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Molecular Ecology of *Listeria monocytogenes*: Evidence for a Reservoir in Milking Equipment on a Dairy Farm▽

Alejandra A. Latorre,[1]* Jo Ann S. Van Kessel,[2] Jeffrey S. Karns,[2] Michael J. Zurakowski,[1] Abani K. Pradhan,[1] Ruth N. Zadoks,[1]† Kathryn J. Boor,[3] and Ynte H. Schukken[1]

Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York 14850[1]; Environmental Microbial Safety Laboratory, ANRI, USDA-ARS, Beltsville, Maryland 20705[2]; and Department of Food Science, Cornell University, Ithaca, New York 14853[3]

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A longitudinal study aimed to detect *Listeria monocytogenes* on a New York State dairy farm was conducted between February 2004 and July 2007. Fecal samples were collected every 6 months from all lactating cows. Approximately 20 environmental samples were obtained every 3 months. Bulk tank milk samples and in-line milk filter samples were obtained weekly. Samples from milking equipment and the milking parlor environment were obtained in May 2007. Fifty-one of 715 fecal samples (7.1%) and 22 of 303 environmental samples (7.3%) were positive for *L. monocytogenes*. A total of 73 of 108 in-line milk filter samples (67.6%) and 34 of 172 bulk tank milk samples (19.7%) were positive for *L. monocytogenes*. *Listeria monocytogenes* was isolated from 6 of 40 (15%) sampling sites in the milking parlor and milking equipment. In-line milk filter samples had a greater proportion of *L. monocytogenes* than did bulk tank milk samples ($P < 0.05$) and samples from other sources ($P < 0.05$). The proportion of *L. monocytogenes*-positive samples was greater among bulk tank milk samples than among fecal or environmental samples ($P < 0.05$). Analysis of 60 isolates by pulsed-field gel electrophoresis (PFGE) yielded 23 PFGE types after digestion with AscI and ApaI endonucleases. Three PFGE types of *L. monocytogenes* were repeatedly found in longitudinally collected samples from bulk tank milk and in-line milk filters.

Listeria monocytogenes can cause listeriosis in humans. This illness, despite being underreported, is an important public health concern in the United States (23) and worldwide. According to provisional incidence data provided by the Centers for Disease Control and Prevention (CDC), 762 cases of listeriosis were reported in the United States in 2007. In previous years (2003 to 2006), the number of reported annual listeriosis cases in the United States ranged between 696 and 896 cases per year (5).

Exposure to food-borne *L. monocytogenes* may cause fever, muscle aches, and gastroenteritis (30), but does not usually cause septicemic illness in healthy nonpregnant individuals (7, 30). Elderly and immunocompromised people, however, are susceptible to listeriosis (22, 10), and they may develop more-severe symptoms (10). Listeriosis in pregnant women may cause abortion (22, 30) or neonatal death (22).

Dairy products have been identified as the source of several human listeriosis outbreaks (4, 7, 10, 22). *Listeria* is ubiquitous on dairy farms (26), and it has been isolated from cows' feces, feed (3, 26), and milk (21, 35). In ruminants, *L. monocytogenes* infections may be asymptomatic or clinical. Clinical cases typically present with encephalitis and uterine infections, often resulting in abortion (26, 39). Both clinically infected and healthy animals have been reported to excrete *L. monocytogenes* in their feces (20), which could eventually cause contamination of the bulk tank milk or milk-processing premises (39).

On-farm epidemiologic research provides science-based information to improve farming and management practices. The Regional Dairy Quality Management Alliance (RDQMA) launched a combined United States Department of Agriculture (USDA)-RDQMA pilot project in January 2004 to scientifically validate intervention strategies in support of recommended best management practices among northeast dairy farms. The primary goal of the project was to track dynamics of infectious microorganisms on well-characterized dairy farms. Target species included *Salmonella* spp. (6, 36, 37), *Mycobacterium avium* subsp. *paratuberculosis* (13, 24), and *L. monocytogenes*.

The objectives of this study were to describe the presence of *L. monocytogenes* on a dairy farm over time and to perform molecular subtyping by pulsed-field gel electrophoresis (PFGE) on *L. monocytogenes* isolates obtained from bulk tank milk, milk filters, milking equipment, feces, and the environmental samples to identify diversity among *L. monocytogenes* strains, persistence, and potential sources of bulk tank milk contamination.

MATERIALS AND METHODS

Farm description. This study was conducted on a New York State dairy farm between February 2004 and July 2007. This dairy farm is considered typical among the better-managed New York State dairy farms in terms of size, management, and milk production. The selection criteria for inclusion of the study farm were the size of the herd, participation in the New York State Cattle Health Assurance Program (NYSCHAP) (http://nyschap.vet.cornell.edu/), and Dairy Herd Improvement Association (DHIA) (http://www.dhia.org/) membership.

* Corresponding author. Mailing address: Cornell University—Quality Milk Production Services, 22 Thornwood Drive, Ithaca, NY 14850. Phone: (607) 255-8202. Fax: (607) 257-8485. E-mail: aal39@cornell.edu.

† Present address: Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian EH25 9RG, Scotland, United Kingdom.

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The availability of a record system, proper identification of the animals, and willingness of the producer to participate were also taken into consideration (28).

The study farm had an average of 330 milking cows. Of these, 300 were housed in a free-stall barn, and approximately 30 were housed in a tie-stall facility. The average milk production was 12,700 kg/cow/year. The herd had monthly veterinary check-ups, annual NYSCHAP evaluation, and monthly DHIA milk testing. No listeriosis cases in the cows have been reported. The milk (approximately 9,071 kg per day) was transported every 48 h to the milk processing plant, where it was pasteurized before distribution.

