Occurrence and Spread of Quinolone-Resistant *Escherichia coli* on Dairy Farms

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ABSTRACT

Quinolone-resistant *Escherichia coli* (QREC) is common in feces from young calves, but the prevalence and genetic diversity of QREC in groups of cattle of other ages and the farm environment are unknown. The aims of the study were to obtain knowledge about the occurrence of QREC on dairy farms, the genetic diversity of QREC within and between farms, and how these relate to the number of purchased animals and geographic distances between farms. We analyzed the within-sample prevalence of QREC in individual fecal samples from preweaned dairy calves and postpartum cows and in environmental samples from 23 Swedish dairy farms. The genetic diversity of the QREC isolates on 10 of these farms was assessed. In general, QREC was more prevalent in the dairy farm environment and in postpartum cows if QREC was commonly found in calves than if QREC was rare in calves. In particular, we found more QREC organisms in feed and water troughs and in environments that may come into contact with young calves. Thus, the results suggest that QREC circulates between cattle and the farm environment and that calves are important for the maintenance of QREC. Some genotypes of QREC were widespread both within and between farms, indicating that QREC has spread within the farms and likely also between farms, possibly through purchased animals. Farms that had purchased many animals over the years had greater within-farm diversity than farms with more closed animal populations. Finally, animals on more closely located farms were more likely to share the same genotype than animals on farms located far apart.

IMPORTANCE

This study investigates the occurrence of a specific type of antimicrobial-resistant bacterium on dairy farms. It contributes to increased knowledge about the occurrence and spread of these bacteria, and the results pave the way for actions or further studies that could help mitigate the spread of these bacteria on dairy farms and in the community as a whole.

Fluoroquinolones are classified as critically important antimicrobials for the treatment of infections in humans (1). Bacteria do not respect species or geographic borders, and quinolone-resistant bacteria are undesirable in any part of the community. Unfortunately, we have observed that quinolone resistance is common in fecal *Escherichia coli* isolates from healthy dairy calves in Sweden (2). We found quinolone-resistant *E. coli* (QREC) in preweaned dairy calves on 60% of 243 farms studied (2). It is not unlikely that QREC spreads from calves to other species, including humans, although to our knowledge, such a link has not been established yet. Presumably, the more QREC bacteria that are present in a population, the higher is the risk of interspecies transmission. Until now, most of the previous investigations on QREC in the dairy population have been with calves (2–5), while the prevalence of QREC in the farm environment and cattle in other age categories is largely unknown. Hence, increased knowledge in this field is needed before best-practice recommendations can be made to mitigate the overall spread of QREC in dairy farms and the community as a whole.

Exposure to quinolone drugs is known to select for bacteria that are resistant (2, 6, 7). However, in our previous study, QREC was isolated from some farms that did not use fluoroquinolones and, in contrast, was not found on some farms that did use those agents (2). This observation, together with the findings of Taylor et al. (7) and Pereira et al. (6), indicates that the occurrence of QREC is not solely dependent on the use of fluoroquinolones. It is therefore possible that there are reasons other than fluoroquinolone exposure for the occurrence of QREC on dairy farms, as

suggested in our previous study (3). One theory is that QREC has been acquired from a source outside the farm, e.g., another farm. The clonal dissemination of QREC from dairy calves has been described previously by Marchese et al. (4), and it is possible that this is also the case on Swedish farms. We have previously found that QREC is more commonly shed by calves on farms in certain regions of Sweden (2) and that breaches in on-farm biosecurity are associated with the shedding of QREC by postpartum cows (3). These findings suggest the dissemination of QREC between farms in close geographic proximity, possibly via purchased cattle entering the farm. An analysis of the genetic diversity of QREC on farms may therefore provide information on the dissemination of QREC both between and within farms.

The primary aims of this study were to obtain baseline knowledge about the occurrence of QREC on dairy farms and to compare the occurrence of QREC on farms where QREC is common in calves with that on farms where QREC is rare in calves. Our

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hypothesis is that if QREC is common in feces from calves, it is also common in the farm environment and in feces from cattle in other age categories on the farm. Secondary aims were (i) to determine the genetic diversity of QREC within farms and (ii) to investigate if the genetic diversity of QREC is related to the number of purchased cattle and geographic distances between farms. Our hypotheses were (i) that the within-farm genetic diversity is larger on farms that have purchased many animals than on farms that have purchased none or a few animals and (ii) that farms that shared QREC genotypes were located closer to each other than farms that had only farm-specific genotypes.

MATERIALS AND METHODS

Selection of farms. This study was part of a project evaluating the spread of, risk factors for, and genetic diversity of QREC. In short, 23 dairy farms were recruited. All farms had a known historic use of fluoroquinolones but various prevalences of QREC in fecal samples from preweaned calves, on the basis of the results of our previous study (2). The inclusion criteria and recruitment process are described elsewhere (3).

Farm visits. Each farm was visited once between November 2013 and March 2014. The following samples were collected during the farm visits by the first author: fecal samples from 15 preweaned calves and five postpartum cows and environmental samples from the calf and the calving areas and from the floor in pens with cattle in other age categories on the farm.

