# Cattle-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria on dairy farms, Minnesota, USA

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## Abstract

The objective of this study was to identify individual cattle-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria (STB), a surrogate for Shiga toxin-producing *E. coli* (STEC), on 28 organic and conventional dairy farms. It was found that small organic herds (fewer than 100 cows) were associated with higher odds of Shiga toxin-encoding bacteria (STB) shedding from 2 (all cattle and all cows) of 3 cattle models, followed by small conventional herds, compared with large conventional herds. Preweaned calves [odds ratio (OR) = 2.6, 95% confidence interval (CI): 1.2, 5.7] had higher odds of shedding STB compared with adult cows. Calves more than 28 days of age (OR = 2.0, 95%CI: 1.0, 4.4) were more likely to shed STB than calves less than 28 days of age. This information may be helpful for identifying potential control strategies such as targeted vaccination or management practices.

## Résumé

*Cette étude avait comme objectif d'identifier au niveau individuel chez des bovins, provenant de 28 fermes laitières organiques et conventionnelles, les facteurs de risque associés avec l'excrétion fécale de bactéries porteuses du gène de la shiga toxine (STB), un substitut pour les* E. coli *producteurs de shiga toxine (STEC). Il a été trouvé que les petites fermes organiques (moins de 100 vaches) étaient associées avec une plus grande probabilité d'excrétion de STB à partir de deux des trois modèles bovins (tout bovin et toutes vaches), suivi des petites fermes conventionnelles, comparativement aux larges troupeaux conventionnels. Les veaux pré-sevrés (OR = 2,6, CI95 % :1,2, 5,7) avaient une plus grande probabilité d'excréter STB comparativement aux vaches adultes. Les veaux âgés de plus de 28 jours (OR = 2,0, CI95 % :1,0, 4,4) étaient plus susceptibles d'excréter STB que les veaux de moins de 28 jours d'âge. Ces informations peuvent être utiles afin d'identifier des stratégies de contrôle potentielles telles que la vaccination ciblée ou des pratiques de régie.*

*(Traduit par Docteur Serge Messier)*

Shiga toxin-producing *Escherichia coli* (STEC) have emerged as a significant public health problem. *Escherichia coli* O157 is the most commonly isolated STEC from human patients in North America, while other STEC infections are likely underreported. According to the Centers for Disease Control (CDC), it is estimated that *E. coli* O157 causes 73 000 cases and 61 deaths each year in the United States and that non-O157 STEC strains account for 36 000 cases and 30 deaths a year in the United States (1).

Domestic ruminants such as cattle and sheep likely serve as the major reservoir of STEC strains that cause human infections (2). Shiga toxin-producing *E. coli* are found in most cattle herds (3), with cited STEC O157 herd prevalences varying from 38.5% to 75% (4–6). This variation is likely due to the various culture methods used, sampling schemes, and seasonal or geographical differences.

There is limited research examining risk factors for STEC either at the individual animal or herd level. Most STEC studies are limited to *E. coli* O157, which is more readily cultured. Previously identified factors include an increased prevalence of STEC O157 during the spring and summer seasons in the northern hemisphere (4,5), and more frequent recovery from young cattle (7–11). Among calves, STEC fecal shedding was more likely to increase after weaning (7,9,12), with 1.4 % prevalence in calves $<$ 8 wk old compared with 4.8% in 8-week-old calves (9). Lactation and stage of the milk production cycle may influence STEC shedding. In one study, lactating cows were more likely to shed STEC O157 than dry cows (43% versus 32%) (13). Another study documented a higher prevalence in culled dairy cows; STEC O157 was identified from 2.8% of culled cows versus 0.9% of milk cows (14).