Sampling. Environmental and fecal samples were collected between February 2004 and April 2007 for detection of pathogens, including *L. monocytogenes*. Fecal samples were collected every 6 months from all lactating cows. Fecal samples were obtained directly from the rectum of each cow, using a separate clean plastic sleeve for each sample. The plastic sleeves were inverted, and the content was aseptically transferred into sterile plastic vials (conical 50-ml propylene screw top; VWR International, Inc., West Chester, PA).

In addition, approximately 20 environmental samples were obtained every 3 months. These environmental samples included feed as presented to the animals, source water used to fill water troughs, and drinking water from water troughs in the pens housing the lactating cows, dry cows, and heifers. Samples were collected from the same locations on each of the samplings. For this purpose, a detailed sampling map was constructed at the initial visit and used at subsequent samplings. Manure composite samples from the walkways, calf area, dry cow pen, precalving pen, and sick cow pen were also collected at all samplings. Samples from specific potential "hot spots" for *Listeria*, such as bedding or, when present, standing water, birds, bird droppings, feral-animal feces, and insects, were also obtained.

Liquid samples were collected into sterile 500-ml bottles. Feed material samples and other solid samples were placed in sterile Whirl-Pak bags (NASCO, Ft. Atkinson, WI). Manure composite samples were obtained from different areas in each pen, using a clean plastic sleeve. The plastic-sleeve content was homogenized, and an aliquot was aseptically transferred into 50-ml plastic vials. Bird dropping samples were obtained by scraping stall dividers and were collected in 50-ml sample vials. Flies were caught in calf hutches, in the lactating cows' pen, and in an outdoor site at least 30 m from the animal-holding facilities. For this purpose, QuikStrike (Wellmark International, Schaumburg, IL) fly abatement strips (for house flies) and previously sterilized sweep nets (for stable flies) were used.

In-line milk filter samples were obtained in October 2004 (two samples) and January 2005 (one sample). Starting from April 2005, in-line milk filter samples were obtained on a weekly basis, until July 2007. Milk filter samples were aseptically transferred into a clean sealable plastic bag for transport. Bulk tank milk samples (100 ml) were aseptically collected on a weekly basis from February 2004 to July 2007.

An additional sampling was carried out in May 2007 to assess the presence of *L. monocytogenes* in the milking machine and milking parlor environment. Forty sampling sites were selected based on areas prone to *Listeria* contamination or on a particular interest to assess the presence of this pathogen in a given sampling site. Sampling sites from the milking equipment included teat cup liners, milk meters, milk pipelines, elbow fittings, and the milk tank outlet. Milk pump surfaces, the motor, and the floor in the storage area for miscellaneous supplies were also sampled. Floors and floor drains, areas previously described as sources of *L. monocytogenes* in food plant environments (34), were also included. Samples were collected using a Bacti-Sponge kit (Hardy Diagnostics, Santa Maria, CA) moistened with 10 ml of neutralizing buffer (Difco; BD Diagnostics, Sparks, MD) (19, 33). For the milking equipment, a sponge was used to wipe the inner surface of the selected site. Sterile cotton swabs were used to sample the milk tank outlet and every other milk meter after the routine washing cycle was complete. Samples from drains were aseptically obtained by rubbing the sponge on the exposed surface and inner portions within reach. For surface sampling, individual sponges were used to wipe an area of approximately 0.6 by 0.6 m (33) on floors and pumps. Sponges were placed in the sterile bags containing neutralizing buffer, and cotton swabs were placed in sterile tubes containing 3 ml of neutralizing buffer.

All samples were packed in coolers with ice packs and transported overnight to the USDA-Beltsville Agricultural Research Center for *L. monocytogenes* detection.

Bacterial analysis. Approximately 25 g of feces or other sampling material, such as composite samples or bedding, was weighed into a filtered stomacher bag (GSI Creos Corporation, Japan), diluted with 50 g of 1% buffered peptone water (BD Diagnostics, Sparks, MD), and pummeled in an automatic bag mixer (Bag-Mixer Interscience Laboratories, Inc., Weymouth, MA) for 2 min. For enrichment of *Listeria* spp., 5 ml of filtrate was added to 5 ml of double-strength modified *Listeria* enrichment broth (MLEB; BD Diagnostics, Sparks, MD) to yield 1× MLEB. For feed samples, larger aliquots (40 to 60 g) were used, and when the samples were low in moisture content, larger volumes of buffered peptone water were used for extraction. For samplings performed between February 2004 and October 2005, every fifth fecal sample was tested for the presence of *L. monocytogenes*. All fecal samples collected in May 2006 and every third fecal sample in April 2007 were tested for the presence of *L. monocytogenes*.

Milk (250 μl) was plated in triplicate directly onto modified Oxford medium (MOX) agar (Difco Laboratories, Detroit, MI) as described by Van Kessel et al. (35). For specific enrichment of *Listeria* spp., 5 ml of milk was added to 5 ml of double-strength MLEB.

In-line milk filters were cut into small (30 to 50 cm^2) pieces and placed in a filtered stomacher bag, diluted (2 to 1 [wt/wt]) with 1% buffered peptone water, and pummeled in an automatic bag mixer for 2 min. The bag was removed from the mixer, filter pieces were repositioned to the bottom of the bag, and the bag was repummeled for two additional minutes. For enrichment of *Listeria* spp., 5 ml of filtrate was added to 5 ml of double-strength MLEB. The extract from the milk filters (250 μl) was also plated directly onto MOX plates.

