Dynamics of *Escherichia coli* Virulence Factors in Dairy Herds and Farm Environments in a Longitudinal Study in the United States

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Pathogenic *Escherichia coli* or its associated virulence factors have been frequently detected in dairy cow manure, milk, and dairy farm environments. However, it is unclear what the long-term dynamics of *E. coli* virulence factors are and which farm compartments act as reservoirs. This study assessed the occurrence and dynamics of four *E. coli* virulence factors (*eae, stx1, stx2*, and the gamma allele of the *tir* gene [\(\gamma-tir\)]) on three U.S. dairy farms. Fecal, manure, water, feed, milk, and milk filter samples were collected from 2004 to 2012. Virulence factors were measured by postenrichment quantitative PCR (qPCR). All factors were detected in most compartments on all farms. Fecal and manure samples showed the highest prevalence, up to 53% for *stx2* and 21% for *\(\gamma-tir\)* in fecal samples and up to 84% for *stx* and 44% for *\(\gamma-tir\)* in manure. Prevalence was low in milk (up to 1.9% for *stx* and 0.7% for *\(\gamma-tir\)*). However, 35% of milk filters were positive for *stx* and 20% were positive for *\(\gamma-tir\).* All factors were detected in feed and water. Factor prevalence and levels, expressed as qPCR cycle threshold categories, fluctuated significantly over time, with no clear seasonal signal independent from year-to-year variability. Levels were correlated between fecal and manure samples, and in some cases autocorrelated, but not between manure and milk filters. Shiga toxins were nearly ubiquitous, and 10 to 18% of the lactating cows were potential shedders of *E. coli* O157 at least once during their time in the herds. *E. coli* virulence factors appear to persist in many areas of the farms and therefore contribute to transmission dynamics.

Diarrheagenic *Escherichia coli* isolates of several pathovars of public health importance, such as enteropathogenic *E. coli* (EPEC), Shiga-toxigenic *E. coli* (STEIC), and enterohemorrhagic *E. coli* (EHEC), have been observed in dairy herds (1–3) as well as in milk (4–10) and other dairy products (4–8, 11, 12). Beef cattle are also known to harbor pathogenic *E. coli* (13–16). Dairy animals also enter the meat production chain, contributing to meatborne infections (17). Infection by these classes of pathogenic *E. coli* can have serious health impacts in humans (8). The cost of human health losses in the United States due to *E. coli* O157 alone was estimated to amount to $405 million per year (18). Numerous *E. coli* outbreaks have been linked to the consumption of milk and dairy products (19–22) and to direct contact with dairy farm animals and environments (23, 24). Milk contamination is usually due to fecal contamination. Intestinal colonization by STEC serogroups such as *E. coli* O157 is usually subclinical in cows and calves and therefore is often undetected. STEC and EHEC have almost never been associated with nonenteric infections in cows, such as mastitis, although other classes of pathogenic *E. coli* have been known to cause mastitis (25).

A virulence factor is a phenotypic trait, usually a large molecule or complex, which determines the ability of *E. coli* and other bacterial pathogens to infect a host. Common phenotypes include the ability to attach to cells of the intestinal lining, the ability to enter such cells, and the ability to cause damage to the cells, e.g., by secreting toxins (26). The genes encoding *E. coli* virulence factors are located either on plasmids, on large genome regions called pathogenicity islands (10 to 200 kb), or on integrated bacteriophages (27), all of which may be exchanged via horizontal gene transfer. The genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*, respectively) are carried by lambdoid phages and can be integrated into the bacterial host genome in multiple copies (28). Genetic elements responsible for attachment to and penetration (effacement) into the host intestinal lining cells are located in the chromosomal locus of enterocyte effacement (LEE), a pathogenicity island which includes the intimin factor *eae* (*E. coli* attachment and effacement) and the *tir* factor (translocated intimin receptor), as well as other virulence elements (29). Other genetic elements are also associated with STEC virulence, including hemolysin, type III secretion factors, and catalase (29, 30).

The traditional classification of enteropathogenic *E. coli* is based on infection mechanisms or symptoms (e.g., EHEC and STEC). To some extent, the presence of certain virulence factors can be linked to infection mechanisms and hence pathogenic classes (31). For example, the presence of the intimin gene *eae*, without any Shiga toxin genes, defines enteropathogenic (EPEC) strains. The presence of *stx* factors defines the STEC class, of which...
enterohemorrhagic *E. coli* (EHEC) is a subgroup. The γ alleles of *eae* and *tir* and the *rfbE*157 gene (involved in O-antigen synthesis) are present in the EHEC serogroup O157. The presence of both *stx* and the gamma allele of the *tir* gene (γ-*tir*) in an isolate is suggestive of one of the most common virulent EHEC serotypes, O157:H7. Translated to an entire sample where more than one strain may occur, the detection of *stx* and γ-*tir* in similar amounts suggests that serotype O157:H7 may be present. In general, information on virulence factors is a better predictor of infection severity than is serotype alone (30). Historically, assay development and monitoring efforts have been devoted to *E. coli* O157:H7, due to its predominance in severe human infections and ease of isolation in clinical labs; however, the risk due to non-O157 STEC and farm environments. Specific objectives were to (i) compare the presence and levels of *E. coli* virulence factors in dairy cow feces, farm environmental compartments (e.g., water, feed, and manure accumulated on floors), and bulk tank milk on the three study farms and (ii) assess time trends of the four virulence factors in the herds and the farm environments.