Sampling of individual cattle. The collection of fecal samples from calves and postpartum cows is described in detail elsewhere (3). In short, rectal swab specimens were collected from 15 calves aged 0 to 30 days and from five postpartum cows 0 to 3 days after calving on each farm. Because all animals in the study were privately owned and no invasive procedures were done, ethical approval for the use of laboratory animals was not needed, according to the Board of Agriculture's regulations and general advice on laboratory animals (SJVFS regulation 2012:26).

Environmental sampling. Samples were collected from the calf pen environment and the calving area. In the calf pen environment, samples were collected from the pen walls in pens with a single calf (one sample per pen in up to three pens, when available) and pens with groups of calves (one sample per pen in up to three pens, when available), from feed and water troughs (one sample per trough in up to three troughs, when available), milk buckets (one sample per bucket in up to three buckets), and from the automatic milk feeder, if present (one sample per station in up to three stations, when available).

In the calving area, samples were taken only from the walls of calving pens or from the headlocks/cubicle partition if the cows calved in a tie stall barn (one sample per pen or cubicle in up to three pens or cubicles). All samples were collected using sterile cloths (Sodibox, Névez, France) that were preimpregnated at the factory with buffered peptone solution containing 10% neutralizing agent (lecithin, Tween 80, L-histidine, and sodium thiosulfate). Samples were obtained by wiping a surface area of approximately 0.25 m² of the pen walls (the cleanest area on all walls), the entire bottom surface of feed, water, and milk troughs; and the entire surface of the nipple in automatic milk feeders. All feed and water troughs, as well as milk buckets, were emptied before sampling. After sampling, each cloth was placed in a separate, plastic stomacher bag. The bag was sealed and stored at 4 to 8°C until it was sent (no later than the following day) by the postal service or brought by car to the laboratory at the National Veterinary Institute, Uppsala, Sweden. The bags were sent by the postal service if more farms were to be visited in the coming days on the same trip and by car if the farm was the last farm to be visited on the trip. Samples arrived at the laboratory, at the latest, on the morning after sampling. All samples were analyzed directly upon arrival.

Overshoe sampling (8) was used for the floors in the following pens (one sample per pen in up to three pens per sampling site): calving area, lactating cow area, dry cow area, and pens containing young stock ages 1

to 6 months and 7 to 24 months. Samples were collected using a pair each of Sterisocks (Sodibox, Névez, France), which are made of jersey material premoistened by the factory with 15 ml of distilled water. For each boot, the jersey material was fitted to the outside of disposable boot protectors. In group pens (loose housing pens with scraper aisles not included), samples were obtained by walking along the pen walls once, through each diagonal, and then back and forth over as much of the pen floor as possible, with a distance of approximately 80 m (100 strides) being covered. In loose housing pens with scraper aisles, samples were obtained by walking back and forth once through all scraper aisles as soon as possible after scraping. In tie stall housing, samples were obtained by walking back and forth behind the hind limbs of the fettered animals. This was done on the cubicle floor (not in the manure gutter), with a distance of approximately 80 m (100 strides) being covered. Care was taken not to step in single fecal pats. After sampling, each sock pair was handled in the same manner as the cloths.

Bacterial isolation, counting, and storage of isolates. The rectal swab specimens were prepared as described by Duse et al. (3). In short, rectal swabs were transferred to 0.9% saline and vortex mixed to release the fecal material. One hundred milliliters of 0.9% saline was added to each stomacher bag containing environmental samples. These bags were then placed in a stomacher (80 Biomaster lab system; Seward Ltd., Worthing, United Kingdom) and treated for 30 s. All environmental samples from the same sampling site within a farm (e.g., a single pen wall) were pooled into a single sample suspension by transferring equal aliquots (2 ml) from each stomacher bag to a single sterile glass tube. The above-described suspensions constituted the nondiluted ones, and 10-fold dilutions of these suspensions were made in 0.9% saline down to the following dilutions: 10⁻⁶ (rectal swabs from calves), 10⁻⁵ (rectal swabs from cows), 10⁻⁵ (environmental samples from pen floors), and 10⁻⁴ (all other environmental samples).

The total number of *E. coli* and QREC isolates in the sample suspensions was determined as described by Duse et al. (3). In short, selected dilutions of each sample suspension were cultured, and colonies were counted on a Petrifilm Select *E. coli* count plate (SEC plate; 3M Microbiology Products, St. Paul, MN, USA) before and after addition of 50 µl of a nalidixic acid solution (672 mg/liter) to 1 ml of the sample suspensions (final concentration on the SEC plates, 32 mg/liter). For each batch of nalidixic acid stock solution that was produced, quality control was conducted using *E. coli* ATCC 25922 as a susceptible reference strain and *E. coli* 4.7 (European Union Reference Laboratory-Antimicrobial Resistance) as a QREC reference strain.

From each of these SEC plates, up to five distinct CFU of QREC per sample was subcultured on horse blood agar (Oxoid, Basingstoke, United Kingdom). From each of the five colonies, pure cultures with a morphology resembling that of *E. coli* and a positive results by the spot indole test (*p*-dimethylaminocinnamaldehyde [DMACA]) (9) were stored at -20°C in 0.5 ml of serum broth with 5% glycerol until genotyping.