Water (250 μl) was plated in triplicate directly onto MOX agar, using an Autoplate 4000 spiral plater (Spiral Biotech, Gaithersburg, MD). Plates were incubated at 37°C and scored for presumptive *Listeria* colonies (black colonies with esculin hydrolysis) at 24 h and 48 h. For enrichment of *Listeria*, water samples (100 ml) were filtered through sterile 0.45-μm cellulose filters (47 mm; Osmonics, Inc., Westborough, MA) with suction, and the filter was placed in 10 ml MLEB.

For all samples, enrichment tubes were incubated at 37°C for 48 h, and broth (10 μl) was streaked onto MOX agar. Cycloheximide-supplemented MOX (50 μg/ml) was used for fecal and milk filter samples to inhibit fungal growth. Plates were incubated at 37°C and scored at 24 and 48 h for presumptive *Listeria* colonies. Isolated, presumptive *Listeria* colonies were transferred from MOX or cycloheximide-supplemented MOX plates onto MOX, PALCAM (polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol; BD Diagnostics), Trypticase soy agar with 0.6% yeast extract, and a chromogenic plating medium, BCM *Listeria* (Biosynth International, Inc., Naperville, IL). Colonies that exhibited the *Listeria* phenotype (as described above on MOX; gray-green colonies with esculin hydrolysis on PALCAM) were preserved for future analysis. Colony biomass was transferred from the PALCAM plates to 1.5 ml tryptic soy broth, incubated at 37°C, and stored at −80°C as previously described (35). Hemolytic activity of select presumptive *L. monocytogenes* isolates (blue colonies on BCM *Listeria* medium) and the Christie, Atkins, Munch-Peterson (CAMP) tests were performed as described by Van Kessel et al. (35).

PFGE. In total, 60 *L. monocytogenes* isolates obtained from different sources at the farm were analyzed by PFGE. Only one *L. monocytogenes* isolate per sample was used for PFGE typing.

First, 36 *L. monocytogenes* isolates were selected to represent the time period between June 2004 and July 2007. Specifically, multiple *L. monocytogenes* isolates isolated in the same month but from different sources were selected. One *L. monocytogenes* fecal isolate from each of the samplings carried out in October 2005, May 2006, and April 2007 was included for PFGE analysis. Furthermore, the first *L. monocytogenes* isolates from the milk filter and from bulk tank milk were included, as well as all *L. monocytogenes* isolates found in milking equipment. A purposive selection of other isolates was used. With the exception of *L. monocytogenes* isolates obtained in June 2004 and May 2005, isolates used for PFGE typing were collected at intervals of 4 months or less for this set of 36 isolates. In addition, 24 *L. monocytogenes* isolates were randomly selected among all previously nonselected *L. monocytogenes*-positive fecal and environmental samples obtained between February 2004 and April 2007. The selection of *L. monocytogenes* isolates was done in proportion to the number of *L. monocytogenes*-positive fecal/environmental samples, available for a particular sampling date.

The standardized CDC PulseNet protocol (http://www.cdc.gov/pulsenet/protocols/pulsenet_listeria_protocol%20.pdf) with modifications was used to do PFGE analysis of *L. monocytogenes* isolates. Bacterial cell suspensions for agarose plug preparation were made using an optical density of 1.50 to 1.59 at a wavelength of 610 nm (SmartSpecPlus spectrophotometer; Bio-Rad Laboratories, Hercules CA). Lysis of agarose plugs was done in a shaking incubator (Labnet 311 DS; Edison, NJ) for 5 h at 54°C and at 170 rpm. The washes were done using sterile distilled water and Tris-EDTA buffer (pH 8.0) in a shaking incubator at 54°C and 70 rpm. DNA digestion using AscI (New England BioLabs, Inc., Ipswich, MA) was carried out at 37°C for at least 5.5 h. The digestion with ApaI (New England BioLabs) was carried out overnight at 30°C using 131 μl of sterile distilled water, 15 μl of NE buffer 4, and 4 μl (50 U/μl) of ApaI endonuclease. *Salmonella*

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TABLE 1. Data for *Listeria* species (including *L. monocytogenes*)- and *L. monocytogenes*-positive samples from the environment and feces of a single dairy herd over a 3-year period

Sampling date (mo/day/yr)	No. (%) of indicated samples						
	Environmental			Fecal			
	Total collected	*Listeria* species positive	*L. monocytogenes* positive	Total collected	Total analyzed	*Listeria* species positive	*L. monocytogenes* positive
2/17/2004	10	3 (30)	3 (30)	308	72 (23.4)	18 (25)	3 (4.2)
6/17/2004	34	6 (17.6)	1 (2.9)				
6/29/2004	3	3 (100)	1 (33.3)				
10/5/2004	23	2 (8.7)	0 (0)	316	65 (20.6)	0 (0)	0 (0)
1/11/2005[a]	19	6 (31.6)	1 (5.3)	12	12 (100)	0 (0)	0 (0)
4/12/2005	24	10 (41.7)	2 (8.3)	335	66 (19.7)	14 (21.2)	7 (10.6)
7/11/2005	29	3 (10.3)	0 (0)				
10/3/2005	26	5 (19.2)	0 (0)	308	63 (20.5)	5 (7.9)	1 (1.6)
2/20/2006	21	10 (47.6)	0 (0)				
5/1/2006	22	7 (31.8)	2 (9.1)	327	327 (100)	67 (20.5)	12 (3.7)
7/10/2006	25	1 (4)	1 (4)				
10/9/2006	23	3 (13)	0 (0)	333	0 (0)	N/A[b]	N/A
1/8/2007	22	9 (40.9)	4 (18.2)				
4/16/2007	22	19 (86.4)	7 (31.8)	333	110 (33)	75 (68.2)	28 (25.5)
Total	303	87 (28.7)	22 (7.3)	2,272	715 (31.5)	179 (25)	51 (7.1)