**MATERIALS AND METHODS**

**Study sites.** The study was conducted at three dairy farms located in the northeastern region of the United States. Farm A was located in New York State, farm B was in Pennsylvania, and farm C was in Vermont. All three farms participated in the state dairy quality assurance program, were National Dairy Herd Improvement (DHIA) members with monthly milk testing, implemented Farm Animal Identification and Records, and maintained computerized on-farm disease records. The herds consisted of approximately 330, 110, and 140 adult dairy cows on farms A, B, and C, respectively. Farms A and C raised heifers on-site. On farm B, weaned calves were sent to an off-site heifer grower and then brought back to the herd prior to giving birth to their first calf and commencing milk production.

**Sampling timeline.** Sampling was carried out from January 2004 to December 2011 on farm A, from March 2004 to August 2012 on farm B, and from November 2004 to September 2010 on farm C, as previously described (53). Samples from the main farm compartments (fetal samples from individual cows; composite manure from pens housing milking cows, nonlactating cows, periparturient cows, and calves as well as the milking alleys; feed; and source water and water from drinking troughs) were collected every 6 months on farms A and C and quarterly on farm B. As much as possible, the same locations were sampled over time. A smaller number of additional samples were collected or sent by the farm manager between the main sample collection sessions, e.g., when a cow was joining or leaving the herd. On farm B, following the detection of *Salmonella* bacteria, samples were collected more frequently, approximately every 3 to 4 months (33). Samples of milk and milk filters were collected weekly.

**Sampling and sample processing.** Fecal samples and samples from the farm environment were collected and handled as previously described (53). In brief, fecal samples were collected directly from the rectum using gloved hands and transferred to 50-ml centrifuge vials. Feed samples (100 to 500 g) were collected in 2-quart zippered bags. Composite manure was collected from the floor and stored in 50-ml centrifuge vials. Water samples were collected in 500-ml or 1-liter bottles. Samples were transported to the laboratory overnight on ice, stored at 4°C, and processed within 24 h.

For fecal and composite manure samples, 25 g of the sample was transferred to a filtered stomacher bag with 50 ml of buffered peptone water (BPW) and pumped for 2 min in a bag mixer (stomacher). For feed samples, 25 g was placed in a filtered stomacher bag with a 1:2 (wt/wt) amount of BPW and pumped for 2 min. For all three sample types, after stomaching, 5 ml of the liquid from the filtered side of the bag was added to 5 ml of double-strength EC broth (Difco Laboratories, Detroit, MI). For water samples, 100 ml of each sample was filtered through a 0.45-μm nitrocellulose filter (43-mm diameter). The filter was put in a tube with 10 ml of 1X EC broth. Tubes were vortexed and incubated at 42°C for 14 to 18 h. After incubation, the tubes were vortexed, and a 1.5-ml aliquot was transferred to a 1.7-ml microcentrifuge tube. Tubes were then centrifuged.
at 12,000 × g for 2 min, the supernatant was discarded, and each pellet was frozen at −20°C.

The collection, shipping, and processing of milk and milk filter samples were described previously (9). In brief, 50 ml of bulk tank milk and in-line milk filters were collected aseptically and shipped with cold packs overnight. For enrichment of E. coli, 5 ml of milk was added to 5 ml of double-strength EC medium and incubated at 42.5°C for 14 to 18 h (10). Milk filters were cut in 30- to 50-cm² pieces, transferred into a large filtered stomacher bag, and mixed 1:1 (wt/vol) with BPW. The bag was stomached for 2 min, briefly massaged by hand to reposition the filter pieces into the liquid, and then pumped again for 2 min. Five milliliters of filtrate from the pummeled filter piece-peptone water mixture was added to 5 ml of double-strength EC broth and incubated at 42.5°C for 14 to 18 h. For both milk and filters, 1.5 ml of the enrichment was centrifuged (12,000 × g for 2 min), the supernatant was removed, and the resulting pellet was frozen at −20°C.

**DNA extraction.** For all samples except milk and milk filters, DNA was extracted from the pellets using a MoBio soil kit (MoBio Inc., Carlsbad, CA). The purified nucleic acids were stored at −20°C until PCR analysis. For milk and milk filter samples, DNA was extracted from the pellets using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s directions. The final DNA preparations (200 μl) were stored at −20°C prior to analysis.

