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Investigating Contamination of Bulk Tank Milk with *Listeria monocytogenes* on a Dairy Farm

J. C. F. PANTOJA,* A. C. O. RODRIGUES, C. HULLAND, D. J. REINEMANN and P. L. RUEGG
Dept. of Dairy Science, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI 53706, USA

SUMMARY

The objectives were to identify the source of *Listeria monocytogenes* in bulk tank milk (BTM), and to assess characteristics of Petrifilm Environmental *Listeria* (PEL) for detection of this pathogen in farm samples. Environmental and milk samples were collected from a dairy during two sampling periods. Follow-up samples of daily BTM and milk filters were collected. Isolates of *L. monocytogenes* were compared by use of Pulsed Field Gel Electrophoresis. Samples were plated on PEL and results classified into positive or negative. Of samples collected during the two sampling periods, *L. monocytogenes* was isolated from 66% of milk filters (19 of 29), 16% of BTM (7 of 44), 6% of water samples (two of 33) and one of 18 in-line milk samples. Except for one isolate, all were identical and of the same molecular type. Contamination of BTM with *L. monocytogenes* most likely originated from a common source, and results indicate that farms can develop persistent sources of contamination. The sensitivity of the PEL was high (100 and 74.1% for environmental and milk samples, respectively), but there was a high proportion of false-positive results and low specificity. These limitations need to be considered when using the PEL for on-farm screening of *L. monocytogenes*.

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*Author for correspondence: Phone: +1 608.263.3498; Fax: +1 608.263.9412
E-mail: josecarlospantoja@hotmail.com

INTRODUCTION

Diseases caused by foodborne pathogens impact the health of millions of people worldwide. It has been estimated that in the United States approximately 48 million foodborne disease cases result in 128,000 hospitalizations and 3,000 deaths each year *(4)*. Among foodborne pathogens, *Listeria monocytogenes* has been considered an emerging public health problem because of its pathogenicity and ability to contaminate food. *Listeria monocytogenes* is capable of multiplying at temperatures ≤ 7°C and surviving in environments with a wide range of pH values (4.3 to 10) and high salt concentrations *(15)*. Various species of *Listeria* are commonly found in soil, decaying vegetation, and water, and well as being part of the fecal flora of animals and humans. In dairy cattle, *L. monocytogenes* can result in several clinical presentations, including encephalitis, septicemia, abortion, and mastitis *(14)*. Mastitis caused by *Listeria* is infrequent, but infected mammary glands can shed this pathogen for periods as long as 12 months *(21)*.

Listeria monocytogenes is present in the dairy farm environment and

can survive in the gastrointestinal tract of cows, thus constituting a source of contamination of bulk tank milk (BTM) *(11)*. The prevalence of isolat-ion of *L. monocytogenes* in BTM samples has ranged from 1.2 to 12.6% *(9, 10, 19)*. Another important characteristic that makes *L. monocytogenes* an emerging concern to public health authorities is its ability to form biofilms and survive on materials commonly used in food processing equipment *(22)*. Colonization of dairy processing equipment can result in cross-contamination of pasteurized milk in processing plants, which has been reported to be an important source of human listeriosis.

Although listeriosis is uncommon in the general population, it can be an important cause of fatal bacteremia and meningoencephalitis in neonates, pregnant women, elderly persons, and immunosuppressed individuals *(12)*. In addition, listeriosis can result in self-limited gastroenteritis in healthy persons *(12)*. In the United States, the Centers for Disease Control and Prevention estimated that approximately 1,600 persons become seriously ill with listeriosis each year, of whom 16% die (*5*). Sporadic outbreaks of listeriosis linked to consumption of unpasteurized or cross-contaminated dairy products have occurred. Of the five outbreaks associated with cheese and fluid milk consumption reported between 1990 and 2006, three were associated with consumption of cheese made from unpasteurized milk. This is a point of concern for public authorities because there has been growing interest in artisanal cheese made with unpasteurized milk. The other two outbreaks were associated with consumption of dairy products made from pasteurized milk *(2)*.