[a] Not a biannual fecal sampling.
[b] N/A, not applicable.

enterica serotype Braenderup (H9812) was used as the reference standard, after digestion with restriction enzyme XbaI (Roche, Indianapolis, IN, or New England BioLabs). The DNA digestion with XbaI from Roche Laboratories was carried out as described in the PulseNet protocol. When using XbaI from New England BioLabs, the DNA digestion was done at 37°C using 132.5 μl of sterile distilled water, 15 μl of NE buffer 2, and 2.5 μl (20 U/μl) of XbaI endonuclease.

The 1% SeaKem Gold agarose (Lonza, Rockland, ME) gel used for DNA separation was run using a contour-clamped homogeneous electric field mapper XA system (Bio-Rad Laboratories). Images were obtained with a Bio-Rad Gel Doc XR system, using the software Quantity One 4.4.1 (Bio-Rad Laboratories), after staining with ethidium bromide (EMD Chemicals, Inc., Gibbstown, NJ). Band patterns were analyzed by two independent observers, using visual inspection. The criteria described by Tenover et al. (32) were used to assign PFGE types/subtypes to *L. monocytogenes* isolates. Comparison of the PFGE patterns was also done using BioNumerics 3.5 software (Applied Maths, Saint-Matins-Latem, Belgium), as described by Fugett et al. (12).

A secondary identification label (Quality Milk Production identification [QMP ID]) was assigned to each of the isolates, and general and source information is available in Pathogen Tracker 2.0 at http://www.pathogentracker.net.

Statistical analysis. Data for the presence of *Listeria* spp. and *L. monocytogenes* were analyzed using statistical software JMP 7.0 (SAS Institute Inc., Cary, NC). Differences among source categories were evaluated using the chi-square test of independence and Fisher's exact test. A significance level (α) of 0.05 was used.

RESULTS

Fecal samples. A total of 2,272 fecal samples were obtained in eight samplings. Of these, 715 samples were analyzed for the presence of *Listeria* spp. and *L. monocytogenes*. One hundred seventy-nine samples (25.0%) were *Listeria* species positive, and 51 (7.1%) were positive for *L. monocytogenes*. A summary of the sampling regimen and the results for fecal samples is presented in Table 1.

Environmental samples. A total of 303 environmental samples were obtained in 14 samplings. Of the 303 samples, 87 (28.7%) were positive for *Listeria* spp., and 22 (7.3%) were positive for *L. monocytogenes*. The total number of samples obtained at each sampling and the number and percentage of *Listeria* species and *L. monocytogenes*-positive samples are summarized in Table 1.

Environmental samples were classified according to their source. The total number of samples obtained from each of the sampled materials is summarized in Table 2. The numbers and percentages of samples positive for *Listeria* species and *L. monocytogenes* found in each of the sampled materials are also shown in Table 2.

During each of the 13 samplings, approximately one source water sample, five drinking water samples, four feed samples, and 10 manure composite samples were obtained. *Listeria* was

TABLE 2. Data for *Listeria* species (including *L. monocytogenes*)- and *L. monocytogenes*-positive environmental samples obtained from different sampling materials over a 3-year period in a single dairy herd

Sampled material	Total no. of samples	No. (%) of samples positive for:	
		Listeria spp.	*L. monocytogenes*
Bedding	15	4 (26.7)	1 (6.7)
Birds/bird droppings	11	2 (18.2)	0 (0)
Manure composite	123	31 (25.2)	3 (2.4)
Other composites[a]	3	2 (66.7)	1 (33.3)
Feed	55	4 (7.3)	1 (1.8)
Flies	16	8 (50)	2 (12.5)
Other insects	5	2 (40)	0 (0)
Source water	13	0 (0)	0 (0)
Drinking water for animals	47	29 (61.7)	14 (29.8)
Water (other)[b]	13	5 (38.5)	0 (0)
Other	2	0 (0)	0 (0)
Total	303	87 (28.7)	22 (7.3)

[a] Waste around a water trough and a feed bunk, and a composite of feral-animal feces that tested positive for *L. monocytogenes* in one of the silage bunkers.
[b] Standing water, runoff silage water, and mud puddles.

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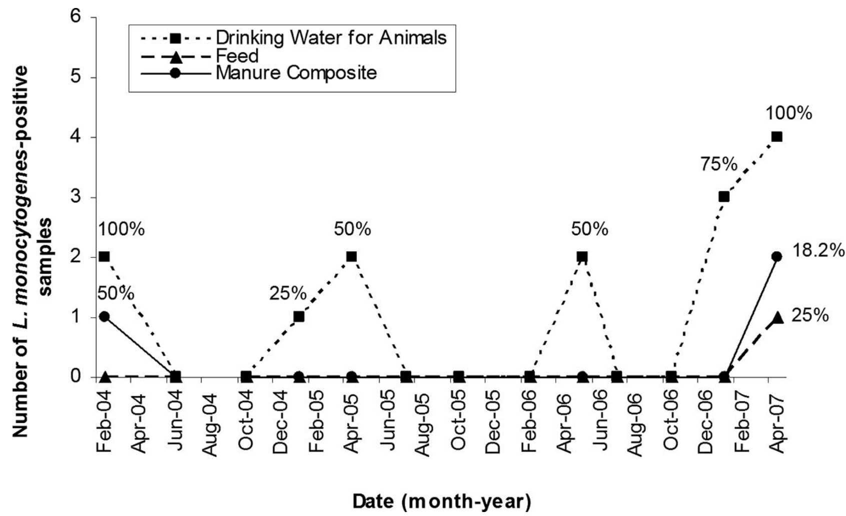