**qPCR assays.** A TaqMan quantitative PCR (qPCR) assay was used to determine the presence and relative abundance of four virulence factors associated with pathogenic E. coli in the nucleic acids extracted from sample enrichments. Specifically, samples were assayed for Shiga toxin genes 1 and 2 (stx1, and stx2, respectively), specific intimin (eae), and the γ allele of the translocated intimin receptor (γ-tir) according to the protocol described elsewhere (9, 10). The relative amplicon amount in the sample was expressed as the number of PCR cycles needed for the fluorescence intensity to rise above a specified threshold (cycle threshold [CT], qPCR was run on Stratagene MX4000 and Mx3005P instruments (La Jolla, CA). Baselines and threshold fluorescence levels were set manually for each dye in an assay using fluorescence plots normalized to the ROX reference dye. As the assay was semiquantitative, no calibration curve was used. These methods are described in detail in the work of Karns et al. (10). From the beginning of the project through winter 2005, samples were analyzed using individual assays for eae, stx1, and stx2; but only samples with strong eae and stx1 signals were tested for γ-tir. Only a relatively small percentage of the total number of samples was not tested for γ-tir (28 from a total of 5,757 samples from farm A, 571 of 4,736 from farm B, and 336 of 2,834 from farm C). Such samples were classified as follows: (i) NLNS, LEE non-stx E. coli —enteropathogenic class. Samples were classified as follows: (ii) LNS, LEE non-stx E. coli —enteropathogenic class.

**RESULTS**

**E. coli virulence factor prevalence by farm and sample type.** All four virulence factors were ubiquitous on the three farms. The overall prevalence significantly differed by farm and sample type (Table 1). The generic eae gene was frequently detected on all three farms (58 to 70% of all samples), while as expected the γ-tir allele was the least frequent (13 to 21% in all samples). Prevalence of the stx genes ranged from 28% to 43% overall. For all farms and samples, composite manure samples collected from floors and farm surfaces consistently showed a significantly higher prevalence than fecal samples from individual cows (15 to 37% higher), with the highest prevalence observed for stx1. Composite samples were the most uniform in prevalence across farms. Feed and trough water were less likely to harbor virulence factors than fecal and composite samples, although the difference in prevalence between fecal samples and water samples was not always significant. In feed samples, a considerably higher prevalence of stx and γ-tir was observed for farm B than for the other two farms, a trend that was not observed in any other compartment.

Prevalence of all virulence factors was drastically lower in milk
TABLE 1 Prevalence of virulence factors by farm and sample type

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Farm</th>
<th>Overall farm</th>
<th>Fecal</th>
<th>Manure composite</th>
<th>Water&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Feed</th>
<th>Milk</th>
<th>Milk filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae</td>
<td>A</td>
<td>63.8 (5,757)</td>
<td>66.2 (4,538)</td>
<td>89.3 (308)</td>
<td>36.4 (121)</td>
<td>62.6 (115)</td>
<td>6.8 (365)</td>
<td>80.6 (310)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>58.3 (4,736)</td>
<td>64.3 (2,847)</td>
<td>91.6 (629)</td>
<td>22.2 (279)</td>
<td>58.9 (207)</td>
<td>4.3 (416)</td>
<td>42.2 (358)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>70.1 (2,834)</td>
<td>76.3 (1,715)</td>
<td>90.8 (412)</td>
<td>26.4 (106)</td>
<td>60.7 (84)</td>
<td>13.4 (269)</td>
<td>76.2 (248)</td>
</tr>
<tr>
<td>stx&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A</td>
<td>29.3 (5,757)</td>
<td>29.5 (4,538)</td>
<td>63.0 (308)</td>
<td>11.6 (121)</td>
<td>24.3 (115)</td>
<td>0.8 (365)</td>
<td>35.2 (310)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>42.2 (4,736)</td>
<td>47.3 (2,847)</td>
<td>83.9 (629)</td>
<td>6.5 (279)</td>
<td>32.9 (207)</td>
<td>0.5 (416)</td>
<td>10.1 (358)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>40.5 (2,834)</td>
<td>41.9 (1,715)</td>
<td>76.5 (412)</td>
<td>8.5 (106)</td>
<td>29.8 (84)</td>
<td>1.9 (269)</td>
<td>20.6 (248)</td>
</tr>
<tr>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>A</td>
<td>28.0 (5,757)</td>
<td>30.1 (4,538)</td>
<td>55.8 (308)</td>
<td>7.4 (121)</td>
<td>20.0 (115)</td>
<td>1.1 (365)</td>
<td>12.6 (310)</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<td>0.3 (416)</td>
<td>6.7 (358)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>41.7 (2,834)</td>
<td>47.1 (1,715)</td>
<td>71.8 (412)</td>
<td>7.5 (106)</td>
<td>13.1 (84)</td>
<td>0.7 (269)</td>
<td>23.0 (248)</td>
</tr>
<tr>
<td>γ-tir&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>13.5 (5,757)</td>
<td>13.8 (4,538)</td>
<td>30.8 (308)</td>
<td>4.1 (121)</td>
<td>2.6 (115)</td>
<td>0.0 (365)</td>
<td>15.2 (310)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.6 (4,736)</td>
<td>11.2 (2,847)</td>
<td>41.6 (629)</td>
<td>3.2 (279)</td>
<td>12.4 (207)</td>
<td>0.0 (416)</td>
<td>0.0 (358)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>21.2 (2,834)</td>
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<td>43.9 (412)</td>
<td>4.3 (106)</td>
<td>6.9 (84)</td>
<td>0.7 (269)</td>
<td>19.8 (248)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Water data in this table refer to trough water, across all age groups.
<sup>b</sup> For the analysis shown in this table, samples with Ct values of >35 were considered nondetects.
<sup>c</sup> The number of samples analyzed for γ-tir was 5,756, 4,193, and 2,498 for farms A, B, and C, respectively, since in the first study year γ-tir was not tested in all samples.

