

Foodborne Pathogens in In-Line Milk Filters and Associated On-Farm Risk Factors in Dairy Farms Authorized To Produce and Sell Raw Milk in Northern Italy

FEDERICA GIACOMETTI,¹ ANDREA SERRAINO,^{1*} GUIDO FINAZZI,² PAOLO DAMINELLI,² MARINA NADIA LOSIO,² PAOLO BONILAUDI,² NORMA ARRIGONI,² ANDREA GARIGLIANI,³ ROBERTO MATTIOLI,³ SILVIA ALONSO,⁴ SILVIA PIVA,¹ DANIELA FLORIO,¹ RAFFAELA RIU,¹ AND RENATO GIULIO ZANONI¹

¹Department of Veterinary Medical Sciences, Faculty of Veterinary Medicine, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy; ²Experimental Institute for Zooprophyllaxis in Lombardy and Emilia Romagna, Via Bianchi 7/9, 25124 Brescia, Italy; ³Bologna Health Trust, Bologna Nord District, Via della Libertà 45, 40016 S. Giorgio di Piano, Bologna, Italy; and ⁴The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK

MS 12-028: Received 18 January 2012/Accepted 20 February 2012

ABSTRACT

All dairy farms authorized to produce and sell raw milk in a province of Northern Italy were investigated to determine the presence of *Campylobacter* spp., verocytotoxin-producing *Escherichia coli* (VTEC), *Listeria monocytogenes*, and *Salmonella* spp. in in-line milk filters and to assess their association with suspected risk factors on farms. A logistic regression model was used to analyze data collected describing the characteristics and management practices of 27 farms and the microbiological status of 378 in-line milk filters by both culture-based and molecular methods. Thermotolerant *Campylobacter*, VTEC, and *L. monocytogenes* were detected in 24 (6.45%), 32 (8.4%), and 2 (0.5%) samples, respectively. No *Salmonella* spp. were detected. For risk analysis, data of *L. monocytogenes* and *Salmonella* spp. were not included in the model because of the low prevalence or absence of these organisms. The univariate analysis disclosed that the presence of VTEC and/or *Campylobacter* spp. in milk filters was associated with lack of cleanliness of bedding, water trough, and feed trough; nonevaluation of water hardness; lack of cleanliness of milk tank; and nonapplication of forestripping. After multivariate analysis, an association was observed with inadequate cleanliness of bedding and milk tank and the nonapplication of forestripping. PCR analysis of milk filters was a rapid and sensitive method for the microbiological evaluation of herd contamination status and should be included among the registration requirements for the authorization to produce and sell raw milk. Specific control actions must be incorporated into the farmer's daily practices to ensure the low-risk production of raw milk.

The sale of raw milk for human consumption by self-service automatic vending machines has been allowed in Italy since 2004. Safe raw milk is essential to ensure consumer safety. The production of uncontaminated raw milk starts at the farm level and is influenced by on-farm management practices. There is clear evidence that dairy farms are major reservoirs of foodborne pathogens like *Campylobacter jejuni*, verocytotoxin-producing *Escherichia coli* (VTEC), *Listeria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica* (32, 33, 41), and other pathogenic bacteria often implicated in outbreaks of milkborne infection. Additionally, the environment of the dairy farm is conducive to contamination by and persistence of many bacterial species. Despite considerable efforts to establish hygienic milking systems and protocols, fecal contamination is inevitable, and milk is therefore at risk of contamination by any pathogen present in the feces or farm environment (60). Knowledge of the microbiological status

of the herd is the first step to control the risks associated with raw milk sale and consumption, and the utility of testing in-line milk filters as a sensitive way to detect pathogens is well documented. Three- to 10-fold-higher pathogen isolation rates have been reported from in-line milk filter samples than from bulk tank milk (36, 39, 52, 61, 66).

From the standpoint of reducing risks at farm level, development of pre- and postharvest control measures to effectively minimize contamination is critical to the control of pathogens in raw milk (38). Identification of on-farm risk factors serve both to devise and monitor farm-specific pathogen reduction programs and to design a control plan based on mutual on-farm risk factors that, as emphasized by Hussein and Sakuma (28), could reduce the presence of several potential foodborne pathogens simultaneously, maximizing the benefits obtained from inputs and minimizing the work and economic burden of farmers.

This study was conducted to (i) determine the prevalence of *Campylobacter* spp., VTEC, *L. monocytogenes*, and *Salmonella* spp. in in-line milk filters of farms authorized to produce and sell raw cow's milk in a province

* Author for correspondence. Tel: (+39) 51 2097332; Fax: (+39) 51 2097346; E-mail: andrea.serraino@unibo.it.

of the Emilia Romagna Region, Northern Italy; and (ii) investigate the association between on-farm management practices and foodborne pathogens, identifying risk practices to be targeted in a shared control plan for the foodborne pathogens considered.