Categorization of farms. Farms were categorized as those with a high (H; *n* = 11) and those with a low (L; *n* = 12) mean within-sample prevalence of QREC in feces from preweaned calves (H farms > 0.5% ≥ L farms). The threshold of 0.5% represents the median value of the mean farm within-sample prevalence of QREC in feces from preweaned calves on the 23 farms. This categorization was done to compare H and L farms with respect to the occurrence of QREC in samples other than those from individual calves on the farm.

MLVA. For multiple-locus variable-number tandem-repeat analysis (MLVA), only QREC isolates from H farms were selected, given that QREC was found in at least one environmental sample from the same farm. One randomly selected isolate from each environmental sample from each of these farms was genotyped. However, due to budget constraints, it was not possible to analyze isolates from all calf and cow samples, and therefore, we randomly selected one isolate from each of four calf samples and two cow samples (when available). The isolates were selected using a random-number generator in Microsoft Excel software (Mi-

crosoft Corporation, Seattle, WA, USA). To determine the diversity within samples, all five QREC isolates from each of a subset of samples from two of the farms (two calves per farm, one cow per farm, and one environmental sample per farm) were analyzed by MLVA. In 75% (6/8) of the samples, all five isolates belonged to the same MLVA type, and in the other 25%, there were two different MLVA types. Hence, we concluded that the analysis of only one isolate per sample would be sufficient to determine the genetic diversity of QREC isolates from different samples and farms.

Bacterial DNA was extracted from each isolate by heating suspensions of bacterial cells at 98°C for 15 min, followed by centrifugation at $10,000 \times g$ for 2 min. Isolates were then genotyped as described by Lindstedt et al. (10) and Løbersli et al. (11) with some modifications. The dyes used for the forward primers were changed from those used by Lindstedt et al. (10) to the following: 6-hexachlorofluorescein phosphoramidite (HEX) for CVN001, *N*-hydroxysuccinimide ester of phosphoramidite (NED) for CVN002, 6-carboxyfluorescein (FAM) for CVN003, HEX for CVN004, FAM for CVN007, FAM for CVN014, NED for CVN015, FAM for CCR001, and for HEX CVN017. The multiplex-2 PCR mixture described by Lindstedt et al. (10) was separated into the multiplex-2A PCR mixture (M2A; 10 pmol each of primers CVN001 and CVN015) and the multiplex-2B PCR mixture (M2B; 10 pmol each of primers CVN004 and CVN007). In contrast to the method used by Lindstedt et al. (10), the amplicons were pooled and diluted as follows. Mix 1 contained 10 μ l of the multiplex-1 PCR product, 4 μ l of M2A, and 86 μ l of water, and mix 2 contained 15 μ l of the singleplex PCR product, 4 μ l of M2B, and 81 μ l of water. In mix 3, the multiplex-3 PCR product was diluted as described by Løbersli et al. (11). In contrast to the method used by Lindstedt et al. (10), the internal size standard was changed to the carboxy-X-rhodamine-labeled GeneFlow625 size standard (CHIMERx, Madison, WI, USA). Moreover, the capillary electrophoresis instrument was changed to an ABI 3100 instrument (Applied Biosystems, Foster City, CA, USA). The sizes of the fragments were determined according to the color and size of the peaks in Peak Scanner software (version 2.0; Applied Biosystems), and an allele number was assigned on the basis of the fragment sizes. If more than one fragment was detected for a single locus, the fragment with the highest level of fluorescence was selected. A minimum spanning tree (MST) of the MLVA types was created using the MST algorithm in BioNumerics software (version 7.1, 2014; Applied Maths, Inc., Austin, TX, USA) by categorizing the isolates by the farm of origin.

Additional data. Data on cattle movements in and out of the farms evaluated in this study were obtained from the central register of bovine animals kept by the Swedish Board of Agriculture (Jönköping, Sweden). This database tracks cattle movements that have taken place since January 1998. Thus, we could obtain the number of in-going movements of cattle to the farm until the date of our farm visit and, moreover, any movements of cattle between farms. The straight-line (bird-flight) distances (in kilometers) between farms were obtained using the distance-measuring function in Google Maps.

Data analysis. Data on the results of bacteriological analyses, cattle trade movements, and between-farm distances were entered in a Microsoft Excel spreadsheet (Microsoft Corporation) and transferred to Stata 13 software (Stata statistical software, release 13.1; Stata, College Station, TX, USA). Descriptive statistics were computed for all sample types on each of the farms. H and L farms were compared with respect to the median within-sample prevalence of QREC in different samples using the Wilcoxon rank-sum test, and the same was done for the proportion of QREC-positive samples, which were analyzed using Fisher's exact test. All analyses were conducted in Stata 13 software (Stata statistical software, release 13.1). A *P* value of less than or equal to 0.05 was considered significant.