FIG. 1. Temporal variation in the number and percentage of *L. monocytogenes*-positive drinking water, feed, and manure composite samples obtained in a dairy farm over 3 years.

never isolated from the source water that was used to supply drinking water troughs. The proportion of *L. monocytogenes*-positive samples was significantly greater for drinking water samples obtained from the water troughs than for feed and manure composite samples ($P < 0.05$). No significant differences between the proportions of *L. monocytogenes* in feed and manure composite samples were found. Temporal variation in the percentages of *L. monocytogenes*-positive samples among drinking water, feed, and manure composite samples is shown in Fig. 1. The prevalence of *L. monocytogenes* in drinking water was generally highest from February through April.

In-line milk filter samples. A total of 108 in-line milk filter samples were obtained over the study period. Seventy-nine (73.1%) of these were positive for *Listeria* spp. and 73 (67.6%) for *L. monocytogenes*. *Listeria monocytogenes* was first isolated from an in-line filter in May 2005. In-line filters were negative for *L. monocytogenes* in the 16 subsequent weekly samplings until September 2005. Starting in September 2005, *L. monocytogenes* was regularly isolated from the in-line filters (Fig. 2). The percentage of *L. monocytogenes*-positive filters on a monthly basis varied between 20% and 100% during this period, except for samples obtained in October 2006 (0%).

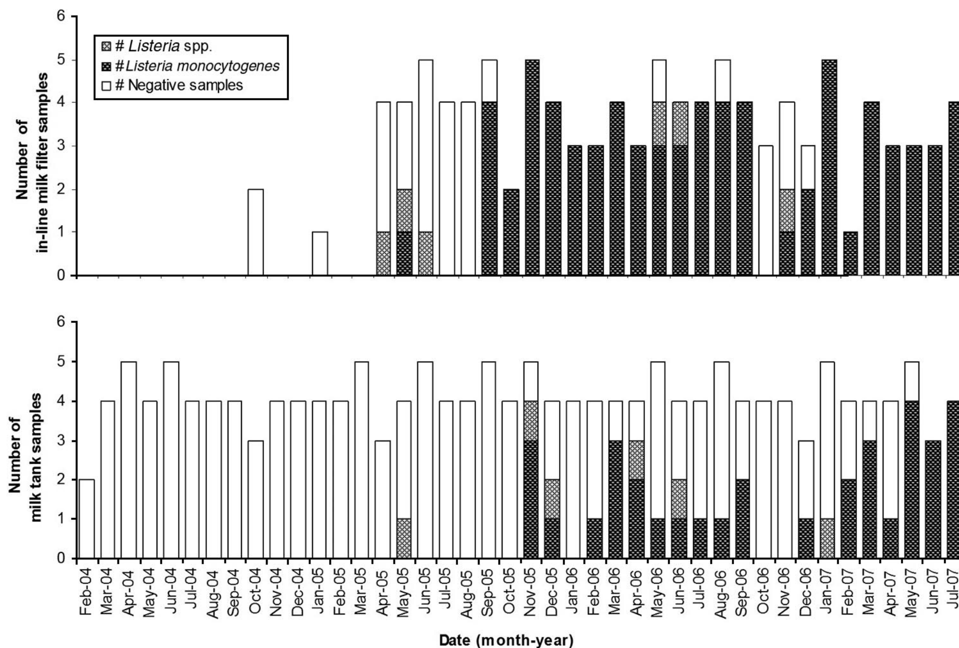

FIG. 2. Number of *Listeria* species- and *L. monocytogenes*-positive samples obtained from in-line milk filters and bulk tank milk during the study period.

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Bulk tank milk samples. A total of 172 milk samples were obtained from the bulk tank between February 2004 and July 2007. Of these samples, 40 (23%) were positive for *Listeria* spp., and 34 (19.7%) were positive for *L. monocytogenes*. *Listeria monocytogenes* was not isolated from any of the bulk tank milk samples analyzed from the beginning of the study until November 2005. A non-*monocytogenes Listeria* species was isolated once from a bulk tank milk sample in May 2005. However, *L. monocytogenes* started to appear regularly in milk samples obtained from the milk tank after November 2005 (Fig. 2).

Milking parlor and milking equipment samples. Samples from 40 sites were analyzed for *Listeria. Listeria* species were found in 14 (35%) of these sites, and *L. monocytogenes* was found in 6 (15%) of these sites. Two samples obtained from the floor (parlor pit and storage area) were positive for *L. monocytogenes*. *Listeria monocytogenes* was also detected in one rubber liner and two milk meters, and one positive sample was obtained from the bulk tank outlet.

In-line milk filters had a significantly greater proportion of *L. monocytogenes*-positive samples than did fecal, environmental, milking parlor, and bulk tank milk samples ($P < 0.005$). Bulk tank milk had a greater proportion of *L. monocytogenes*-positive samples than did fecal and environmental samples ($P < 0.05$).