MATERIALS AND METHODS

Selection of farms and data collection. All 33 farms authorized to produce and sell raw cow's milk in a province of the Emilia-Romagna Region were considered in the study. Twenty-seven of 33 farmers agreed to participate and formed the study sample. Information was collected and verified in all farms by the same person during a visit to each farm, using a standardized form to evaluate farm characteristics and practices including general management, milk quality data, health status, feeds and feeding, housing conditions of cows, hygienic conditions of facilities, good milking practices, and milking machine management. A total of 71 variables were considered and investigated as binary variables (presence versus absence, yes versus no, sufficient versus insufficient, adequate versus inadequate, or "greater than" versus "less than" a critical limit). The data were not included in the analysis when the answers were homogeneous, meaning all farms had exactly the same answer.

In-line milk filter sampling. The sample size was designed using EpiTools epidemiological calculators (3); the expected prevalence value was calculated on the basis of average prevalence data of the pathogens in milk determined by official provincial testing. Samples were collected at each farm 14 times, twice a week for seven successive weeks between January 2010 and June 2010 for a total of 378 filters; the filters collected represented the group of lactating cows at the time of sampling. Immediately after the end of milking, the fresh milk filter was collected and placed in a sterile plastic bag adsorbed with enough milk from the bulk tank to completely cover the sock filter; the remaining air in the plastic bag was eliminated. Samples were placed in refrigerated coolers at $6 \pm 2^\circ\text{C}$, transported to the laboratory, and processed within 6 h of sampling.

Laboratory investigation. Immediately after arrival at the laboratory, milk filters were aseptically weighted and cut into four portions for *Campylobacter* spp., VTEC, *L. monocytogenes*, and *Salmonella* species detection. Cultural methods were performed according to protocols outlined in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (58). The milk filters were put into a volume of each enrichment medium in a ratio of 1:10. Two main modifications were made to the U.S. Food and Drug Administration's procedures: (i) for the isolation of *Campylobacter* spp., Bolton broth was incubated at both 42 and 37°C ; (ii) Aloa agar was used to replace Palcam agar as the isolation medium for *L. monocytogenes*. Oxoid media were used with two exceptions: Aloa Agar (Biolife) and RF *E. coli* O157:H7 agar (RF Laboratories).

Detection of pathogens by PCR was performed starting from each enriched broth prepared according to the modified *Bacteriological Analytical Manual* procedures; 1.5 ml of each enriched broth was centrifuged for 15 min at $400 \times g$, the supernatant was discarded, and the DNA was extracted according to the "GenElute™ Bacterial Genomic DNA Mini Kit (SIGMA) directions for gram-negative or gram-positive bacteria. Specific PCR protocols were used for each of the pathogens considered: for thermotolerant *Campylobacter*, the DNA preparations were

analyzed for the presence or absence of *Campylobacter coli* and *C. jejuni* via qualitative booster-PCR as described by Inglis and Kalischuk (31). For detection of *Salmonella* spp., *L. monocytogenes*, and VTEC a real-time PCR was carried out using the Real Time PCR AB7300 System according to the manufacturer's instructions on the Taqman Universal PCR Master Mix kit (Applied Biosystems). For *Salmonella* spp. and *L. monocytogenes*, probes and primers designed by Applied Biosystems were used (TaqMan *Salmonella enterica* detection kit and TaqMan *Listeria monocytogenes* detection kit). For VTEC, virulence genes (*vtx*₁ and *vtx*₂) were detected with probes and primers described by Perelle et al. (49), while the virulence gene *eae* was detected with primers described by Nielsen and Andersen (47). Lastly, in samples positive for *eae* gene and *vtx*₁ and/or *vtx*₂ genes, a serotype identification (O26, O103, O111, O145, and O157) was performed with primers described by Perelle et al. (49).

Isolates were identified as follows: colonies presumptive for *Campylobacter* spp. were subcultured to obtain pure cultures, subjected to DNA extraction using the Nucleo Spin Tissue kit (Macherey Nagel), and submitted to a multiplex PCR protocol as described by Denis et al. (17). From samples positive for *Campylobacter* genus but negative for *C. jejuni* and *C. coli*, the 16S rDNA partial sequence using universal primers 1492-r, p27-f (10) was amplified; the amplicons were sent to a sequencing service (PRIMM, Milan, Italy) and sequenced by the ABI3730 DNA analyzer. The sequences obtained were analyzed by Vector NTI Advance 10.0 software (Invitrogen) and compared with the sequences present in the data bank by BLAST. The serotype and the presence of *vtx*₁ and *vtx*₂ genes and the *eae* gene in isolates with cultural characteristics ascribable to *E. coli* O157:H7 were confirmed by PCR as described above.

Data analysis. Data analysis was carried out as a two-step process: independent variables were subjected to univariable analysis (linear regression) with the following dependent variables: (i) positive-filter probability for every pathogen considered in the study (fraction of filters positive for each pathogen, of 14 filters examined) and (ii) positive-filter probability for any of the pathogens considered (fraction of filters detected positive for at least one pathogen of the 4 filters examined). Variables with *P* values of <0.10 in the univariable model were further considered for subsequent multivariable analysis using the backward stepwise method. A pair of independent variables were tested to detect colinearity, and interactions were tested if two or more factors were significant. Risk factors obtaining *P* values of <0.1 in the multivariate analysis were kept in the final model. All analyses were carried out with Intercooled STATA 7.0.