To determine the overall and within-farm genetic diversity, a Simpson's index of diversity (*D*) was calculated as $1 - \sum [n_i(n_i - 1)]/[n(n - 1)]$, where n_i is the number of isolates with subtype *i* and *n* is the total number of isolates (12). The correlation between the within-farm *D* value and the

number of purchased cattle was assessed using the Spearman rank-order correlation coefficient in Stata 13 software (Stata statistical software, release 13.1), to test the hypothesis that the within-farm genetic diversity increases with the number of purchased cattle. We also hypothesized that more closely located farms were more likely to have indistinguishable MLVA types than farms located far apart since farms closely located were assumed to be more connected epidemiologically. Therefore, the straight-line (bird-flight) distance between pairs of farms with at least one shared MLVA type was compared to the straight-line distance between pairs of farms with only unique MLVA types using a two-sided *t* test in Stata. A *P* value of less than or equal to 0.05 was considered significant.

RESULTS

Samples. In total, 345 fecal samples from preweaned calves, 115 fecal samples from postpartum cows, and 257 environmental samples were collected on the 23 dairy farms.

Occurrence of QREC. The growth of *E. coli* was observed in all but two calf fecal samples, three cow fecal samples, and two environmental samples. All farms except one (farm T) had at least one QREC-positive sample. Quinolone-resistant *E. coli* was isolated from 60% (calf) and 28% (cow) of the fecal samples and 44% of the environmental samples. The within-sample prevalence of QREC per sampling site and farm is described in Table 1.

Occurrence of QREC on H and L farms. The proportion of samples with QREC from calf feed troughs (*P* = 0.003), calf water troughs (*P* < 0.001), milk buckets (*P* = 0.001), the walls of pens with a single calf (*P* = 0.03), the walls of pens with groups of calves (*P* = 0.012), individual fecal samples from postpartum cows (*P* < 0.001), and the floors in pens with young stock 1 to 6 months old (*P* = 0.009) was significantly higher on H farms than L farms (Fig. 1). There were no statistically significant differences between H and L farms in the proportion of samples with QREC from automatic milk feeders (*P* = 0.08), calving pen walls (*P* = 0.67), or the floors in the dry cow pens (*P* = 0.63), the lactating cow pens (*P* = 0.21), or the pens with young stock aged 7 to 24 months (*P* = 0.4) (Fig. 1).

The within-sample prevalence of QREC in individual fecal samples from cows (*P* = 0.008) and samples from calf feed troughs (*P* ≤ 0.001), calf water troughs (*P* ≤ 0.001), milk buckets (*P* ≤ 0.001), automatic milk feeders (*P* = 0.02), the walls of pens with a single calf (*P* = 0.01), the walls of pens with groups of calves (*P* ≤ 0.001), and the floors in the calving pens (*P* = 0.005) and pens with young stock aged between 1 and 6 months (*P* = 0.005) was significantly higher on H farms than L farms (Fig. 2). There were no statistically significant differences between H and L farms in the within-sample prevalence of QREC in samples from the calving pen walls (*P* = 0.57) or the floors in the lactating cow pens (*P* = 0.15), dry cow pens (*P* = 0.46), or pens with young stock aged 7 to 24 months (*P* = 0.47) (Fig. 2).

Genetic diversity of QREC on H farms. Ten of the 11 H farms fulfilled the criteria for inclusion of samples in the MLVA analyses (H farms where QREC was found in at least one environmental sample). On these 10 H farms, 136 isolates from the 138 samples that yielded QREC were subjected to genotyping by MLVA. A pure colony of QREC could not be isolated from the remaining two samples due to overgrowth of *Proteus* spp. Twenty-four unique MLVA types were identified (Table 2). The three most common MLVA types were type 1 (36% of all 136 isolates), type 2 (20%), and type 3 (9%). Six MLVA types were identified on more than one farm (Table 2 and Fig. 3), whereas the remaining types were found only on a single farm. Of the MLVA types that were