than any other sample type for all farms. However, prevalence in milk filters was in most cases significantly higher than in milk and at similar levels as in fecal samples for γ-tir. Prevalence of all factors in milk filters was also notably higher for farms A and C than farm B. It has to be noted that milk and milk filter samples were collected weekly throughout the study, while other sample types were collected only 2 to 4 times a year. Hence, available data for these two matrices may represent different occurrence and transmission patterns compared to other sample types.

The distribution of qPCR Ct levels in positive samples showed a high degree of variability within each sample type (Fig. 1 for stx<sub>2</sub> and γ-tir; also see Fig. S1 in the supplemental material for eae and stx<sub>1</sub>). Samples were enriched prior to qPCR analysis; thus, Ct values are indicative only of relative abundance, not absolute concentrations in the original samples. For all farms, a small number of fecal samples had Ct values below 20, corresponding to a very high postenrichment concentration, possibly due to “supershedding” events. Positive composite manure samples presented Ct ranges similar to those of fecal samples, and a slightly lower variability, possibly as a result of dilution and die-off. Other sample types had generally lower maximum levels (i.e., higher Ct values), although rare high levels were also observed in other compartments. Positive trough water samples, although infrequent, presented Ct levels comparable to fecal and composite positive samples, and slightly higher levels of eae. For all farms, stx<sub>1</sub> and γ-tir (less so for eae and stx<sub>2</sub>) positive milk samples had a much lower variability in Ct values than other compartments, in part likely due to the low number of positive samples. The Ct distribution in positive milk filter samples was notably different from milk samples in both range and shape.

Occurrence in different farm locations and age groups. Composite, feed, and water samples were collected from different locations on the farms. For composite manure, which was mostly collected from floors, the prevalence of γ-tir was distributed differently across farm locations (see Table S1 in the supplemental material). For farm A, γ-tir prevalence was in the 25 to 37% range, and not significantly different across barns that housed cows of different ages (calves, heifers, adult cows, and dry cows). For farms B and C, however, γ-tir prevalence was significantly higher in calves than in adult cows (61% versus 38% for farm B and 51% versus 37% for farm C, respectively). Heifer manure showed a prevalence similar to that of manure from calves, and prevalence was significantly higher than that in adult cow manure (results are meaningful only for farms A and C, where heifers were housed on-site). Manure collected from the manure pit showed a γ-tir prevalence comparable to that of other locations (31% for farm A and 51% for farm C), while for farm B it was notably lower (28%) than that in manure collected in pens housing either calves or adult cows. Comparable trends were observed for stx<sub>1</sub> and stx<sub>2</sub> in composite manure, where prevalence was considerably higher in manure from calves and heifers than in manure from adult cows on all farms.

Feed samples included ingredients, formulated adult feed (total mixed ration [TMR]), haylage or silage, and feed for calves, although not all categories were present on all farms. TMR and calf feed were collected at the feed bunk and hence could have been in contact with animals and manure. γ-tir was detected in feed for both calves (7 to 8%) and adult cows (4 to 10%). γ-tir was also occasionally detected in some feed components such as haylage (0 to 10%), corn (7% on farm C), and other feed ingredients (10% on farm B). stx<sub>1</sub> and stx<sub>2</sub> were generally found at higher prevalence in ingredients (e.g., corn, silage, and haylage) than in finished feed such as TMR sampled in the pens, on farms B and C. eae was detected in most feed categories on all farms. Overall, only a moderate number of feed samples were collected (121, 279, and 106 from farms A, B, and C, respectively), and hence, prevalence estimates in feed subcategories should be considered preliminary.

Virulence factors were also unevenly distributed in water samples (source drinking water, trough water, and on-farm streams). eae was detected in virtually all water categories on all three farms. stx<sub>1</sub> and stx<sub>2</sub> were detected at similar frequencies in water from calf and adult cow troughs (21 to 30% of all trough water samples were positive for stx<sub>1</sub> and 13 to 22% were positive for stx<sub>2</sub>), and at a lower frequency in source drinking water from local wells, collected at taps. γ-tir was detected in 0 to 5% of source water samples (0 of 31 samples from farm A, 1 of 34 from farm B, and 1 in 21 from farm C) and at 0 to 9% in trough water (trough water prevalence is shown in Table 1). In positive trough water samples, Ct levels were generally comparable to levels in manure positive samples (Fig. 1). On farm B, stx<sub>1</sub>, stx<sub>2</sub>, and γ-tir were also detected in...
standing water and in a nearby stream. The number of water samples collected was 146, 547, and 108 for farms A, B, and C, respectively, 50 to 79% being trough water samples.