The high fatality rate, occurrence of outbreaks, and ability of *L. monocytogenes* to contaminate both unpasteurized and pasteurized foods has led the FDA to adopt a "zero tolerance" policy for the presence of *L. monocytogenes* in ready-to-eat foods *(6)*. In order to comply with this regulatory policy and minimize the risk of human listeriosis, a reduction in the initial contamination of farm BTM has been an important objective of governmental agencies *(9)*. Nonetheless, few studies have been conducted to identify risk factors for the isolation of *L. monocytogenes* from BTM or in-line milk filters *(1, 8, 16)*. Results of these observational studies suggest that environmental and milking hygiene-related risk factors such as milking of cows directly into buckets, lack of pre-milking teat disinfection *(8)*, poor cow cleanliness, and use of dirty towels in teat pre-milking disinfection *(16)* were associated with the isolation of *L. monocytogenes* from BTM *(16)* or in-line milk filters *(8)*. Antognoli et al. *(1)* reported that large herds (> 500 milking cows; n = 850) were five times more likely than small herds to have *L. monocytogenes* isolated from their BTM.

Results of a more recent longitudinal study *(11)* indicate that *L. monocytogenes* can be persistently present in BTM of individual farms. The authors concluded that the milking machine was the most likely source of *Listeria* on a dairy farm, because strains found in BTM and milk filters were similar to each other and yet different from heterogeneous strains isolated from environmental samples. A better understanding of sources of *L. monocytogenes* on dairy farms could help identify management strategies that reduce the risk of BTM contamination and therefore protect public health. Moreover, a rapid and simple diagnostic test that would reduce both the cost and time for identification of contaminated milk would be useful for identification of this zoonotic pathogen on dairy farms. Current tests based on traditional microbiology or molecular methods are still expensive and time consuming, which may preclude their use in such programs.

The primary objective of this study was to determine the source of contamination with *L. monocytogenes* in BTM on a dairy farm with a history of recent isolation. A secondary objective was to assess diagnostic test characteristics of the Petrifilm Environmental *Listeria* (PEL) system to detect *L. monocytogenes* in environmental and milk samples.

MATERIALS AND METHODS

Farm selection and description

A dairy farm with a recent isolation of *L. monocytogenes* from unpasteurized BTM was used for this study. The herd contained 711 lactating Holstein cows that produced an average of 36 kg of milk per day with mean BTM SCC of 250,000 cells/mL. Cows were milked three times per day (seven hours per milking) in a parallel parlor equipped with 24 milking units, automatic unit removers, and electronic milk meters. Milk passively flowed from the parlor to the milk house and entered a receiver jar, from which it was pumped through a milk filter, into a plate milk cooler, and subsequently into two bulk tanks.

Milking machine sanitation (post-milking rinse, detergent wash, acid rinse, and pre-milking sanitation) was performed using an automatic wash controller after each milking. Each month the milking equipment was inspected and maintained by a manufacturer's authorized dealer. The milking routine consisted of teat disinfection using a 0.5% iodine solution, stripping of foremilk on the milking platform, drying of teats using a single cloth towel, and finally unit attachment. After milking, teats were disinfected using a 1% iodine dip solution. Cows were separated into nine groups based on stage of lactation and reproductive status. A separate group consisting of sick cows (hospital group) was milked last, with milk being diverted from the bulk tank. Free stalls were bedded with sand during spring and summer and with dried manure solids during fall and winter.