TABLE 1 Within-sample prevalence of QREC in different samples from dairy farms^a

Farm	Farm category	Mean within-sample prevalence (%) of QREC for all individual samples (no. of QREC-positive samples/total no. of samples tested)		Within-sample prevalence of QREC (%) in one pooled sample per sample type												Mean within-sample prevalence (%) of QREC for all samples (no. of QREC-positive samples/total no. of samples tested)
		C	PPC	F	W	A	M	S	G	CPW	CPF	L	D	YY	OY	
B	H	31 (14/14 ^b)	31 (5/5)	71	11	26	33	15	11	1	30	5	9	0.2	0	26 (30/31)
K	H	24 (12/15)	<0.01 (1/5)	10	5	NA	0	8	0.2	40	2	0	0	0	0	14 (19/31)
F	H	17 (14/15)	0.01 (1/5)	38	33	NA	19	0	0	0 ^c	<0.01	0.03	0	0	0	12 (20/30)
I	H	16 (13/15)	0.2 (1/5)	18	5	NA	30	17	2	0.9	3	0	2	0	0.2	10 (23/31)
G	H	11 (15/15)	0.03 (2/5)	5	38	9	NA	6	1	0	0.2	0.1	20	0.3	0.1	8 (27/31)
H	H	11 (15/15)	0.9 (4/5)	0	2	0.6	2	0.02	5	4	5	0	0	2	0.1	6 (28/32)
E	H	7 (14/15)	<0.01 (2/5)	1	0.8	0	31	4	1	0	0	0.02	0	0.04	0.1	5 (24/32)
A	H	7 (12/15)	0.2 (2/5)	100	3	NA	0.4	3	49	0	<0.01	0	0 ^c	0.07	0	8 (21/31)
L	H	6 (14/15)	0 (0/3 ^b)	12	0.03	0.2	0	12	0.8	5	<0.01	4	0	<0.01	0	4 (23/30)
D	H	4 (7/14 ^b)	0 (0/5)	0	NA	NA	0	0	0	0	0	0	0	0	0	2 (7/29)
J	H	0.7 (15/15)	0.2 (3/5)	7	5	6	5	0.2	1	0.6	0.08	0.1	X ^d	0.07	0.07	1 (29/31)
M	L	0.5 (8/15)	0 (0/5)	0	0	0	0	0.1	0	15	0	0	0	0	0	0.7 (10/32)
N	L	0.3 (11/15)	<0.01 (2/5)	0	0	0	0	0.1	0	1	0.4	0.03	0	0	2	0.3 (18/32)
X	L	0.2 (9/15)	5 (5/5)	0.2	NA	NA	0	45	0.2	11	0.5	4	0.9	0	0	3 (22/31)
O	L	<0.01 (3/15)	0 (0/4 ^b)	0	0 ^c	NA	0	NA	0	40	0.01	0	NA	0	2	2 (6/27)
P	L	<0.01 (4/15)	0 (0/5)	0	0	NA	0	0	0	0	0	0	0	0	0	<0.01 (4/32)
Q	L	<0.01 (7/15)	0 (0/5)	0	0	NA	0	0	0.03	0	0	0	0	0	0	<0.01 (8/31)
R	L	<0.01 (4/15)	0 (0/5)	0	0	NA	0	0	0	0	0	0	NA	0	0	<0.01 (4/30)
S	L	<0.01 (3/15)	0.1 (1/5)	0	0	NA	0	0	0	<0.01	0	0.1	13	0	0	0.4 (7/31)
U	L	<0.01 (1/15)	0 (0/5)	0.04	0.05	0.01	0	0	0.3	0	0	0	0	0	0	0.01 (5/31)
V	L	<0.01(11/15)	0 (0/5)	0	0	0	0	0	0	0	0	0	0	0	0	<0.01 (11/32)
C	L	<0.01 (1/15)	<0.01 (2/5)	0	0	NA	0	0	0	0	0	0	0	<0.01	<0.01	<0.01 (5/32)
T	L	0 (0/15)	0 (0/5)	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/32)
All		6 (207/343)	2 (31/112)	11	5	4	6	5	3	5	2	0.5	2	0.1	0.2	4 (351/712)

^a Data are for 23 dairy farms. Abbreviations: H, farms where QREC was common in feces from preweaned calves; L, farms where QREC was rare in feces from preweaned calves; F, calf feed troughs; W, calf water troughs; M, milk buckets; A, automatic milk feeder; S, wall of a pen with a single calf; G, wall of a pen with groups of calves; C, individual calf samples; PPC, individual postpartum cow samples; CPW, calving pen wall; CPF, calving pen floor; L, lactating cow pen floor; D, dry cow pen floor; YY, pen floor of younger young stock (age, 1 to 6 months); OY, pen floor of older young stock (age, 7 to 24 months); NA, not available for sampling.

^b *E. coli* could not be isolated from some of the fecal samples.

^c *E. coli* colonies could not be isolated from the sampling site.

^d X, dry cows were kept only in the calving pens.

identified on more than one farm, type 1 was found on six farms (farms F, G, and I to L), type 2 was found on five farms (farms A, B, and I to K), type 3 was found on two farms (farms A and H), type 4 was found on two farms (farms G and H), type 7 was found on two farms (farms G and J), and type 11 was found on two farms (farms G and H). In general, one or two MLVA types dominated on individual farms. Type 1 was the dominating genotype on four of the six farms where it was found. Type 2 was the dominating genotype on two of the five farms where it was found, and type 3 was the dominating type on both of the two farms where it occurred (Table 2 and Fig. 3). The within-farm *D* value for each farm is given in Table 2. The *D* value for the genetic diversity of all QREC isolates was 0.82.

Genetic diversity, distance between farms, and number of purchased cattle. Pairs of farms with at least one shared MLVA type were located, on average, 196 km (95% confidence interval [CI], 148 to 244 km) from each other, whereas pairs of farms with no shared MLVA types were located, on average, 276 km (95% CI, 230 to 323 km) from each other ($P = 0.02$). There was a strong positive correlation ($\rho = 0.82$, $P = 0.004$) between the number of cattle purchased since 1998 and the within-farm *D* value (Fig. 4).