RESULTS

The 27 participating farms had an average of 60 milking cows (from a minimum of 9 to a maximum of 216 milking cows) and sold about 3,500 liters of raw milk per day in 60 vending machines. A total of 378 in-line milk filters were collected and examined for pathogen detection: 58 milk filters (15.3%) and 12 farms (44.4%) were positive for at least one pathogen. VTEC were detected in 32 (8.4%) samples from eight (29.6%) farms; *Campylobacter* spp. were detected in 24 (6.4%) samples from six (22.2%) farms; *L. monocytogenes* was detected in 2 (0.5%) samples from one farm; and *Salmonella* spp. were not detected in any farm. The results of culture and PCR examinations are summarized in Table 1. All 18 thermotolerant *Campylobacter* organisms

TABLE 1. Results of culture and PCR assays for farms positive for at least one pathogen^a

Positive farm	<i>L. monocytogenes</i>			<i>Campylobacter</i> spp.			VTEC			
	Total	PCR	BAM	Total	PCR ^b	BAM	Total	PCR	BAM	Serotype(s) detected by PCR (no.)
A	ND	ND	ND	2	2	ND	4	4	2	O157 (4)
B	ND	ND	ND	ND	ND	ND	2	2	2	O157 (2)
C	ND	ND	ND	ND	ND	ND	6	6	0	O103 (2); undetermined (4)
D	ND	ND	ND	ND	ND	ND	2	2	0	O157 (2)
E	ND	ND	ND	ND	ND	ND	8	8	0	O103 (2); undetermined (6)
F	ND	ND	ND	ND	ND	ND	2	2	0	O103 (2)
G	ND	ND	ND	6	4	4 ^c	ND	ND	ND	
H	2	2	0	ND	ND	ND	2	2	0	O157 (2)
I	ND	ND	ND	6	4	2 ^d	ND	ND	ND	
L	ND	ND	ND	2	ND	2 ^e	ND	ND	ND	
M	ND	ND	ND	2	ND	2 ^f	ND	ND	ND	
N	ND	ND	ND	8	8	4 ^g	6	6	0	O103 (2); O145 (4)
Total	2	2	0	24	18	14	32	32	4	

^a Values represent numbers of samples positive for indicated organisms. All VTEC isolated were O157:H7. ND, not detected; BAM, assay by culture.

^b All thermotolerant *Campylobacter*s detected by PCR were *C. jejuni*.

^c Two samples were positive for *C. jejuni*, and two were positive for *C. hyointestinalis*.

^d Two samples were positive for *C. jejuni*.

^e Two samples were positive for *C. sputorum*.

^f Two samples were positive for *C. hyointestinalis*.

^g Four samples were positive for *C. jejuni*.

detected by PCR were *C. jejuni*; of 14 samples in which *Campylobacter* spp. were detected by culture, *C. jejuni* was identified in 8 samples while in the other 6 samples four *Campylobacter hyointestinalis* isolates and two *Campylobacter sputorum* isolates were identified. Samples positive for *C. jejuni* by culture examination were confirmed by PCR detection. All *E. coli* O157:H7 isolates ($n = 4$) by the culture method contained the virulence genes investigated (*eae*, *vtx*₁, and *vtx*₂ genes). Samples positive by culture examination were confirmed by PCR detection. Only the O157 serotype was detected by PCR in four farms (farms A, B, D, and H); only the O103 serotype was detected in one farm (farm F); O103 and O145 serotypes were detected once (farm N); and in the other positive farms, the O103 serotype and virulence factors not associated with any of the serotypes tested were detected twice (farms C and E) (Table 1). For VTEC, serotypes and virulence factors of isolates and positive samples detected by PCR are listed in Table 2.

Only data for VTEC and *Campylobacter* spp. were considered in the risk analysis; *Salmonella* spp. and *L.*

monocytogenes were omitted because of their absence or low prevalence. Table 3 summarizes the R^2 and P values of the univariate and multivariate regression analysis between positive filter probability and the different risk factors investigated. The general herd status of cleanliness (bedding cleanliness, teat cleanliness, and water trough and feed trough cleanliness) and the operations connected with milking hygiene (milking system maintenance and hygiene, fore-stripping, water hardness evaluation, and tank cleanliness) were factors associated with positive herds for *Campylobacter* spp. or VTEC or both; in addition the presence of pests and the manure composting time were associated with herds positive for *Campylobacter* spp. and VTEC, respectively.

DISCUSSION

This is the first study to investigate foodborne pathogens in in-line milk filters of dairy farms authorized to produce and sell raw cow's milk in Italy. Milk filter screening was confirmed to be a sensitive method to investigate several foodborne pathogens in dairy herds, and PCR proved a rapid and sensitive method to assess the microbiological status of the herd for target pathogens. In addition to confirming the results of isolation, PCR disclosed pathogens not detected by the culture method: 6 further samples were positive for O157, VTEC were detected in another 12 samples (8 samples positive for O103 and 4 samples positive for O145), 10 further samples were positive for *C. jejuni*, and 2 samples were positive for *L. monocytogenes*. In this regard the ability of PCR for detecting even nonviable target bacteria must be taken into account. A further advantage of PCR compared with the culture method was the detection of several non-O157 VTEC serotypes that were often not isolated because it was

TABLE 2. VTEC serotypes and virulence factors detected in positive samples^a

Serogroup	<i>vtx</i> ₁ gene	<i>vtx</i> ₂ gene	<i>eae</i> gene	No. of positive samples
O103	+	+	+	6
	—	+	+	2
O145	—	+	+	4
O157	+	+	+	10
Undefined	+	+	+	2
	—	+	+	8

^a +, gene detected; —, gene not detected.