Unlike *E. coli* O157:H7, other STEC serotypes have no distinct biochemical markers that could facilitate their identification, and this limitation frequently leads to low isolation rates (15). One of the screening steps for the detection of STEC in environmental samples is the detection of Shiga toxin genes from *Enterobacteriaceae* obtained on selective media such as sorbitol MacConkey (16). Because of the low

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**Table I. List of potential risk factors, total cattle, and test results**

| Variable | Levels | Number positive | Number of cattle | % positive |
|---|---|---|---|---|
| Farm size | 30–49 cows | 22 | 460 | 4.8 |
| | 50–99 cows | 31 | 577 | 5.4 |
| | 100–199 cows | 8 | 569 | 1.4 |
| | 200 or more cows | 10 | 602 | 1.7 |
| Farm type | Conventional | 41 | 1750 | 2.3 |
| | Organic | 30 | 458 | 6.6 |
| Cattle group | Sick cow | 1 | 97 | 1.0 |
| | Periparturient | 8 | 261 | 3.1 |
| | Healthy lactating cow | 41 | 1445 | 2.8 |
| | Cull cow | 0 | 20 | 0.0 |
| | Preweaned calf | 21 | 385 | 5.5 |
| Treatment with antibiotics (within 14 d) | Yes | 0 | 80 | 0.0 |
| | No | 71 | 2128 | 3.3 |
| Calf age | 0–14 d old | 3 | 83 | 3.6 |
| | 15–28 d old | 3 | 85 | 3.5 |
| | 29–42 d old | 5 | 82 | 6.1 |
| | > 42 d old | 10 | 135 | 7.4 |
| Parity number | 1st | 16 | 621 | 2.6 |
| | 2nd | 9 | 496 | 1.8 |
| | 3rd | 8 | 296 | 2.7 |
| | 4th and up | 16 | 379 | 4.2 |
| Health status | Diarrhea | 2 | 57 | 3.5 |
| | Metritis | 0 | 13 | 0.0 |
| | Mastitis | 1 | 51 | 2.0 |
| | Respiratory problem | 1 | 26 | 3.9 |
| | Lame | 0 | 45 | 0.0 |
| | Other disease | 0 | 24 | 0.0 |
| | Healthy | 67 | 1976 | 3.4 |

isolation rates of STEC from Shiga toxin positive plates, the use of a general group described as "Shiga toxin-encoding bacteria" serves as an indicator for the presence of STEC. The objective of our study was to identify individual cattle-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria (STB) on 28 organic and conventional dairy farms in Minnesota, USA.

*Herd selection* — Holstein dairy farms in Minnesota were selected on the basis of farm type (8 organic versus 20 conventional) and farm size (based on the number of milking and dry cows, selected within the following size categories: 30–49, 50–99, 100–199, and 200 cows). Fecal samples were collected over an 8-month period as part of another multi-state project (17). Farm selection criteria included that each farm had at least 30 milking or dry cows, had at least 90% of cows of Holstein breed, and raised their own calves for replacement animals. Conventional farms indicating interest in participation based on a mailing to farms within approximately 100 miles from the University of Minnesota were randomly selected based on meeting the farm selection criteria. Organic farms certified by an independent organic certification agency recognized by the United States Department of Agriculture were selected based on the herd selection criteria and their desire to participate.

*Sample collection* — Enrolled farms (8 organic and 20 conventional) were sampled at 2-month intervals (approximately) up to 3 times (average: 2.0 visits per farm) from April to October in 2001 as previously described (16). A total of 2208 fecal samples were collected from 28 farms distributed over 8 counties (Goodhue, Stearns, Wright, Wabasha, Sibley, Mille Lacs, Douglas, and Scott) in central and southeastern Minnesota. Approximately 10 g of feces were collected directly from the rectum of individual cattle using either plastic sleeve gloves for adult cows or disposable gloves for calves and transported on ice to the laboratory where they were stored overnight prior to bacterial culture.

*Target cattle groups* — Fecal samples were systematically collected from healthy lactating cows, preweaned calves (heifer calves receiving milk or milk replacer), periparturient cows (cows due to calve within 14 d or within 14 d after calving), sick cows (cows described as sick by farm personnel on the day of sample collection), and to-be-culled cows (cows to be culled within 14 d). The target number