**Classification into potential *E. coli* pathogenic classes.** The occurrence of *E. coli* potential pathogenic classes in enrichments, according to the operational definitions adopted in this study, varied by farm and sample type, as well as over time (Fig. 2; see also Table S2 in the supplemental material). On all three farms, 23 to 29% of all samples did not contain any of the considered virulence factors and were classified as containing “non-LEE non-stx” *E. coli* (NLNS). For fecal samples, the percentage of NLNS samples was much lower for farms B and C (12.1 to 12.7%) than for farm A (21.0%). All three farms had a sizeable portion of LNS samples (20 to 31%), i.e., which contained *eae* and/or *γ-tir*, but no *stx*, and could not be classified in any higher-pathogenicity class. For fecal samples, farm A had a higher proportion of LNS, followed by lower proportions of STEC (occurrence of *stx* and/or *stx*$_2$) and p-EHEC (occurrence of *eae* and *stx*$_1$ and/or *stx*$_2$ at approximately the same C$_T$ levels). Conversely, farm B had the highest proportion of STEC samples among the three farms (40.4%). Farm C had comparable prevalences of LNS and STEC and the highest prevalence of p-EHEC among the three farms (27.7%). For all farms, only a minor proportion of samples could be classified as potentially containing *E. coli* O157 (p-O157, defined by the occurrence of *stx*$_2$ and *γ-tir* in similar abundance, with *eae* also present in the enrichment), with the vast majority being from manure composite samples. The proportion of individual cows that excreted p-EHEC or p-O157 at any point in the study is higher than the prevalence in all fecal samples, providing further evidence of intermittent shedding (Table 2). Notably, p-EHEC and p-O157

![FIG 1 Distribution of C$_T$ values in positive samples by sample type, for *stx*$_2$ and *γ-tir*. MC, manure composite; filter, milk filters. Thick line, median; box, interquartile range; whiskers, quartile ± 1.5 interquartile range (nominal data range); circles, data points beyond the nominal data range. Higher C$_T$ values correspond to lower concentrations in the samples.](http://aem.asm.org/)
were found more frequently in manure composite than fecal samples. Conversely, trough water had a very low proportion of potentially pathogenic *E. coli*, with no p-O157 except for two samples from farm B and one from farm C. *E. coli* in feed followed a different distribution from other sample types, with sample proportion declining from lower-pathogenicity LNS to p-EHEC and p-O157 on all farms. Milk and milk filter samples showed a relative trend similar to what was observed between fecal and composite manure samples (reflecting the fact that milk filters represent a composite sample of the milk processed during a milking session): the distribution of *E. coli* classes was highly skewed toward NLNS in milk (85 to 95%), while milk filters showed a higher proportion of samples of higher potential pathogenicity.

**Seasonality.** For all three farms and all virulence factors, both season and year effects accounted for a significant amount of the variability in prevalence and *C*<sub>T</sub> levels. However, no individual year or season was consistently higher or lower for all farms, virulence factors, or sample types. For fecal samples, the interaction between year and season was statistically significant for all farms and virulence factors, making any potential seasonal effect more difficult to parse out. Logistic regression performed on prevalence or on *C*<sub>T</sub> categories yielded substantially the same results, and hence, the term “levels” is used here. Due to the different sampling frequencies for the three farms, fecal samples for all four seasons were available only for farm B (527, 849, 619, and 852 fecal samples, respectively, for winter, spring, summer, and fall). On farm A, samples were collected only in winter, spring, and fall (308, 1,912, and 2,318 fecal samples, respectively), while on farm C, samples were collected in spring and fall (886 and 829 fecal samples, respectively). Seasonal trends were not consistent across farms or virulence factors. For eae and γ-tir, levels were significantly higher in spring than in winter on farms A and B, and they were higher in fall than in spring on farm C. For stx<sub>1</sub>, levels in fall were higher than in winter on farms A and B, and levels in fall were higher than in spring on farm C. However, levels in spring were lower than in winter on farm B. For stx<sub>2</sub>, higher levels were observed in the warmer seasons of spring and summer than in winter on farm B. However, contrary to what was observed for stx<sub>1</sub>, stx<sub>2</sub> levels were lower in fall than in winter on farm A and lower in fall than in spring on farm C. A concordant pattern between stx<sub>1</sub> and stx<sub>2</sub> was observed only for farm B. No consistent trend was observed between overall warmer (summer and fall) and colder (winter, and possibly spring) seasons, although seven of the significant differences were compatible with, and three contrary to, the hypothesis that warmer seasons are associated with higher prevalence or levels of pathogenic *E. coli*. The same seasonality analysis applied to composite manure samples yielded different results than those for fecal samples. Namely, no significant season effect was observed for any of the factors on farms A and B, and only a few years had significantly higher or lower levels than others. For farm C, however, levels of all virulence factors were significantly higher in fall than in spring.