TABLE 3. Univariate and multivariate regression analysis between positive filter probability and the different risk factors investigated^a

	Risk factor	R ²	P value univariate	P value multivariate
<i>Campylobacter</i> spp. ^b	Presence of pests ^c	0.18	<0.05	<0.01
	Milking system maintenance	0.13	<0.1	<0.05
VTEC ^d	Milking system hygiene	0.11	<0.1	
	Bedding cleanliness	0.31	<0.01	<0.05
	Water trough cleanliness	0.29	<0.01	
	Feed trough cleanliness	0.29	<0.01	<0.1
	Manure composting time	0.16	<0.05	
	Water hardness evaluation	0.14	<0.1	
	Forestripping	0.21	<0.05	<0.1
	Teat cleanliness	0.12	<0.1	
	Bedding cleanliness	0.27	<0.01	<0.05
	Water trough cleanliness	0.27	<0.01	
<i>Campylobacter</i> spp. and VTEC ^e	Feed trough cleanliness	0.18	<0.05	
	Water hardness evaluation	0.19	<0.05	
	Forestripping	0.23	<0.05	<0.01
	Tank cleanliness	0.16	<0.05	<0.05

^a Linear regression and multivariate analysis (backward stepwise method, $P < 0.1$) between probabilities of filters positive for *Campylobacter* spp. (number of positive samples of 14 examined), VTEC (number of positive samples of 14 examined), and *Campylobacter* spp. and/or VTEC (number of positive samples of 28 examined). No colinearity and significant interaction between these risk factors were detected.

^b ($R^2 = 0.38$): $Y = -0.092 - 0.15 \times \text{pests} + 0.128 \times \text{milking system maintenance}$.

^c Mainly birds and rodents.

^d ($R^2 = 0.52$): $Y = -0.041 + 0.127 \times \text{bedding cleanliness} + 0.104 \times \text{forestripping} + 0.108 \times \text{feed trough cleanliness}$.

^e ($R^2 = 0.52$): $Y = -0.03 + 0.084 \times \text{bedding cleanliness} + 0.077 \times \text{tank cleanliness} + 0.109 \times \text{forestripping}$.

very difficult to differentiate and determine the individual serotypes with available selective-differential media. On the other hand, culture examination isolated six other thermotolerant *Campylobacter* isolates referring to two species, *C. hyointestinalis* and *C. sputorum* bv. *sputorum*.

The results of this study show that VTEC and thermotolerant *Campylobacter* contamination is a major public health concern of dairy farms authorized to produce raw milk in the province considered. Not surprisingly, the reported outbreaks associated with raw milk consumption in the same province were solely due to *C. jejuni* and *E. coli* O157:H7 (1, 2, 57).

Although thermophilic campylobacters (*C. jejuni* and *C. coli*) are the most common cause of bacterial gastroenteritis in humans and there is growing evidence that raw milk consumption might also be significant for human infection (45, 46, 56), only one study has investigated *C. jejuni* in milk filters (39). Our study found a lower prevalence than that reported by Leone et al. (39), but *C. jejuni* was confirmed as the most prevalent thermotolerant *Campylobacter* species in the dairy farms considered. The presence of *C. hyointestinalis* and *C. sputorum* bv. *sputorum* in our study is in line with their wide distribution in livestock reported in northern Spain (48). *Campylobacter* species other than *C. jejuni* and *C. coli* have been isolated with increasing frequency from a variety of human infections, but until their pathogenic potential is clarified, our results provide valuable epidemiological data and emphasize the importance of these emerging gastrointestinal pathogens.

The prevalence of VTEC contamination of milk filters observed in this study does not differ from literature data

(13, 42, 44, 60), and the circulation of *E. coli* O157 in Italian farms was confirmed by other studies (6, 7, 14, 15). Our data indicate that non-O157 VTEC strains are more representative in dairy cattle than *E. coli* O157:H7 (4, 26–28). In addition, there was a total absence of *E. coli* O26, a strain considered among the most important both in the United States (40) and in continental Europe (12). We also found *E. coli* O103, O111, and O145, whereas these serogroups were not reported in a previous Italian study (6). In conclusion, the abundance of non-O157 VTEC and the presence in 10 samples of VTEC virulence factors not ascribable to any of the tested serotypes (O26, O103, O111, O145, and O157) emphasize that testing only for *E. coli* O157:H7 conceals a significant public health concern and results in an inadequate approach to consumer safety for raw milk consumption.

