</sub><sup>157</sup> and the flagellar **fliC**<sub>157</sub> genes were amplified using previously published primer pairs (27, 47). The PCR assays were carried out in a 25-μl reaction volume containing 1 μl of the lystate, 1× Taq buffer (20 mM Tris-HCl, pH 8.0, and 50 mM KCl; Invitrogen, Paisley, UK), 0.75 U of Taq DNA polymerase (Invitrogen), 500 μM concentrations of the deoxynucleoside triphosphates (dNTPs), 3 mM MgCl<sub>2</sub>, and 1.7 μM concentrations of each primer (Invitrogen). Samples were subjected to an initial denaturation of 1 min at 95°C and then 30 cycles each consisting of 15 s of denaturation at 95°C, 15 s of annealing at 50°C, and 30 s of elongation at 72°C. The final cycle was followed by an elongation step at 72°C for 8 min, and the final PCR products were held at 4°C. The presence of the desired products was determined by analyzing 8 μl of PCR product on a 1.5% agarose gel that was then stained with ethidium bromide; resulting bands were compared with that of a molecular weight marker (Track-It 100 bp DNA ladder, Invitrogen). Isolates that were negative for the **fliC**<sub>157</sub> gene were restested with a **fliC** PCR restriction fragment length polymorphism (PCR-RFLP) assay using the enzyme **HhaI** according to the method described by Machado et al. (25).
Detection of virulence genes. For each isolate identified as *E. coli* O157, a multiplex virulence PCR assay was performed using the primers for *vt*₁ (15), *eaeA* (15), *hlyA* (15), and *vt*₂ (32). PCR assays were carried out in a 25-µl reaction volume containing 1 µl of the lysates, 1× *Taq* buffer, 0.75 U of *Taq* DNA polymerase, 500 µM concentrations of the dNTPs, 3 mM MgCl₂, 1.5 µM concentrations of the *vt*₁, *eaeA*, and *hlyA* primers and a 0.6 µM concentration of the *vt*₂ primers. Amplifications were performed with an initial 95°C denaturation step for 3 min followed by 30 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final cycle was followed by a 72°C elongation for 8 min. PCR products were analyzed as in the identification step.

**Statistical analysis.** Statistical analysis was carried out with Stata/SE 10.1 (40). A logistic regression model was used when testing the various putative risk factors, taking into account the survey design where required (the primary sampling unit was the farm). Analysis started with the saturated model, and nonsignificant interactions and single variables were discarded until the top interaction was significant or until only significant single variables remained. A significance level of 0.05 was used throughout. Veal calves were excluded from the statistical analysis of the risk factors because the presence of only one age category and the large number of purchases had too much influence on the interactions. Spatial analysis was performed in ArcView 3.2a (Environmental Systems Research Institute Inc., Redlands, CA).

**RESULTS**

Of the 820 pens sampled, *E. coli* O157 was detected in samples from 180 pens belonging to 68 different farms (37.8%). Spatial analysis indicated a higher proportion of *E. coli* O157-positive farms in Antwerp (47.2%) and Limburg (50.0%) than in Flemish Brabant (31.6%), East Flanders (33.3%), and West Flanders (27.8%). However, a nearly significant difference was found between West Flanders and Limburg (odds ratio = 2.6, 95% confidence interval = 0.98 to 6.92).

Table 1 shows the prevalence rates related to each type of cattle farm. Dairy farms had a significant higher prevalence than did beef and veal farms (*P* < 0.001 and *P* = 0.011, respectively). The prevalence on mixed dairy and beef farms was significantly higher than that on beef farms (*P* = 0.0137). The lowest prevalence was found on the veal farms, but this prevalence was not significantly different from the prevalence found on the mixed dairy and beef farms and the beef farms.

The influence of different variables on the prevalence of *E. coli* O157 at the pen level was evaluated. A significant correlation was found between age category and the prevalence of *E. coli* O157 (Table 1). In mixed dairy and beef farms and dairy farms, a higher prevalence of *E. coli* O157 was found in older animals than in the youngest animals (<8 months). This age effect was absent in beef farms. Neither introduction of new animals onto the farm nor farm size resulted in a higher prevalence of *E. coli* O157.

All 324 *E. coli* O157 isolates selected for further investigation harbored the flagellar antigen gene *flIC*<sub>VT</sub>. The *hlyA* and the *eaeA* genes were present in all isolates. Fifty isolates (15.4%) carried *vt*₁ and *vt*₂, 245 (75.6%) carried only *vt*₂, and 29 (8.95%) had neither of the *vt* genes.
DISCUSSION

In the present study, the prevalence of E. coli O157 on different types of cattle farms in Flanders was determined. All selected farms located in the five provinces were visited randomly during the summer season to reduce the seasonal effect on the prevalence of E. coli O157. In several other studies, this sampling period (or at least a part of this period) was the season with the highest E. coli prevalence; the highest prevalences were observed from June until August (44) and in summer and early autumn (2).