**Time trends and autocorrelation.** The trend of *C*<sub>T</sub> categories in fecal samples over time-ordered sampling sessions is shown in Fig. 3 for stx<sub>2</sub> and γ-tir and in Fig. S2 in the supplemental material for eae and stx<sub>1</sub>. On all three farms, *C*<sub>T</sub> levels fluctuated visibly over time. *C*<sub>T</sub> levels appear somewhat clustered in time at a visual inspection.

### TABLE 2 Highest-pathogenicity *E. coli* class shed by individual cows <sup>a</sup>

<table>
<thead>
<tr>
<th>Farm</th>
<th>NLNS (no. of fecal samples)</th>
<th>LNS (no. of fecal samples)</th>
<th>STEC (no. of fecal samples)</th>
<th>p-EHEC (no. of fecal samples)</th>
<th>p-O157 (no. of fecal samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.3 (21.0)</td>
<td>6.9 (33.8)</td>
<td>26.6 (26.0)</td>
<td>55.0 (17.8)</td>
<td>9.2 (1.4)</td>
</tr>
<tr>
<td>B</td>
<td>0.0 (12.4)</td>
<td>1.0 (21.4)</td>
<td>13.4 (40.4)</td>
<td>67.7 (24.0)</td>
<td>17.9 (1.8)</td>
</tr>
<tr>
<td>C</td>
<td>0.7 (12.7)</td>
<td>2.8 (28.0)</td>
<td>21.2 (25.9)</td>
<td>64.6 (31.2)</td>
<td>10.6 (2.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> *E. coli* classes were ranked from lowest potential pathogenicity (“non-LEE non-stx” *E. coli* [NLNS]) to highest (p-O157), from left to right. Individual cows were classified according to the highest potential pathogenicity class observed among their fecal samples; e.g., if the highest-pathogenicity sample from a cow was classified as p-EHEC, that cow was counted in the p-EHEC class.

<sup>b</sup> Values in parentheses represent the percentage of all fecal samples classified in each *E. coli* pathogenic class (see also Fig. 2, as well as Table S2 in the supplemental material).
spection, a trend that is more visible for stx₁ and stx₂ for all farms. No significant correlation was observed between prevalence in fecal and milk filter samples, or between composite manure and milk filter samples, for any of the factors. No consistent trend in autocorrelation of prevalence was observed across farms or factors. A few instances of positive autocorrelation of Cₜ means at lag 1 (i.e., contiguous sampling sessions) were observed in fecal samples for eae (farm B), stx₁ (farm C), and stx₂ (farms B and C).

FIG 3 Cycle threshold (Cₜ) categories over time in fecal samples, for stx₂ and γ-tir.

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which may indicate sporadic long “waves” of occurrence at similar levels spanning two sampling sessions. However, in most cases no autocorrelation was observed at lag 1. More instances of positive autocorrelation at lag 1 were observed in manure composite, for both prevalence and C_p means and for eae and stx, but never for γ-tir. Other instances of autocorrelation at different time lags do not correspond to any yearly or seasonal cycles and are consistent with the high degree of variability over time observed for all factors.

**DISCUSSION**

**Prevalence of virulence factors and *E. coli* pathogenic classes.** To our knowledge, this is the first study to assess the dynamics of *E. coli* virulence factors on dairy farms over a time frame of several years. This large longitudinal data set provides a long-term perspective on the temporal patterns of prevalence and relative abundance of virulence factors associated with diarrheagenic *E. coli*, in the major matrices of three dairy farms located in different northeastern U.S. regions. While several past studies assessed the occurrence of *E. coli* pathovars or virulence factors at a single point in time or over a short period of up to a few months (35, 55–60), intensive long-term studies are necessary to understand patterns of a pathogen’s introduction, epidemic curve, persistence, and resurgence on a farm. Large longitudinal data sets, in addition to short-term surveys focusing on specific factors, are also crucial for building and validating mathematical models to predict pathogen dynamics and test mitigation strategies. Direct identification of pathogenic *E. coli* in dairy farm samples through culture of the organism is difficult due to the wide degree of *E. coli* diversity in manure and feces. Except for O157:H7, isolating STEC strains is difficult due to the lack of metabolic differences that can be exploited for their selection. Even O157 is difficult to isolate from manure and fecal samples, often requiring time-consuming extraction with immunomagnetic beads and expensive chromogenic agars to increase the probability of successful isolation. Using quantitative PCR to detect four virulence factor genes associated with enteropathogenic *E. coli* after enrichment provides a direct semiquantitative comparison of the relative abundance of these virulence factors within the community of *E. coli* in a sample. Because such comparison describes the community and not individual cells, it cannot predict with certainty the presence of specific pathogenic serotypes, but more generally, it describes the population of each virulence factor within the mix of *E. coli* and other strains that can multiply in enrichments designed for *E. coli* growth. Therefore, while the presence of a virulence factor combination in a sample does not necessarily mean that a specific pathogenic *E. coli* strain is present, it implies the possibility for such occurrence. The operational definition of *E. coli* O157 and other *E. coli* categories used in this study is an attempt to describe this possibility.