FIG 1 The proportion of samples positive for QREC for all environmental samples and individual fecal samples from postpartum cows, summarized for farms where QREC was common in feces from preweaned calves (H farms; gray bars) and farms where QREC was rare in feces from preweaned calves (L farms; black bars). *, significant ($P \leq 0.05$) difference, using Fisher's exact test, between H and L farms.

FIG 2 Box-and-whisker plot of the minimum, 25th percentile, median, 75th percentile, and maximum within-sample prevalence of quinolone-resistant *Escherichia coli* in environmental samples and individual fecal samples from postpartum cows for farms where QREC was common in feces from preweaned calves (H farms) and farms where QREC was rare in feces from preweaned calves (L farms). *, significant ($P \leq 0.05$) difference, using the Wilcoxon signed rank sum test, between the H and L farms investigated.

Except for the movement of 10 animals between farms K and F in 2009, no cattle had been traded directly between the farms in the study.

DISCUSSION

In this study, QREC was found on all but one farm, but the prevalence in samples from individuals and from the farm environment differed between farms. Quinolone-resistant *E. coli* was isolated in all types of samples and was therefore not specific to the calves. However, QREC was isolated in more samples on farms where QREC was common in feces from calves (H farms) than on farms where QREC was rare in feces from calves (L farms). On H farms, a high within-sample prevalence of QREC was observed in the calf feed and water troughs and in milk bucket samples. The MLVA was not used to test movement within farms; however, we can assume, based on these results, that the bacterial flora surrounding the calves may play an essential role in the presence of bacteria in their gastrointestinal (GI) tract, which was also observed in a recent study by Liu et al. (13). A high prevalence of QREC in feed and water troughs may ease the fecal-oral circulation of QREC, which was also suggested by Yamamoto et al. (14) to be important for the on-farm dissemination of multidrug-resistant *E. coli*. The higher that the proportion of QREC isolates in the calf feed and water troughs is, the more likely it is that QREC establishes itself in the calf gut and the more likely it is that the calf recontaminates the pen environment. Snow et al. (15) concluded that infrequent disinfection of milk feeding equipment is a risk factor for the shedding of extended-spectrum-beta-lactamase (ESBL)-producing *E. coli* on dairy farms. It is reasonable to assume that the cleaning and disinfection of feed, water, and milk troughs would reduce the level of contamination with QREC in

the farm environment, but such conclusions cannot be drawn from the findings of our study. We also observed that QREC was more common in areas where young calves were kept, at least transiently (e.g., in the calf pens, pens with young stock aged 1 to 6 months, and the calving area) than in pens where only older cattle were kept. Previous studies have indicated that resistant *E. coli* strains may outcompete susceptible *E. coli* strains in the young calf gut but not in the guts of older cattle, even in the absence of a selective pressure (16). If QREC is favored in the calf gut, the calf will excrete feces in which QREC is more concentrated than it is in an older animal, which is also indicated by the results of our previous study (3). This led us to hypothesize that young calves may be important for the maintenance and multiplication of QREC on the dairy farm. Newborn calves are likely to be colonized with QREC from the immediate environment, and thus, the degree of QREC contamination in the calving area may be important for shedding of QREC by calves. Whether the presence of QREC in the calving area is due to contamination by calves or cows, exposure to a QREC-contaminated calving area by calves or cows may lead to the subsequent dissemination of QREC on the farm when the animal is moved to other pens. Watson et al. (17) also suggested that transition via the calving area may be crucial for the on-farm dissemination of ESBL-producing *E. coli* and that improved cleaning and disinfection of the calving area can reduce the burden of ESBL-producing *E. coli*. Our results also suggest that actions taken in the calving area might be important for the load of QREC in the dairy farm environment, but further studies should be performed to investigate if increased cleaning and disinfection in the calving area or calf pens would lead to a reduction in the prevalence of QREC on dairy farms.

TABLE 2 Distribution of MLVA types in 136 QREC isolates from different types of samples on 10 H farms^a