PFGE. Sixty of 186 *L. monocytogenes* isolates obtained between February 2004 and July 2007 were typed by PFGE. Thirteen PFGE types and eight subtypes were distinguished with the restriction endonuclease AscI through visual inspection. Fourteen PFGE types and seven subtypes were found by visual inspection when using the restriction endonuclease ApaI. Analysis of the combined AscI and ApaI restriction profiles by automated cluster analysis using the Dice coefficient (tolerance of 1.5%) and unweighted-pair group method with arithmetic averages (UPGMA) showed 23 PFGE types, using a similarity score value of 100% as the cutoff.

Cluster analysis of the combined AscI and ApaI restriction digest profiles showed that PFGE types F, T, and D were predominant from September 2005 through February 2006, from March 2006 to May 2007, and from May 2007 through July 2007, respectively. Types F, T, and D accounted for 13.3%, 25%, and 15% of *L. monocytogenes* isolates subjected to PFGE typing, respectively.

The PFGE type F was first detected in an in-line milk filter sample from September 2005 and, subsequently, in additional milk filter samples collected in September (several sampling dates), October and December 2005, and February 2006. PFGE type F was also isolated from a bulk tank milk sample in November 2005. This sample was the first bulk tank milk sample from which *L. monocytogenes* was isolated.

PFGE type T was first detected in a feral-animal feces composite in June 2004. Type T was not detected in 2005 on this set of isolates. PFGE type T was subsequently found in in-line milk filter samples obtained in March, July, September, and December 2006 and in February and April 2007 (Fig. 3). Furthermore, PFGE type T was found in bulk tank milk samples in May, July, September, and December 2006, and February and May 2007 (Fig. 3). In May 2007, type T was also obtained from a milk meter and from the milk tank outlet (Fig. 4). PFGE type S was closely related to PFGE type T. It was found

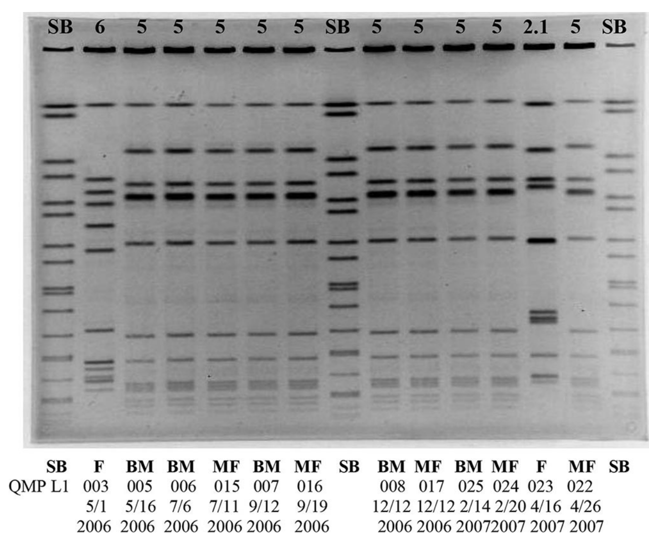

FIG. 3. Examples of *L. monocytogenes* PFGE types/subtypes among isolates obtained between June 2004 and April 2007, using restriction endonuclease AscI. The first row (top panel) indicates the (sub)type assigned to PFGE patterns based on the criteria from Tenover et al. (32). First and second rows (bottom panel) indicate the source (feces [F], bulk tank milk [BM], and milk filter [MF]) and sample identification (QMP ID, L1), respectively. Samples are in chronological order from left to right, with sampling date (month/day) and year shown on the third and fourth rows (bottom panel), respectively. Lanes 1, 8, and 15 contain *Salmonella enterica* serotype Braenderup (SB; standard).

in a manure composite sample obtained in February 2004 and in water samples in February 2004 and January 2007.

PFGE type D was first detected in a bulk tank milk sample in May 2007 (approximately 2 weeks before the sampling of the milking equipment) and subsequently in one of the milk meters (May 2007) and in in-line milk filter and bulk tank milk samples obtained between May and July 2007. PFGE type E, which was closely related to PFGE type D, was found in a sample obtained from a rubber liner.

AscI and ApaI restriction digest profiles of the 60 *L. monocytogenes* isolates are shown in Fig. 4. Clustering of these *L. monocytogenes* isolates and the PFGE types assigned by visual inspection and by automated cluster analysis are also presented.

DISCUSSION

Detection of *L. monocytogenes* in bulk tank milk has been previously reported. In a regional survey of 131 dairy herds in South Dakota and Minnesota, *L. monocytogenes* was isolated from 4.6% of the bulk tank milk samples (21). In a national survey, *L. monocytogenes* was isolated from 6.5% of collected bulk tank milk samples (35). Even on this high-prevalence farm where *L. monocytogenes* was on average isolated in one of five weekly samples (19.8%), a single point time survey might have missed *L. monocytogenes* in raw milk. Hence, repeated sampling over time is a more reliable method to gauge the potential presence of *L. monocytogenes*.