The reported prevalence of *L. monocytogenes* and *Salmonella* spp. in milk filters varies widely, ranging from 1.2 to 67% for *L. monocytogenes* (24, 36, 60) and from 1.5 to 66% for *Salmonella* spp. (22, 24, 52, 60, 61, 66). The very low prevalence of *L. monocytogenes* observed to our knowledge could be related to the fact that the presence of *L. monocytogenes* in raw milk was associated with the poor quality of silage and poor milking practices (25, 30, 53). In our study, the animal feed was based mainly on hay in contrast to other places in Italy where the main forage is corn silage (i.e., only 8 farms of 27 used silage). In addition, to obtain the authorization to produce and sell raw milk, these dairy farms must implement higher standards of good dairy farming practices than other dairy farms.

Several studies have attempted to identify management practices and herd characteristics associated with shedding

of *Salmonella* spp. in cattle. However, the results have been inconsistent with the exception of herd size (5, 22, 29, 52, 59, 65, 67). The fact that the farms considered in the study had an average of 60 lactating cows (a minimum of 9 and a maximum of 216) may play a role in the low prevalence detected.

Several factors appeared to be associated with the probability of detecting foodborne pathogens in milk filters of dairy farms; despite our relatively small sample size, we consider our combined model to be correct on the basis of R^2 and Y value and associated with well-established scientific knowledge.

The general status of herd cleanliness in our study, meaning cleanliness of bedding and feed and water troughs, proved to be one of the most important factors in reducing VTEC contamination of dairy farms and consequently raw milk. Wet and dirty bedding and dirty and encrusted water troughs can be reservoirs for VTEC because they create an optimal environment for their growth and survival (21, 37, 50, 54, 64). Daily routine interventions such as a frequent cleaning of water troughs, regular cleaning of feed bunks, and the assessment of dryness of bedding offer significant potential to decrease VTEC contamination (28, 37) and might also reduce the risk for other pathogens (19). Manure and its handling play a critical role in VTEC prevalence and in the safety of food from dairy cattle (23, 28). In our study, farms that composted the manure less than 6 months were more likely to have at least one pathogen-positive milk filter. Forestripping was significantly inversely correlated with the probability of detecting VTEC in milk filters. This practice ensures that abnormal milk is diverted from the human food chain and should be a standard food safety practice on all farms (51, 62, 68). The nonsystematic application of premilking forestripping reflects a lack of farmers' education on good milking practices.

Very little is known about the dynamics and transmission of *Campylobacter* among cattle. A recent study (18) observed a surprising increase in the risk of *Campylobacter* related to dairy cows, suggesting that the risk was associated with management practices rather than the cows' age or potential immune status. In our study the detection of *Campylobacter* spp. in milk filters was associated with the presence of pests (mainly birds) on the farm and with poor hygiene and maintenance of milking systems. *Campylobacter* spp. are common in the environment (34, 35, 43), and their wide distribution among wild birds is well known (8, 9, 63). Inadequate hygiene and maintenance of milking systems are linked to the ability of some *Campylobacter* strains to survive in extreme conditions by forming a biofilm on stainless steel (11, 55). All these aspects may play a critical role in the presence of *Campylobacter* spp. in the dairy milking environment.

The lack of association between the burden of VTEC and *Campylobacter* may be due to the different survival and infection mechanisms of *Campylobacter* from *Enterobacteriaceae* such as *E. coli* (43).

The analysis of risk factors linked to the presence of *Campylobacter* spp. and/or VTEC confirmed the association with poor general cleanliness on the farm (bedding and water

and feed troughs) and lack of forestripping. However, the probability of detecting *Campylobacter* spp. or VTEC was higher on farms in which water hardness had not been evaluated and on farms with inadequate milk tank cleanliness. Water is the most important component in cleaning and sanitizing solutions, and its properties can affect the cleaning process and milk quality (20). Hard water can reduce the effectiveness of cleaning chemicals and may lead to the formation of films or deposits in the milking system (16). The importance of cleaning and sanitizing the milking and storage equipment is clear, and the association with inadequate cleaning conditions of milk tanks highlights the importance of farmers' education.

A positive milk filter is a clear index of the presence of the pathogen in the herd and the failure of preharvest practices and milking hygiene, and it has to sound as an alarm bell for a potential and probable milk contamination. Microbiological testing of milk or milk filters cannot guarantee that raw milk is safe for consumption; nevertheless, in order to reduce the risk for consumer, the evaluation of microbiological herd contamination status by PCR analysis of in-line milk filters can be useful to define a risk-based planning for official controls and the implementation of on-farm management practices, and it should be considered a requirement for the authorization to produce and sell raw milk.

Official analytical control is currently based on testing milk. Testing milk filters is a more sensitive indicator of the bacteria entering the bulk tank than sampling milk alone (60). The addition of regular milk filter sampling to official testing of raw milk would be a more sensitive method to verify the application of good farming and milking practices.

The identification of risk factors associated with the probability of detection of foodborne pathogens in milk filters and the following adoption and/or implementation of specific control actions are critically needed to support a low-risk production of raw milk: farm-specific interventions need to be implemented at the farm level and incorporated into specific farmer's daily practices in order to reduce the contamination of raw milk and to minimize the risks for raw milk consumers.