Farm	QREC prevalence (%) ^b	MLVA type	MLVA allelic string ^c	Sample type ^d (number of individuals)	Simpson's index of diversity (D)
B	31	2	06-02-NA-12-03-13-05-01-01-NA	F, W, M, S, C (1), PPC (2), CPW, CPF, L, D, YY	0.425
		6	05-02-NA-09-03-02-05-01-03-NA	A, G, C (3)	
K	24	1	05-02-NA-12-03-06-05-01-NA-NA	F, W, S, G, C (4), CPW, CPF	0.182
		2	06-02-NA-12-03-13-05-01-01-NA	PPC (1)	
F	17	1	05-02-NA-12-03-06-05-01-NA-NA	F, W, M, C (3), PPC (1), CPF, L	0.200
		23	05-02-NA-12-03-05-05-01-NA-NA	C (1)	
I	16	1	05-02-NA-12-03-06-05-01-NA-NA	F, W, M, S, G, C (3), PPC (1), CPF, CPW, D, OY	0.143
		2	06-02-NA-12-03-13-05-01-01-NA	C (1)	
G	11	1	05-02-NA-12-03-06-05-01-NA-NA	F, W, C (2)	0.917
		4	06-02-NA-12-03-08-05-01-02-NA	A, C (1)	
		7	05-02-NA-12-03-08-05-01-NA-NA	C (1), YY	
		11	06-02-NA-12-03-06-05-01-02-NA	G	
		12	07-19-NA-12-03-06-05-NA-NA-NA	L, OY	
		13	07-19-NA-12-03-08-05-NA-NA-NA	CPF, D	
		14	05-02-NA-12-03-04-05-01-NA-NA	S	
		19	07-19-NA-12-03-04-05-01-00-NA	PPC (1)	
H	11	4	06-02-NA-12-03-08-05-01-02-NA	W, M, A, G, CPF	0.725
		3	06-02-NA-12-03-06-05-01-01-NA	S, C (1), PPC (2), YY, OY	
		10	06-02-NA-12-03-04-05-01-02-NA	C (2)	
		11	06-02-NA-12-03-06-05-01-02-NA	C (1)	
E	7	17	06-02-NA-12-03-07-05-01-01-NA	D	0.603
		5	05-02-NA-12-03-07-05-01-10-NA	W, M, S, G, C (3), PPC (1)	
		9	08-02-NA-12-03-08-05-02-15-NA	PPC (1), YY, OY	
		15	05-02-NA-12-03-06-05-01-10-NA	F	
A	7	18	06-07-NA-12-03-04-05-01-NA-NA	C (1)	0.673
		2	06-02-NA-12-03-13-05-01-01-NA	PPC (1)	
		3	06-02-NA-12-03-06-05-01-01-NA	F, S, G, C (2), CPF	
		8	06-02-NA-12-02-14-05-02-01-NA	C (2), PPC (1)	
L	6	16	06-00-NA-12-03-09-05-02-NA-NA	YY	0.167
		1	05-02-NA-12-03-06-05-01-NA-NA	F, W, A, S, G, C (3), PPC (1), L, YY	
J	0.7	21	07-02-NA-12-03-11-05-01-01-NA	C (1)	0.507
		1	05-02-NA-12-03-06-05-01-NA-NA	CPF	
		2	06-02-NA-12-03-13-05-01-01-NA	F, W, M, A, S, G, C (1), PPC (1), CPW, L, YY, OY	
		7	05-02-NA-12-03-08-05-01-NA-NA	C (1), PPC (1)	
		22	07-02-NA-12-03-13-05-01-01-NA	C (1)	
		24	05-02-NA-12-03-08-05-01-01-NA	C (1)	

^a Each color represents a unique MLVA type.^b Mean within-sample prevalence (%) of QREC in feces from 15 preweaned calves.^c Allele number for the 10 loci in the following order: CVN001, CVN002, CVN003, CVN004, CVN007, CVN0014, CVN0015, CCR001, CVN0016, and CVN001; NA, not available.^d F, calf feed troughs; W, calf water troughs; M, milk buckets; A, automatic milk feeder; S, wall of a pen with a single calf; G, wall of a pen with groups of calves; C, individual calf samples (number of samples); PPC, individual postpartum cow samples (number of samples); CPW, calving pen wall; CPF, calving pen floor; L, lactating cow pen floor; D, dry cow pen floor; YY, pen floor of younger young stock (age, 1 to 6 months); OY, pen floor of older young stock (age, 7 to 24 months).

Our hypothesis that QREC would be more prevalent within the H farms than the L farms was confirmed. With some exceptions (farms D and V), the prevalence of QREC in fecal samples from preweaned calves was a good indicator of the degree of on-farm

occurrence of QREC. Although the majority of calves on farms D and V shed QREC, it was rarely found in any of the other samples on the farm. Possible explanations for these differences could be that different cleaning strategies or selection pressures that either

FIG 3 Minimum spanning tree of the diversity and relatedness of the MLVA types of quinolone-resistant *E. coli* isolates, categorized by farm. Each pie wedge represents one isolate, each circle represents one unique MLVA type, and each color represents one farm.

inhibit or promote the spread of QREC apply on different farms. It could also be speculated that the QREC strains on farms D and V were either less viable outside the calf's gut or that they only passed through the GI tract without increasing in number, as opposed to the characteristics of QREC strains on farms where they were found in the environment. The success of the circulation of QREC between animals and the farm environment—that is, how well adapted the QREC strains are to the farm environment and how well they outcompete susceptible *E. coli* isolates in the calf's GI

tract—may be key factors in the degree of on-farm dissemination. Studies with humans reveal that some QREC mutants have properties that facilitate their overall survival and passage through the GI tract (18). Also, some mutations mediating quinolone resistance are associated with higher levels of bacterial fitness, which could increase the ability of the organism to survive outside the calf (19). Unfortunately, the genetic basis for quinolone resistance was not investigated in this study.