The proportion of *L. monocytogenes*-positive in-line filter samples (67.6%) was even greater than the proportion of *L.*

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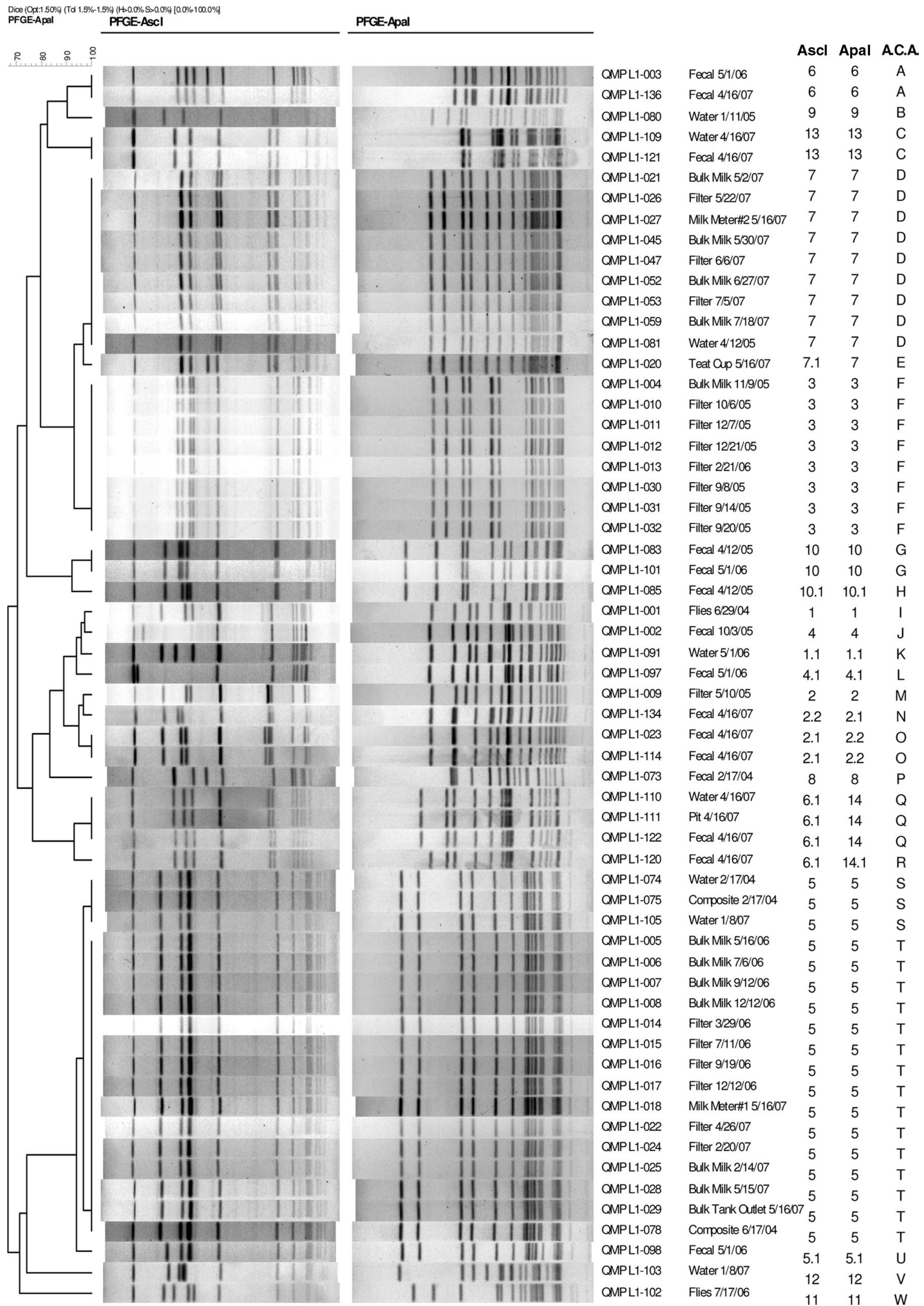

FIG. 4. Automated cluster analysis of the 60 *L. monocytogenes* isolates selected for PFGE typing, after digestion with restriction endonucleases AscI and ApaI. PFGE types assigned by visual inspection (AscI-ApaI*)*, and PFGE types assigned by automated cluster analysis (A.C.A.) of the combined AscI and ApaI restriction digest profiles are shown to the right of the dendrogram.

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monocytogenes-positive bulk tank milk samples (19.8%), suggesting that testing in-line filters could be a more sensitive means to detect pathogens than bulk tank milk samples. In-line milk filters have previously been used in a survey of New York State dairy farms (17). In this survey of 404 farms, *L. monocytogenes* was isolated from 12.6% of the filters. Higher sensitivity of detection in in-line milk filters than that in bulk tank milk has also been reported for *Salmonella* spp. (37).

The milk produced on the study farm is pasteurized before its distribution to consumers, and the presence of *Listeria* in bulk tank milk is therefore unlikely to pose a human health hazard. Pasteurized milk from retail stores in the United States (11) and in England and Wales (14), however, tested positive for *L. monocytogenes* in 0.018% and 1.1% of samples, respectively. Outbreaks of human listeriosis have been attributed to the consumption of pasteurized milk (7, 10) or dairy products manufactured with improperly pasteurized milk (22). Raw milk contaminated with *Listeria* could be a source of contamination for a milk processing plant (38). Hence, prevention of *Listeria* contamination is important for milk that is consumed raw, as well as for milk that will be pasteurized before consumption.

In the current study, weekly samplings for in-line milk filters were not part of the original sampling protocol and started in April 2005; therefore, it is not certain whether *L. monocytogenes* was endemic before this time or only sporadically present. The three in-line milk filter samples obtained in October 2004 and January 2005 were negative for *L monocytogenes*, so it would appear that the contamination was established after January 2005. Starting in September 2005, *L. monocytogenes* was isolated from the in-line milk filter samples on a regular basis. Standard plate counts of bulk tank milk were unusually high during that month (peak, 593,000 CFU/ml) (unpublished data). High standard plate counts have been associated with deficiencies in the cleaning of the milking equipment, because the presence of milk residues may provide ideal conditions for bacterial growth (25).