This study confirms the widespread presence of potentially pathogenic *E. coli* in dairy herds and dairy farm environments. All four *E. coli* virulence factors considered in this study were consistently observed in all sample types and on all three farms, with the exception of γ-tir in milk and milk filters, and most frequently in fecal and manure composite samples. The prevalence of STEC genes (stx1 and stx2), an overall 28 to 43% for the three study farms, was consistent with and somewhat higher than ranges observed in other STEC virulence factor surveys in U.S. dairy herds (2, 8). However, stx prevalence higher than that in this study, up to 58% in manure, was also observed in the United States (33). The γ allele of the tir virulence factor associated with *E. coli* O157:H7 has also been commonly detected on dairy farms, in the United States and other countries (3).

A comparison between survey data obtained by culture-based identification methods and those obtained by PCR targeting virulence factors is not directly appropriate, as significantly different results have been observed (30). In addition, several past studies reported prevalence of *E. coli* strains in reference to the number of unique isolates, not individual samples. However, under the assumption that the STEC and p-O157 potential pathogenic classes adopted in this study can be taken as a whole-sample proxy for the presence of STEC and O157 strains, the prevalence ranges here reported are consistent with the wide range of STEC prevalence (0.17 to 40%) observed in fecal samples from adult dairy cows in the United States (8, 30, 33, 40, 61–63). These figures are also consistent with the range of STEC prevalence in dairy cattle observed in other countries (13, 30, 39, 64). Also, *E. coli* strain O157:H7 has been frequently isolated in dairy herds in the United States (34–36, 58) and in other countries (14, 39, 65). It is known that young cattle harbor STEC at significant prevalence, often higher than that in adult cows (13, 60, 63, 65–67). Also, one study observed that soil and floor areas where calves were raised had a higher prevalence of STEC than areas housing adult cows (68). The higher prevalence of stx1, stx2, and γ-tir observed in calf than in adult cow composite manure on two of the farms appears to confirm this trend. However, on farm A the considered virulence factors were detected at similar frequencies in composite manure from all age groups.

In this study, stx genes were occasionally detected in bulk tank milk from all three farms and γ-tir was detected in the milk from one farm. Virulence factor patterns compatible with STEC pathovars were detected at low prevalence (1.4 to 1.7%) in milk but at higher prevalence in milk filters (10.1 to 22.6%). STEC strains or stx virulence factors have also been detected at low prevalence in milk from U.S. farms (5, 8, 9, 69) and European farms (20, 70, 71). *E. coli* O157 or the associated virulence factors have also been detected in raw bulk milk at prevalences in the range of 0.2% to 9.1% (10, 72). Other surveys, however, detected no *E. coli* O157:H7 in raw milk in the United States (6, 35, 69, 73) and abroad (43, 74). In this study, virulence factor patterns compatible with the presence of *E. coli* O157:H7 were not detected in any of the milk samples, but they were detected in 0 to 2.0% of milk filters. Overall, existing evidence points to the fact that even when STEC and EHEC are present in dairy cow feces or the farm environment, appropriate sanitary practices can effectively lower the risk of milk contamination. Milk filters sampled in this study showed a much higher prevalence of all four virulence factors than did bulk milk, highlighting the impact of different sampling strategies and sample volume on assay sensitivity and observed prevalence, as seen in studies on other pathogens (75–77). Other surveys of milk filters observed an stx prevalence of 13.6% over 15 farms (33) and 51% in a large survey of dairy herds in the United States (9). *E. coli* O157:H7 was not detected by culture in any milk filters sampled by Hancock (35), while virulence factors compatible with the potential presence of *E. coli* O157:H7 were detected in 4.2% of raw milk samples in the 2002 National Animal Health Monitoring System (NAHMS) survey (based on the detection of eae, stx1 or stx2, and γ-tir but not using the semiquantitative criterion adopted in the present study; only 0.1%, i.e., 1 of 859
This study supports a body of evidence showing that *E. coli* virulence factors associated with STEC, EHEC, and EHEC O157:H7 are commonly present in the dairy farm environment, besides in cow manure. STEC or stx genes have been observed in dairy cow feed in some studies (63, 68, 78) but not in others (37). It is unclear if feed may be a source of pathogenic *E. coli* contamination, or if it becomes contaminated after being in contact with the animals or other farm environments. STEC has been detected in drinking water used by dairy cows (36, 63–65, 68, 79, 80). *E. coli* O157:H7 has also been detected in dairy cow feed (78) and trough water (36, 78). The very low frequency of detection of low levels of STEC virulence factors in source water in this study indicates that well water is unlikely to be a vehicle introducing STEC into the farms. Trough water is most likely contaminated by the cows, as shown by the much higher prevalence of STEC virulence factors in trough water than in source water across all farms and cow ages. Some studies have found a correlation between trough water contamination by *E. coli* O157 and infection in cattle herds, but the direction of pathogen spread is unclear (36, 78). Although trough water is very unlikely to be the vehicle introducing pathogens into the herd, *E. coli* O157:H7 has been shown to survive for weeks in trough water (81) and for several months in simulated trough sediments, even when chlorine was added to the water (82). Hence, water can potentially act as a reservoir and as a vehicle spreading pathogens from cow to cow (78).