**Table II. Potential risk factors for Shiga toxin-encoding bacteria fecal shedding cattle in univariable logistic regression models**

| Model | Variable | Levels | Number positive | Number of cattle | % positive | OR | *P*-value |
|---|---|---|---|---|---|---|---|
| All cattle | Farm type/size | Small organic | 30 | 458 | 6.6 | 4.5 | 0.04 |
| | | Small conventional | 23 | 579 | 4.0 | 2.7 | |
| | | Large conventional | 18 | 1171 | 1.5 | 1.0 | |
| | Cattle group | Preweaned calf | 21 | 385 | 5.5 | 2.1 | 0.17 |
| | | Adult cattle | 50 | 1823 | 2.7 | 1.0 | |
| | Season | Fall | 23 | 307 | 7.5 | 3.4 | 0.38 |
| | | Spring | 26 | 969 | 2.7 | 1.1 | |
| | | Summer | 22 | 932 | 2.4 | 1.0 | |
| | Status of diarrhea | Yes | 2 | 57 | 3.5 | 1.1 | 0.93 |
| | | No | 69 | 2151 | 3.2 | 1.0 | |
| All calves | Calf age | < 29 d old | 6 | 168 | 3.6 | 0.5 | 0.13 |
| | | 29–56 d old | 15 | 217 | 6.9 | 1.0 | |
| | Farm type/size | Small organic | 4 | 48 | 8.3 | 2.0 | 0.70 |
| | | Small conventional | 6 | 84 | 7.1 | 1.7 | |
| | | Large conventional | 11 | 253 | 4.4 | 1.0 | |
| All cows | Number of parity | ≤ 3rd | 33 | 1413 | 0.9 | 0.5 | 0.20 |
| | | 4th or more | 16 | 379 | 4.2 | 1.0 | |
| | Stage of lactation | 0–30 d | 11 | 256 | 4.3 | 1.5 | 0.49 |
| | | 31–120 d | 10 | 403 | 2.5 | 0.9 | |
| | | 121–240 d | 10 | 509 | 2.0 | 0.7 | |
| | | ≥ 241 d | 16 | 548 | 2.9 | 1.0 | |
| | Farm type/size | Small organic | 26 | 410 | 6.3 | 8.8 | 0.01 |
| | | Small conventional | 17 | 495 | 3.4 | 4.6 | |
| | | Large conventional | 7 | 918 | 0.8 | 1.0 | |

OR — odds ratio.
Variables with $P < 0.20$ were eligible for inclusion in the multivariable model.
*P*-value originated from F statistics (Type 3 GEE analysis).

of samples per cattle group was determined as previously described (17). Up to 30, 40, 50, and 55 total cattle fecal samples per visit were collected from herds with 30–49, 50–99, 100–199, and 200 cows, respectively. The majority of samples from each farm were obtained from healthy lactating cows.

*Management questionnaire* — A questionnaire was administered to farm personnel at the initial visit and a shorter questionnaire was completed at each sampling visit to document changes in rations fed and herd inventory. The following information was collected for each sampled animal at each visit: cattle group (preweaned calves, sick cows, culled cows, periparturient cows, and healthy lactating cows), calf age (in days), antibiotic treatment in the previous 14 d (Yes/No), observed or reported specific diseases, parity (number), and stage of lactation (days in milk).

*Bacterial culture and detection of Shiga toxin-encoding bacteria (STB)* — One gram of feces samples was aseptically diluted in EC broth supplemented with novobiocin (20 µL/mL) for an approximate 1:10 ratio and incubated at 37C for 18–24 h. *Escherichia coli* enrichment cultures were streaked on sorbitol MacConkey (SMAC) agar plates and incubated for 24 h. Shiga toxin-encoding bacteria were detected using a polymerase chain reaction (PCR) colony-sweep assay as described in previous studies (16,18). Briefly, a representative sample of bacterial cells was taken by randomly sweeping areas of heavy growth on primary SMAC plate agar using a 10-µL disposable inoculating loop and then suspended in 200 µL distilled water. These cell suspensions were frozen at -20°C. For DNA extraction, the suspensions were boiled for 10 min and immediately cooled on ice for 5 min. Cell pellets were spun down and supernatants were used as the DNA template. All samples were screened by PCR with *stx*1 and *stx*2 primer pairs run in duplex. *Escherichia coli* strains ATCC 43890 (*stx*1 gene only) and ATCC 43895 (both *stx*1 and *stx*2 genes) were used as positive controls, and *E. coli* K-12 W3110 was used as a negative control (run concurrently with all test samples/each gel).