TABLE 1. Sampling methodology and number of samples collected during weekly farm visits

Source	Type	Size	Method	Total per visit
Feces	Composite	Full 4-L plastic bag	Collected from 10 areas of each pen's floor.	5
Bedding	Composite	Full 4-L plastic bag	Collected from the rear of every 5th stall of each pen.	1
Water troughs	Composite	200 mL sterile plastic vial	Collected in 50 mL sterile plastic vials, from all water troughs on each pen.	1
Silage	Composite	Full 4-L plastic bag	Collected from 10 points of the face of each silage bunker (grass and corn).	2
Bulk tank milk	Single	30 mL	Sterile 50 mL-syringe and pipette.	1
Liners	Composite	4 mL per swab	Swab of 10 liners on each side of the parlor during the final 30 min. of milking.	2
Milk meters hose	Composite	4 mL per swab	Swab of inner surface of 10 hoses per side of the parlor, at the connection between milk meters and milk hoses.	2
Hose insertion to the milk line	Composite	4 mL per swab	Swab of the inner surface of 10 hoses per side of the parlor, at the insertion to the milk line.	2
Pre-pump gaskets	Composite	4 mL per swab	Swab of 3 rubber gaskets on the milk line before the receiver jar.	1
Receiver jar	Single	4 mL per swab	Swab of the bottom of receiver jar.	
Post-pump gaskets	Composite	4 mL per swab	Swab of 4 rubber gaskets on the milk line located between the milk pump and the bulk tanks.	1
Pre-pump milk line	Composite	4 mL per swab	Swabs of the inner surface of the milk line when connections were disassembled to sample gaskets.	1
Post-pump milk line	Composite	4 mL per swab	Swabs of the inner surface of the milk line.	1
In-line milk filters	Single	4-L plastic bag	Collected using plastic gloves, immediately after milking.	1
Source water	Single	30 mL	Collected in a sterile plastic vial from a hose in the parlor or a well located in the milk house (common source).	1
Total				31

Sampling procedures

Milk and environmental samples were collected during weekly farm visits (n = 6) conducted during 3 successive weeks in September and November, 2010. During each farm visit, a variety of environmental and milk samples (milk filters and BTM) were obtained to assess the presence of *L. monocytogenes* (Table 1). Environmental samples were obtained from feces, bedding, silage, and water (troughs, hoses and well), while swabs were obtained from the inner surface of milking liners, milk hoses, milk meters, selected points in the milk line, milking equipment gaskets, and receiver jar. Liner swabs were collected using large obstetric rayon swabs (Puritan Medical Products Company

TABLE 2. Distribution of samples collected by sampling periods and sample type

	Study Period				
		Period 2[b]			
Sample Type	Period 1[a] (Sep. 2010) N	(Oct. & Nov. 2010) N	Follow-up[2] (Dec. 2010) N	Follow-up[2] (Mar 2011) N	Total N
Bulk tank milk	17	27	23	14	81
Milk filter	12	17	15	15	59
Milking machine	33	34	0	0	67
Feces	13	15	0	0	28
Bedding	13	15	0	0	28
Silage	6	6	0	0	12
Water	15	18	0	0	33
In-line milk	0	18	0	0	18
Quarter milk	0	40	0	0	40
Total	109	190	38	29	366

[a]Samples collected by study personnel on three successive weekly visits

[b]Daily samples collected by farm personnel

LLC, Guilford, ME), as described by Zadoks et al. *(23)*. Milking machine swabs were collected individually upon completion of wash cycles using cotton swabs (Puritan Medical Products Company LLC, Guilford, ME) transported in 4 mL of Neutralizing Buffer (Difco; BD Diagnostics, Sparks, MD). During the period between weekly visits, daily BTM and milk filters were collected and frozen by farm personnel.

To identify cows with intramammary infections possibly shedding *L. monocytogenes* in milk, separate milk samples (500 mL) were collected during milking from each of the 9 groups of cattle using an in-line sampling system (BoldBioTech, Fernandina Beach, FL). Samples were collected from the milk line at a point located between the receiver jar and the milk filter, on the first and second weekly visits during October. When *L. monocytogenes* was isolated from the in-line milk samples of a group, aseptic quarter milk samples were collected from all cows housed in that group during the following weekly visit.