REFERENCES

1. Amato, S., M. Maragno, P. Mosele, M. Sforzi, R. Mioni, L. Barco, M. C. Dalla Pozza, K. Antonello, and A. Ricci. 2007. An outbreak of *Campylobacter jejuni* linked to the consumption of raw milk in Italy. *Zoonoses Public Health* 54(Suppl. 1):23.
2. Arrigoni, N., G. Scavia, and M. Tamba. 2009. Latte crudo: esperienze e problematiche igienico-sanitarie in Regione Emilia-Romagna. *Large Anim. Rev.* 15:215–219.
3. AusVet Animal Health Services. EpiTools epidemiological calculators. Available at: <http://epitools.ausvet.com.au/content.php?page=home>. Accessed 12 November 2009.
4. Bettelheim, K. A. 2001. Development of a rapid method for the detection of verocytotoxin-producing *Escherichia coli* (VTEC). *Lett. Appl. Microbiol.* 33:31–35.
5. Blau, D. M., B. J. McCluskey, S. R. Ladely, D. A. Dargatz, P. J. Fedorka-Cray, K. E. Ferris, and M. L. Headrick. 2005. *Salmonella* in dairy operations in the United States: prevalence and antimicrobial drug susceptibility. *J. Food Prot.* 68:696–702.
6. Bonardi, S., E. Foni, C. Chiapponi, A. Salsi, and F. Brindani. 2007. Detection of verocytotoxin-producing *Escherichia coli* serogroups