We used genetic fingerprinting by MLVA to determine if the widespread dissemination of QREC on the H farms could be due to the clonal dissemination of a particular QREC strain. Even though the species *E. coli* is very diverse (20), the on-farm diversity was relatively low on most farms (the *D* value was below 0.5 on half of the farms). On five of the farms, only two unique genotypes were found, which indicates that the same clones circulate within the farm rather than that different QREC strains emerge in different cattle categories and environments. This is similar to the results of Hoyle et al. (5), who observed that the same clone of QREC disseminates throughout the herd over time. The dominance of some MLVA types on some farms in our study further emphasizes that some QREC clones are more successful than others. The discriminatory power of MLVA for *E. coli* is comparable to or sometimes superior to that of pulsed-field gel electrophoresis, which is often considered the “gold standard” genotyping method in epidemiological investigations (21). Hence, the low diversity of strains in this study is probably not due to the low discriminatory power of the genotyping method used.

In a report by Duse et al. (3), we discussed the possibility that

FIG 4 Simpson's index of diversity of quinolone-resistant *E. coli* isolates within farms as a function of the number of cattle purchased since 1998. Dotted line, linear prediction of the function.

QREC is introduced onto the farm from an exogenous source, rather than solely being a result of emergence on the farm itself. In this study, we found the same MLVA types on several farms, strongly indicating the clonal dissemination of QREC between farms. This is in line with the results of Lim et al. (27) and Marchese et al. (4). Farms located within a shorter distance from each other were also more likely to be contaminated with the same clone of QREC than farms located far apart. Although the distances between farms in our study were much longer than those in the study of Lim et al. (27), a similar pattern emerged. This was a rather rough and primitive method of investigating a genetic-geographic relationship, and more advanced analyses could yield more confident results. Moreover, the results of our previous study (2) indicated clustering of QREC isolates within certain regions. This could be due to regional differences in the use of fluoroquinolones, but testing of such a hypothesis would require very accurate estimates of the historic use of fluoroquinolones. It can also be assumed that the shorter the distance between farms is, the more likely it is that the farms will be connected epidemiologically. The most obvious such connection would be the movement of animals between farms. Likewise, the purchase of cattle is a risk factor for the presence of QREC on the farm (3). In the present study, we also found that the on-farm genetic diversity was strongly correlated to the number of purchased cattle, indicating that the genetic heterogeneity of QREC may be due to the introduction of new QREC strains along with new cattle rather than due to mutation of susceptible *E. coli* strains already present on the farm. Likewise, the QREC strains from human volunteers in the study of de Lastours et al. (22) were genetically different from the susceptible *E. coli* strains, indicating that emergence toward quinolone resistance was due to the acquisition of exogenous QREC strains rather than the mutation of already existing *E. coli* strains. However, the movement of animals is not the only plausible transmission pathway. Farms can also be connected epidemiologically via the movement of personnel, utensils, shared equipment, birds or insects, milk trucks, and professionals visiting the farms (veterinarians, artificial insemination technicians, feed counselors, etc.). Investigation of such relationships is difficult and was beyond the scope of this study. Hence, our data can neither dismiss nor confirm the possibility that QREC strains were transferred between farms through cattle movements or in some other way.

There are some drawbacks to this study. First, the small sample used in this study may have introduced some type II errors into the analyses comparing the prevalence of QREC on H and L farms. A second possible bias is that we were not able to control the ambient temperature while the samples were sent to the laboratory, but we believe that the short duration (approximately half a day) in which the samples were in the custody of the postal service at various ambient temperatures would have a negligible impact on the samples. Third, the use of 32 mg/liter as the cutoff for QREC was not optimal for the detection of plasmid-mediated quinolone resistance (23), but although such a resistance mechanism is of primary concern, it is still very rare in QREC from cattle (4, 24). Another limitation is that we were unable to quantify the load of QREC in samples because the amount of fecal material was not standardized in the analysis. However, we assumed that the ratio of resistant to susceptible isolates is at least as important for the spread of QREC between individuals. Finally, although the use of selective medium for quantifying the load of QREC in samples was useful for the purpose of this study, the results are difficult to

interpret in relation to those of studies or surveillance programs that use approaches with nonselective medium, e.g., the approach used by the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) program (25). While such methods are good at estimating the prevalence of resistance at the bacterial population level, they offer less confidence than approaches with selective medium when estimating the prevalence of bacterial resistance at the animal or sample level, at least for rare phenotypes (26). This difference should be taken into consideration when interpreting the results of this study in a broader sense.

Conclusions. In this study, we observed that QREC is abundant on some dairy farms and rare on others. Quinolone-resistant *E. coli* was, in general, more prevalent in samples related to the calf, in particular, in the calf feed and water troughs, indicating that the fecal-oral route may be important in the on-farm dynamics of QREC. Also, the higher that the within-sample prevalence of QREC in feces from preweaned calves was, the more widespread that QREC was on the farms. A high prevalence of QREC in calf-related samples implies that calves may play an important role in the maintenance of QREC on dairy farms. Some MLVA types of QREC were widespread both within and between farms, indicating the clonal dissemination of QREC. Farms that were located within a shorter distance from each other were more likely to share the same MLVA types, and the genetic diversity within farms increased with the total number of purchased cattle.

Our study presents knowledge about the dissemination of QREC within and between farms that could be valuable in reducing the burden of QREC. Increased hygiene in the calf and calving pens and increased biosecurity may be valuable for accomplishing that.

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