This study is the first to be conducted on a large scale with the overshoe sampling technique to estimate the prevalence of E. coli O157 on different types of cattle farms. In a previous study, the presence of E. coli O157 in a group of beef cattle was determined most efficiently by this overshoe method (9). Previously, prevalence studies at the farm level were based on data from rectal fecal contents (11, 19, 31, 37) or fecal pats (38, 43), which means that many samples must be collected and analyzed, which is very labor-intensive. By taking samples from overshoes that have been in contact with the bedding material in the stables, the fecal material of several animals can be analyzed by analyzing one pair of overshoes, and therefore fewer samples must be analyzed. Although the farms were visited only once, the overshoe method also may overcome the problem of intermittent shedding and the fact that E. coli O157 can survive and replicate in various bedding materials in the presence of urine (10, 13, 23).

The results of this study indicated that E. coli O157 is widespread on Flemish cattle farms (37.8% of farms sampled). The distribution according to farm type revealed that dairy farms were more often positive for this pathogen than were the other farm types. However, in general the prevalence of E. coli O157 in healthy cattle was within the range of that reported in other European countries. For example, in The Netherlands, E. coli O157 was detected on 7 of 10 dairy farms (19). A lower prevalence was found in Denmark, where E. coli O157 was found on 17% of 60 sampled dairy farms (31). In Scotland and Wales, E. coli O157 was isolated from 34.5% of dairy and 53.3% of fattening herds (41), whereas in the present study a lower prevalence on beef farms than on dairy farms was found. In Scotland, an estimated 22.8% of the beef farms have at least one animal in the herd that is shedding E. coli O157 (18). Differences in isolation rates between the different types of farms may be due to management variables, such as feeding programs or housing systems.

From the 11 veal farms visited, E. coli O157 was isolated from the overshoes from only 1 of the 110 pens sampled, thereby confirming that veal calves rarely carry E. coli O157. Low prevalence was recently found in a Canadian study in which the prevalence rate was 3.2% in 62 calves (11). In Italy, E. coli O157 is rarely isolated from veal calves (5). The low prevalence on veal farms may be due to the particular nutrition status of these animals. Calves usually are fed liquid milk replacers until slaughtering. Because of this diet, the fermentative activity in the rumen does not develop; thus, the very low pH in the abomasum may hinder the survival of E. coli (5).

Factors that may affect the prevalence of E. coli O157 at the pen level on cattle farms were evaluated. Age category was positively associated with the presence of E. coli O157. On mixed dairy and beef farms and dairy farms, the prevalence increased with animal age. On mixed farms, most samples were from dairy cattle because most of the beef cattle were on pasture, resulting in results more or less analogous to those from dairy farms.

In previous studies, the impact of age has been mainly studied on dairy farms, and samples evaluated were collected from individual animals or fecal pats rather than overshoes. In a Danish study, prevalence rates were 23.3% for calves (<6 months), 31.6% for heifers (6 to 24 months), and 14.4% for cows (31). Testing of cattle on 10 Dutch dairy farms revealed E. coli O157 prevalence rates ranging from 0.8 to 22%, with the highest prevalence rate in calves (4 to 12 months old) (19). In another study, prevalence rates for dairy calves were about 6% (37). In the United Kingdom, testing of fecal samples from cow-calf operations revealed prevalence rates of 0.6% for calves and 12.8% for cows (33).

In our study, prevalence was not associated with farm size, which was consistent with the findings of a Danish research group (31). Because the purchase of animals within the year before sampling was not associated with the prevalence of E. coli O157, environmental survival of E. coli O157 and dissemination between animals may play an important role in E. coli O157 prevalence. In contrast, other researchers have found that the introduction of animals from other farms was associated with a higher prevalence of E. coli O157 (31, 48).

Most of the E. coli O157 isolates present on the Belgian cattle farms in this study were positive for eaeA, hlyA, and at least one of the vt genes. The presence of these genes is typical for human pathogenic E. coli strains, and these isolates may be considered as potential human pathogens. Although E. coli O157 strains that do not ferment sorbitol and have been isolated from cattle are typically described as being able to produce verocytotoxins, in this large-scale study sorbitol-negative E. coli O157 without vt genes may frequently be found in bedding material. Because the public health significance of the presence of atypical strains in animal reservoirs or in food is unclear, these findings emphasize the usefulness of both phenotypic and genotypic assays to identify E. coli O157 strains.

The main objective of this study was to determine the prevalence of E. coli O157 on different types of cattle farms in the northern part of Belgium and to identify factors associated with the E. coli O157 status of these cattle. Dairy cattle farms were more likely to harbor VTEC than were other types of farms, and older animals were at higher risk of infection than were younger animals (<8 months of age). The results obtained during this study should be interpreted as indicating possible risk factors that can be useful for further investigation.

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