- O157 and O26 in the cecal content and lymphatic tissue of cattle at slaughter in Italy. *J. Food Prot.* 70:1493–1497.
7. Bonardi, S., E. Maggi, G. Pizzin, S. Morabito, and A. Caprioli. 2001. Faecal carriage of *Escherichia coli* O157 and carcass contamination in cattle at slaughter in northern Italy. *Int. J. Food Microbiol.* 66:47–53.
 8. Broman, T., H. Palmgren, S. Bergström, M. Sellin, J. Waldenström, M. L. Danielsson-Tham, and B. Olsen. 2002. *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): prevalence, genotypes, and influence on *C. jejuni* epidemiology. *J. Clin. Microbiol.* 40:4594–4602.
 9. Broman, T., J. Waldenström, D. Dahlgren, I. Carlsson, I. Eliasson, and B. Olsen. 2004. Diversities and similarities in PFGE profiles of *Campylobacter jejuni* isolated from migrating birds and humans. *J. Appl. Microbiol.* 96:834–843.
 10. Brosius, J., J. L. Palmer, H. P. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16s ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 75:4801–4805.
 11. Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. M. McGiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and-rRNA staining. *Appl. Environ. Microbiol.* 64:733–741.
 12. Caprioli, A., and A. E. Tozzi. 1998. Epidemiology of Shiga toxin-producing *Escherichia coli* infections in continental Europe, p. 38–48. In J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157: H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, DC.
 13. Cizek, A., M. Dolejská, R. Novotná, D. Haas, and M. Vyskocil. 2008. Survey of Shiga toxigenic *Escherichia coli* O157 and drug-resistant coliform bacteria from in-line milk filters on dairy farms in the Czech Republic. *J. Appl. Microbiol.* 104:852–860.
 14. Conedera, G., P. A. Chapman, S. Marangon, E. Tisato, P. Dalvit, and A. Zuin. 2001. A field survey of *Escherichia coli* O157 ecology on a cattle farm in Italy. *Int. J. Food Microbiol.* 66:85.
 15. Conedera, G., P. A. Chapman, S. Marangon, A. Zuin, and A. Caprioli. 1997. Atypical strains of verocytotoxin-producing *Escherichia coli* O157 in beef cattle at slaughter in Veneto Region, Italy. *J. Vet. Med. B* 44:301–306.
 16. Cords, B. R., G. R. Dychdala, and F. L. Richter. 2001. Cleaning and sanitizing in milk production and processing, p. 547–585. In J. Steele and E. Marth (ed.), *Applied dairy microbiology*. Marcel Dekker Inc., New York.
 17. Denis, M., C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat, and P. Colin. 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *Campylobacter coli*. *Lett. Appl. Microbiol.* 29:406–410.
 18. Ellis-Iversen, J., G. C. Pritchard, M. Wooldridge, and M. Nielsen. 2009. Risk factors for *Campylobacter jejuni* and *Campylobacter coli* in young cattle on English and Welsh farms. *Prev. Vet. Med.* 88:42–48.
 19. Ellis-Iversen, J., R. P. Smith, L. C. Snow, E. Watson, M. F. Millar, G. C. Pritchard, A. R. Sayers, A. J. Cook, S. J. Evans, and G. A. Paiba. 2007. Identification of management risk factors for VTEC O157 in young-stock in England and Wales. *Prev. Vet. Med.* 82:29–41.
 20. Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, and B. M. Jayarao. 2009. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1. Overall risk factors. *J. Dairy Sci.* 92:2634–2643.
 21. Faith, N. G., J. A. Shere, R. Brosch, K. W. Arnold, S. E. Ansay, M. S. Lee, J. B. Luchansky, and C. W. Kaspar. 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Appl. Environ. Microbiol.* 62:1519–1525.
 22. Fossler, C. P., S. J. Wells, J. B. Kaneene, P. L. Ruegg, L. D. Warnick, L. E. Eberly, S. M. Godden, L. W. Halbert, A. M. Campbell, C. A. Bolin, and A. M. Geiger Zwald. 2005. Cattle and environmental sample-level factors associated with the presence of *Salmonella* in a multi-state study of conventional and organic dairy farms. *Prev. Vet. Med.* 67:39–53.
 23. Hancock, D. D., T. E. Besser, and D. H. Rice. 1998. Ecology of *Escherichia coli* O157:H7 in cattle and impact of management practices, p. 85–91. In J. B. Kaper and A. B. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, DC.
 24. Hassan, L., H. O. Mohammed, and P. L. McDonough. 2000. A cross-sectional study on the prevalence of *Listeria monocytogenes* and *Salmonella* in New York dairy herds. *J. Dairy Sci.* 84:292–298.
 25. Hassan, L., H. O. Mohammed, and P. L. McDonough. 2001. Farm-management and milking practices associated with the presence of *Listeria monocytogenes* in New York state dairy herds. *Prev. Vet. Med.* 51:63–73.
 26. Hussein, H. S. 2007. Ongoing research and outbreak efforts targeted at non-O157 STEC. Presented at the Public Health Significance of Non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC), Public Meeting, Arlington, VA, 17 October 2007.
 27. Hussein, H. S. 2007. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. *J. Anim. Sci.* 85:E63–E72.
 28. Hussein, H. S., and T. Sakuma. 2005. Shiga toxin-producing *Escherichia coli*: pre- and postharvest control measures to ensure safety of dairy cattle products. *J. Food Prot.* 68:199–207.
 29. Huston, C. L., T. E. Wittum, B. C. Love, and J. E. Keen. 2002. Prevalence of fecal shedding of *Salmonella* spp. in dairy herds. *J. Am. Vet. Assoc.* 220:645–649.
 30. Hsu, J. R., J. T. Seppanen, S. K. Sivela, and A. L. Rauramaa. 1990. Contamination of raw milk by *Listeria monocytogenes* on dairy farms. *J. Vet. Med. B* 37:286–275.
 31. Inglis, G. D., and L. D. Kalischuk. 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. *Appl. Environ. Microbiol.* 69:3435–3447.
 32. Jayarao, B. M., S. C. Donaldson, B. A. Straley, A. A. Sawant, N. V. Hegde, and J. L. Brown. 2006. A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *J. Dairy Sci.* 89:2451–2458.
 33. Jayarao, B. M., and D. R. Henning. 2001. Prevalence of foodborne pathogens in bulk tank milk. *J. Dairy Sci.* 84:2157–2162.
 34. Jones, K. 2001. *Campylobacter* in water, sewage and the environment. *J. Appl. Microbiol.* 90:S68–S79.
 35. Kemp, R., A. J. Leatherbarrow, N. J. Williams, C. A. Hart, H. E. Clough, J. Turner, E. J. Wright, and N. P. French. 2005. Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. *Appl. Environ. Microbiol.* 71:1876–1882.
 36. Latorre, A. A., J. A. S. Van Kessel, J. S. Karns, M. J. Zurakowski, A. K. Pradhan, R. N. Zadoks, K. J. Boor, and Y. H. Schukken. 2009. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. *Appl. Environ. Microbiol.* 75:1315–1323.
 37. LeJeune, J., T. E. Besser, and D. D. Hancock. 2001. Cattle water troughs as reservoirs of *Escherichia coli* O157. *Appl. Environ. Microbiol.* 67:3053–3057.
 38. LeJeune, J. T., and P. J. Rajala-Schultz. 2009. Unpasteurized milk: a continued public health threat. *Clin. Infect. Dis.* 48:93–100.
 39. Leone, P., P. Cremonesi, and A. Stella. 2010. Molecular-based identification of pathogens in raw milk and milk filter residuals. *Renc. Rech. Ruminants* 17:108.
 40. Mathusa, E. C., Y. Chen, E. Enache, and L. Hontz. 2010. Non-O157 Shiga toxin-producing *Escherichia coli* in foods. *J. Food Prot.* 73:1721–1736.
 41. Murinda, S. E., L. T. Nguyen, S. J. Ivey, B. E. Gillespie, R. A. Almeida, F. A. Draughon, and S. P. Oliver. 2002. Prevalence and molecular characterization of *Escherichia coli* O157:H7 in bulk tank milk and faecal samples from cull cows: a 12-month survey of dairy farms in East Tennessee. *J. Food Prot.* 65:752–759.
 42. Murphy, B. P., M. Murphy, J. F. Buckley, D. Gilroy, M. T. Rowe, D. McCleery, and S. Fanning. 2005. In-line milk filter analysis: *Escherichia coli* O157 surveillance of milk production holdings. *Int. J. Hyg. Environ. Health* 208:407–413.
 43. Murphy, C., C. Carroll, and K. N. Jordan. 2006. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100:623–632.