Most past surveys assessed prevalence of generic or pathogenic *E. coli*, but concentrations were determined in only a few studies, for instance, in milk (72, 83). One study on fecal shedding by calves observed concentrations of *E. coli* O157 up to 10^5 CFU/g (84). Since pathogenic *E. coli* is common on dairy farms, prevalence alone has not proven sufficient to identify critical reservoirs or the likelihood of transmission between reservoirs. Studies directly measuring concentrations or relative abundance, such as the present study, are crucial in both understanding which farm compartments are reservoirs of *E. coli* pathogenic elements and pinpointing when and how frequently spikes in virulence factor abundance occur.

**Seasonality.** Temperature-dependent seasonality in the prevalence of pathogenic *E. coli*, including *E. coli* O157:H7, in dairy herds has been suggested, although only a few studies have investigated time trends in details. Specifically, a higher prevalence of *E. coli* O157 was observed during warmer weather, approximately from May to September in the northern hemisphere (30, 43, 58, 85–90), which is also the time when human outbreaks have occurred at higher frequency (91, 92). Significant seasonal effect on the occurrence of non-O157 STEC or stx genes has also been observed in some studies (37, 93). An increase in pathogenic *E. coli* prevalence in the warmer months was also documented in feedlot cattle herds (37, 94–98). Based on the analysis of data from this 7- to 9-year longitudinal study, no consistent seasonality was observed across farms, while a significant interaction effect between seasons and years was present. The autocorrelation analysis also did not provide strong evidence for or against seasonal or yearly cycles. It is possible that the sampling frequency (two sampling sessions per year for farms A and C and 3 to 4 sampling sessions per year for farm B) masked seasonal patterns. However, although the evidence is not conclusive, seven out of 10 significant seasonal differences, spanning all four factors, were in favor of the hypothesis that warmer seasons are associated with higher levels of *E. coli* virulence factors.

**Time trends and transmission dynamics.** In a short-term study of shedding in naturally infected calves, intermittent but ongoing shedding of *E. coli* O157 over a time frame of a few weeks was observed in dairy calf feces, and a small proportion of high-shedding individual calves were observed to shed almost continuously (84). Other studies observed intermittent shedding of STEC by adult dairy cows, each animal potentially shedding for several months (63, 99–101). Similar intermittent shedding patterns have been observed in feedlot cattle (58, 102). Individual and herd shedding patterns have been associated with several factors, including diet, herd size, housing type, and age of the animal (3).

While all pathogenic classes were observed consistently over time, in this study prevalence and CF7 levels fluctuated widely for all farms and all factors, a pattern that is compatible with intermittent shedding of variable intensity in individual animals but ongoing shedding at herd level. Occasional time clusters observed in fecal and composite samples are compatible with long shedding peaks spanning consecutive sampling sessions (i.e., time lag 1), although frequent shorter-term peaks cannot be excluded. This pattern is also compatible with the ongoing presence of a factor, with periodic flares in prevalence and levels not clearly associated with seasons. Overall, the long-term nature of the study and the frequency of sampling that was logistically feasible allowed analyzing time dynamics at a maximum time resolution of 3 to 6 months. Hence, while the observed long-term dynamics are compatible with underlying shedding patterns identified over the time frames of days and weeks in other studies, no definitive conclusions can be drawn on short-term dynamics occurring between sampling sessions.

Data on prevalence of virulence factors and *E. coli* pathogenic classes in different farm compartments and time dynamics can provide insight into prevention and mitigation strategies to reduce the persistence and movement of zoonotic pathogens on dairy farms. For example, the presence of *E. coli* virulence factors observed in manure, feed, and, to a lesser extent, water makes these compartments potential candidates for transmission reduction measures. Furthermore, the variability in prevalence observed between different age groups may support physical separation as a measure to reduce transmission. As demonstrated by the low prevalence and low pathogenicity observed in milk samples in this study, hygienic measures to prevent fecal contamination of milk appear to be effective, although exceptions (and not knowing the cause of such exceptions) can still result in significant risk. While it was outside the scope of this study to test the effect of farm practices, there is evidence that different farm management strategies such as spreading manure on pastures, extent of contact between animals, organic versus conventional approaches, diet, and hygiene may impact the risk of pathogenic *E. coli* occurrence and transmission (3, 33, 35, 103, 104). However, such evidence is still ambiguous, and climatic and geographical variables are also potential risk factors (79). While eradication of pathogenic *E. coli* on dairy farms still appears a far-fetched goal due to the high prevalence and widespread geographical distribution of the pathogen, as well as its persistent fluctuating occurrence in several farm compartments, a better understanding of the ecology of pathogenic *E. coli* and the mobile genetic elements associated with its virulence can lead to improved strategies to control *E. coli* pathogenicity on farms.
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