Excretion of *L. monocytogenes* in milk has been reported for cows suffering from mastitis (9, 29, 40). In our study, milk from all clinical mastitis cases from the dairy farm was cultured as part of the routine examination of mastitis cases, and *Listeria* was never isolated from these samples. We did not specifically culture samples from cows with subclinical mastitis; however, the mammary gland would not be a specific target for *L. monocytogenes* in cattle (9), and isolation of the organism from nonclinical milk samples is extremely rare (QMPS, unpublished data). Thus, milk from individual cows is unlikely to have been an important source of *L. monocytogenes* in the bulk tank of the study farm.

Milk and milk filters were positive for *L. monocytogenes* more frequently than expected, based on the low incidence of the pathogen in fecal samples (37). In our study, 7.1% of fecal samples were positive for *L. monocytogenes*, with a range from 0 to 25.5% at any given sampling time. When 15 *L. monocytogenes* isolates obtained from fecal samples were characterized by PFGE, 12 PFGE types were observed, demonstrating a high level of heterogeneity among fecal isolates. If the presence of *L. monocytogenes* in bulk tank milk was due to fecal contamination, we would have expected to find heterogeneity among *L. monocytogenes* isolates (3, 18) in the bulk tank milk as well. However, only three *L. monocytogenes* PFGE types, each persisting over time, were observed in milk. Furthermore, the PFGE types of fecal isolates were different from those observed in bulk tank milk and milk filters. By visual inspection, the PFGE types N/O (three fecal samples obtained in April 2007) and U (one fecal sample obtained in May 2006) were "closely related" but not identical (32) to PFGE types M (one isolate from a milk filter in May 2005) and T (15 isolates from milk and milk filter in March 2006 to May 2007). It is possible that some strains present in feces were not detected with our study design (8).

In addition to feces, a variety of environmental sources harbored *L. monocytogenes*, and some strains found in bulk tank milk were previously isolated from other sources on the farm. Our data suggest that the presence of *L. monocytogenes* in the milk system was initially caused by fecal or environmental contamination and that specific strains could have subsequently established themselves in the milking system as a biofilm. *Listeria monocytogenes* has the ability to form biofilms (16, 31) on stainless steel surfaces and other materials (1) that can be present in dairy operations. Bacterial cells can detach from biofilms (15), and this could explain the presence of the same *L. monocytogenes* PFGE types in bulk tank milk and filters for prolonged periods of time. Persistent *L. monocytogenes* strains can be defined as those strains in a particular dairy premise that are repeatedly found over time in bulk tank milk samples (2). This definition agrees with our finding. In previous studies, persistent strains have shown a better ability to form biofilms than transient strains (2, 27). Although the biofilm-forming ability of the persistent PFGE types from our study has not yet been assessed, formation of biofilm could potentially explain our observations.

In this study, the low number of manure composite samples positive for *L. monocytogenes* suggests low levels of fecal shedding in *L. monocytogenes*-positive animals. Samples of silage and other feeds were negative, except in April 2007, when one feed sample (25%) was positive for *L. monocytogenes*. On the same sampling date, the highest percentages of *L. monocytogenes*-positive fecal (25.5%) and drinking water (100%) samples were also reported. Fecal contamination of the feed cannot be ruled out, since *L. monocytogenes*-positive samples were obtained from feedstuffs that were fed to animals ("feed as presented to the animals"). Because all source water samples were negative throughout the study, fecal contamination could be the likely source of *L. monocytogenes* in water. Results of PFGE typing of *L. monocytogenes* isolates obtained from both fecal and water samples also suggest feces as the source of water contamination.

PFGE results were concordant for most of the 60 *L. monocytogenes* isolates regardless of which restriction endonuclease (AscI and ApaI) was used. A few discrepancies were observed between the analysis using visual inspection and automated cluster analysis. For example, PFGE type S, which was observed in three environmental samples obtained in February 2004 (drinking water and manure composite samples) and January 2007 (drinking water), was considered indistinguishable from PFGE type T by visual inspection, whereas types S and T were closely related but distinguishable based on computer-assisted analysis. PFGE types I/K and J/L were considered a main type and a subtype, respectively, when analyzing

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digestion profiles by visual inspection, whereas they were considered to be distinct profiles based on computer-assisted data analysis. The differences in interpretation of banding patterns based on visual or automated comparison are subtle and do not affect the interpretation of the study results.

In conclusion, our study on this farm shows high heterogeneity of *L. monocytogenes* isolates in a variety of on-farm sources and predominant homogeneity of the *L. monocytogenes* population in in-line milk filters and bulk tank milk, implying a potential presence of *L. monocytogenes* biofilm in the milking equipment.

Even though we report a suggested presence of *L. monocytogenes* biofilm in the milking system of just one dairy farm, the relatively high prevalence of this organism in bulk tank milk surveys combined with the documented ability of *L. monocytogenes* to form biofilms on stainless steel (1, 27) would suggest that this is not an isolated finding. Further research to quantify the importance of biofilms in milk harvesting equipment and methods to prevent buildup of such biofilms is needed.

To our knowledge, this is the first report to indicate the potential presence of *L. monocytogenes*-containing biofilms in dairy farm milk harvesting equipment. Measures to prevent *L. monocytogenes* contamination and persistence on dairy operations, as well as the communication of the risk attributed to the consumption of contaminated raw milk or dairy products made with nonpasteurized milk, are encouraged.

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