*Data analysis* — A total of 2208 fecal samples from a total of 57 visits on 28 dairy farms were used for data analysis. Enrolled farms included 8 herds ($n$ = 460 samples) with 30–49 milking cows, 9 herds ($n$ = 577) with 50–99 milking cows, 6 herds ($n$ = 569) with 100–199 milking cows, and 5 herds ($n$ = 602) with 200 or more

**Table III. Risk factors for Shiga toxin-encoding bacteria fecal shedding in cattle in multivariable logistic regression models**

| Model | Variable | Level | Number of cattle | % positive | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|---|
| All cattle | Cattle group | Preweaned calf | 385 | 5.5 | 2.6 | 1.2–5.7 | 0.02 |
| | | Other adults | 1823 | 2.7 | 1.0 | | |
| | Farm type/size | Small organic | 458 | 6.6 | 4.5 | 2.2–9.5 | < 0.01 |
| | | Small conventional | 579 | 4.0 | 2.6 | 1.0–6.6 | 0.05 |
| | | Large conventional | 1171 | 1.5 | 1.0 | | |
| All calves | Calf age | 29–56 d old | 217 | 6.9 | 2.0 | 1.0–4.4 | 0.06 |
| | | < 29 d old | 168 | 3.6 | 1.0 | | |
| | Farm type/size | Small organic | 48 | 8.3 | 2.0 | 0.4–10.5 | 0.40 |
| | | Small conventional | 84 | 7.1 | 1.6 | 0.4–7.5 | 0.53 |
| | | Large conventional | 253 | 4.4 | 1.0 | | |
| All cows | Number of Parity | ≥ 4 | 379 | 4.2 | 1.7 | 0.9–3.1 | 0.11 |
| | | < 4 | 1413 | 0.9 | 1.0 | | |
| | Farm type/size | Small organic | 410 | 6.3 | 7.7 | 2.6–22.3 | < 0.01 |
| | | Small conventional | 495 | 3.4 | 4.4 | 1.2–16.4 | 0.03 |
| | | Large conventional | 918 | 0.8 | 1.0 | | |

OR — odds ratio.

95% CI — 95% confidence interval.

Farm type/size and season were forced to be included as covariates in all 3 models.

milking cows. The database was managed using MS Access 2000 (Microsoft, Redmond, Washington, USA) and the risk factors were analyzed using a computer program (SAS Ver 8.2; SAS Institute, Cary, North Carolina, USA). Data were analyzed using separate logistic regression models for all cattle, all cows, and all calves. Logistic regression using generalized estimating equations (GEE) was used for analysis, accounting for correlation of observations within herds (*Proc genmod*, repeated subject = herd in SAS). Variables were initially analyzed individually for association with fecal shedding of STB within each herd in separate univariable models.

Farm type/size (organic herds with < 100 cows versus conventional herds with < 100 cows versus conventional herds with ≥ 100 cows) and season (April to June = Spring; July to August = Summer; September to October = Fall) were included as independent variables in all models because these factors were assumed to be important potential confounding variables. The outcome variable for all models was the test result of STB fecal shedding from the individual cattle (the presence or absence of Shiga toxin gene in a fecal sample). Variables with $P < 0.20$ based on F test (Type 3 GEE analysis) were eligible for inclusion in the multivariable models. The model parameters were estimated with F statistics using previously defined methods (19). The final GEE model was developed using a forward selection procedure. From the final model, odds ratios (OR) and 95% confidence intervals (CI) were calculated, controlling for the presence of other variables simultaneously.

Descriptive herd characteristics from the study farms are shown in Table I. Separate models for each independent variable of interest were analyzed for all cattle, all cows, and all calves in both univariable and multivariable analysis of risk factors associated with STB shedding (Table II and Table III, respectively). For the "all cattle" analysis, the outcome variable was the test result of STB shedding (*stx* gene positive or negative) for 2208 fecal samples from healthy cows, preweaned calves, periparturient cows, sick cows, and cull cows. In the "all calves" analysis, the outcome variable was the test result of STB shedding for 385 preweaned calves. In the "all cows" analysis, the outcome variable was the test result of STB shedding for 1823 fecal samples from cows (healthy lactating, periparturient, sick, or to-be-culled cows).