44. Murphy, M., J. F. Buckley, P. Whyte, W. O'Mahony, W. Anderson, P. G. Wall, and S. Fanning. 2007. Surveillance of dairy production holdings supplying raw milk to the farmhouse cheese sector for *Escherichia coli* O157, O26 and O11. *Zoonoses Public Health* 54: 358–365.
45. Nachamkin, I. 2007. *Campylobacter jejuni*, p. 237–248. In M. P. Doyle and L. R. Beuchat (ed.), *Food microbiology: fundamentals and frontiers*, 3rd ed. ASM Press, Washington DC.
46. Neimann, J., J. Engberg, K. Molbak, and H. C. Wegener. 2003. A case-control study of risk factors for sporadic campylobacter infections in Denmark. *Epidemiol. Infect.* 130:353–366.
47. Nielsen, E., and M. Andersen. 2003. Detection and characterization of verocytotoxin producing *Escherichia coli* by automated 5' nuclease PCR assay. *J. Clin. Microbiol.* 41:2884–2893.
48. Oporto, B., and A. Hurtado. 2011. Emerging thermotolerant *Campylobacter* species in healthy ruminants and swine. *Foodborne Pathog. Dis.* 8:807–813.
49. Perelle, S., F. Dilasser, J. Grout, and P. Fach. 2004. Detection by 5'-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. *Mol. Cell. Probes* 18:185–192.
50. Rice, E. W., and C. H. Johnson. 2000. Short communication: survival of *Escherichia coli* O157:H7 in dairy cattle drinking water. *J. Dairy Sci.* 83:2021–2023.
51. Ruegg, P. L. 2003. Practical food safety interventions for dairy production. *J. Dairy Sci.* 86(Suppl.):E1–E9.
52. Ruzante, J. M., J. E. Lombard, B. Wagner, C. P. Fossler, J. S. Kams, J. A. S. Van Kessel, and I. A. Gardner. 2010. Factors associated with *Salmonella* presence in environmental samples and bulk tank milk from US dairies. *Zoonoses Public Health* 57:e217–e225.
53. Sanaa, M., B. Poutrel, J. L. Menard, and F. Serieys. 1993. Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms. *J. Dairy Sci.* 76:2891–2898.
54. Shere, J. A., K. J. Bartlett, and C. W. Kaspar. 1998. Longitudinal study of *Escherichia coli* O158:H7 dissemination on four dairy farms in Wisconsin. *Appl. Environ. Microbiol.* 64:1390–1399.
55. Somers, E. B., J. L. Schoeni, and A. C. Wong. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*. *Int. J. Food Microbiol.* 22:269–276.
56. Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous campylobacter infection: a Swedish case control study. *Epidemiol. Infect.* 125:269–275.
57. Tonucci, F. (Experimental Institute for Zooprophyllaxis in Umbria and March). 2011. Personal communication.
58. U.S. Food and Drug Administration. 1998. Microbiological methods: bacteriological analytical manual. Available at: <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm>. Accessed 21 November 2009.
59. Vaessen, M. A., J. Veling, K. Frankena, E. A. M. Graat, and T. Klunder. 1998. Risk factors for *Salmonella dublin* infection on dairy farms. *Vet. Q.* 20:97–99.
60. Van Kessel, J. A. S., J. S. Kams, J. E. Lombard, and C. A. Kopral. 2011. Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* virulence factors in bulk tank milk and in-line filters from U.S. dairies. *J. Food Prot.* 74:759–768.
61. Van Kessel, J. S., J. S. Kams, and D. R. Wolfgang. 2008. Environmental sampling to predict fecal prevalence of *Salmonella* in an intensively monitored dairy herd. *J. Dairy Sci.* 71:1967–1973.
62. Wagner, A. M., and P. L. Ruegg. 2002. The effect of manual forestripping on milking performance of Holstein dairy cows. *J. Dairy Sci.* 85:804–809.
63. Waldenström, J., T. Broman, I. Carlsson, D. Hasselquist, R. P. Achterberg, J. A. Wagenaar, and B. Olsen. 2002. Prevalence of *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. *Appl. Environ. Microbiol.* 68:5911–5917.
64. Ward, W. R., J. W. Hughes, W. B. Faull, P. J. Cripps, J. P. Sutherland, and J. E. Sutherst. 2002. Observational study of temperature, moisture, pH and bacteria in straw bedding, and fecal consistency, cleanliness and mastitis in cows in four dairy herds. *Vet. Rec.* 151:199–206.
65. Warnick, L. D., L. M. Crofton, K. D. Pelzer, and M. J. Hawkins. 2001. Risk factors for clinical salmonellosis in Virginia, USA, cattle herds. *Prev. Vet. Med.* 49:259–275.
66. Warnick, L. D., J. B. Kaneene, P. L. Ruegg, S. J. Wells, C. Fossler, L. Halbert, and A. Campbell. 2003. Evaluation of herd sampling for *Salmonella* isolation on midwest and northeast US dairy farms. *Prev. Vet. Med.* 3:195–206.
67. Wells, S. J., P. J. Fedorka-Cray, D. A. Dargatz, K. Ferris, and A. Green. 2001. Fecal shedding of *Salmonella* spp. by dairy cows on farm and at cull markets. *J. Food Prot.* 64:3–11.
68. Zucali, M., L. Bava, A. Tamburini, M. Brasca, L. Vanoni, and A. Sandrucci. 2011. Effects of season, milking routine and cow cleanliness on bacterial and somatic cell counts of bulk tank milk. *J. Dairy Res.* 78(4):436–441.