Small organic herds were associated with higher odds of STB shedding from 2 (all cattle and all cows) of 3 cattle models, when compared with large conventional herds (Table III). For example, in the all cattle model, cattle that shed STB had increased odds of being in small organic herds (< 100 cows, OR = 4.5, 95% CI: 2.2, 9.5) or small conventional herds (< 100 cows, OR = 2.6, 95% CI: 1.0, 6.6) compared with large conventional herds (≥ 100 cows) after adjusting for cattle group and herd effects.

This difference may be due to distinct herd management practices between the 2 types of farms (organic versus conventional), such as pasture access, limited administration of antimicrobials, use of a total mixed ration, and housing type. Cattle group and calf age were also significantly associated with STB fecal shedding, with calves being more likely to be STB-positive (OR = 2.6, 95% CI: 1.2, 5.7) compared with adult cows, and calves 29 to 56 d old (OR = 2.0, 95% CI: 1.0, 4.4) were more likely to shed STB compared with calves < 29 d old. In the "all cows" model, there was a nonsignificant trend with parity appearing to be associated with STB shedding in cows (Table III). Cows with parities of ≥ 4 were 1.7 times more likely to shed STB, compared with cows that had parities of ≤ 3 (OR = 1.7, 95% CI: 0.9, 3.1). In addition, farm type/size were significantly associated with STB shedding. The odds of STB shedding in cows on small organic

farms and small conventional farms were 6.3 times and 4.4 times higher respectively, compared with those on large conventional farms after adjusting for parity and herd effects. Previous studies have demonstrated a relationship between animal age and STEC shedding, with STEC O157 and other STEC recovered more often in young cattle, especially recently weaned animals in a variety of studies (7–12). In a recent STEC risk factor study performed with a similar PCR method, increased parity was associated with an increased risk for being a STEC carrier animal (20). Similarly, our study showed a trend towards higher odds of STB shedding from older cows with a parity of 4 or greater compared with cows that had a parity of $< 4$. The reason for this observation is unclear but may be due to waning immunity or increased lactation stress.

No association between cattle having diarrhea and STB shedding was observed. Similar findings have also been observed by others (2). Shiga toxin-encoding *E. coli* O157 and other STEC are not associated with clinical illness and appear to be transient commensal bacteria in cattle.

There are few studies documenting risk factors for STEC from individual cattle on dairy farms (20), and most of the few available STEC risk factor studies are limited to *E. coli* O157 (4,9,10). Herds were randomly selected using a stratified sampling process to obtain representation of both large and small dairy producers. However, there were no enrolled organic farms with more than 100 cows in this study, limiting our ability to evaluate STB on large organic farms.

The methods herein were focused on identifying Shiga toxin-encoding bacteria. It is possible that bacteria other than *E. coli* may produce Shiga toxins. With our culture methods and selection of PCR primers, we hoped to limit the detection of other Shiga toxin-producing bacteria. While it is theoretically possible that *Shigella dysenteriae* type 1 can be found in bovine feces this would be unlikely. Current evidence suggests that most Shiga toxin-producing bacteria in the bovine gastrointestinal system are likely STEC. Using these methods, we have previously documented the recovery of 43 STEC isolates belonging to 26 different serotypes, including *E. coli* O157 (16).

Another possible limitation of the prospective study design was that samples from an individual animal may have been tested multiple times. It is believed that the likelihood is remote and the impact minimal. This situation would only apply for adult cows from smaller herds and not for calves, because calves were removed from the eligible sampling population after weaning at around 60 d of age, and herds were re-sampled at 2-month intervals.

This study enhances the understanding of STB shedding among cattle groups (calves, healthy lactating cows, periparturient cows, cull cows, and sick cows) on dairy farms. Managing herd-level factors along with identified individual-level factors may synergistically reduce the occurrence of STB on dairy farms, contributing to a decrease in contaminated food in the food chain and ultimately a decrease in human STEC infections. Unfortunately, the identified animal-level factors in our study are not readily manageable; therefore, there are no clear recommendations that we can provide to dairy producers to reduce STEC shedding. With the on-going development of an STEC O157 vaccine, targeted vaccination of high-risk groups of cattle may be applicable to reduce STEC occurrence on the farm. Further research involving a larger number of animals and an examination of herd-level risk factors may provide additional prevention or control strategies.

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