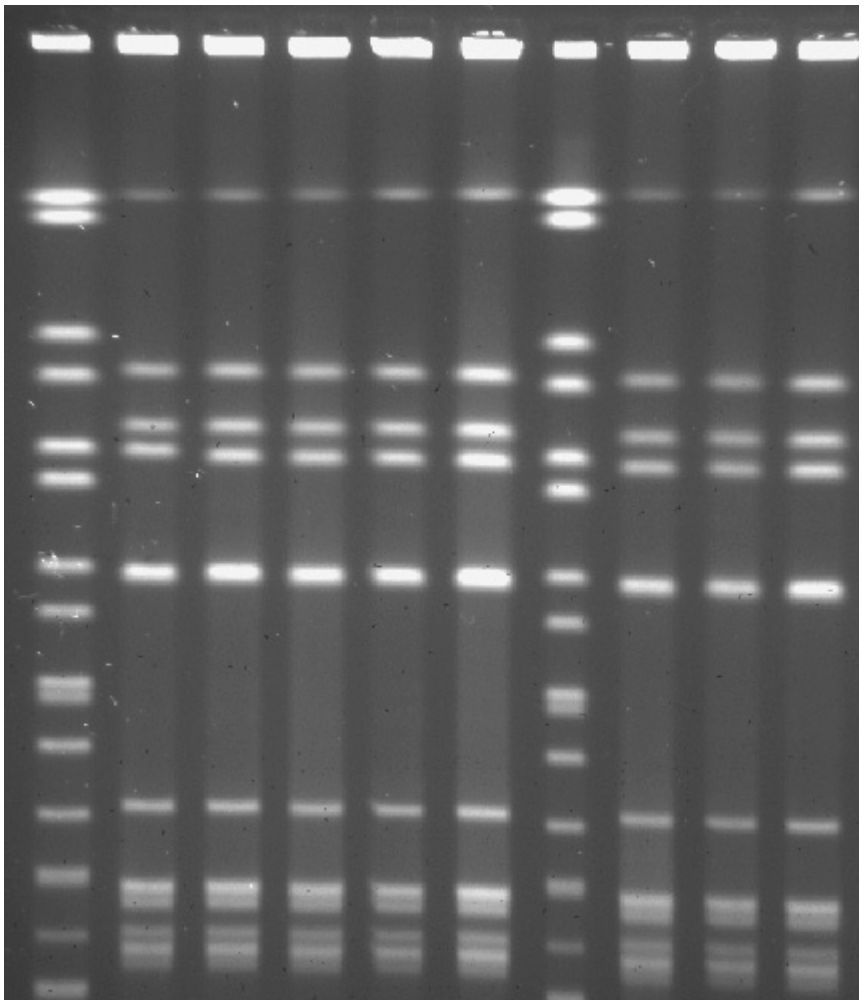
Follow-up sampling was performed during successive three-week periods occurring in December, 2010, and March, 2011. During this periods, farm personnel collected daily BTM samples and milk filters. These samples were frozen and sent to the University of Wisconsin's Milk Quality Laboratory.

Laboratory methods

Environmental and milk samples were processed as described by Latorre et al. *(11)*. Silage, bedding, and fecal samples (50 g) were mixed with 200 mL of peptone water in two-chamber filter bags (Filtra-Bag; LABPLAS inc., Sainte-Julie, Quebec, Canada). Samples were stomached for one minute, after which five mL of the filtered sample were reserved for further incubation in Modified *Listeria* Enrichment Broth (Difco; BD Diagnostics, Sparks, MD). The same procedure was repeated for milk filters, which were cut into 50 cm^2 pieces, weighed, and mixed with twice as much weight of peptone water in a filter bag. Milking equipment swabs for each sampling location (Table 1) were composited in the laboratory by adding four mL of Neutralizing Buffer included in each tube to a sterile plastic vial.

Five mL of BTM, water, milking equipment swabs, and all other filtered samples were mixed with five mL of concentrated (two times the concentration recommended by the manufacturer) Modified *Listeria* Enrichment Broth and incubated at 30°C for 24 and 48 h, at which times an aliquot of 10 microliters was plated onto Oxford medium (Oxoid Ltd., Basingstoke, England) and incubated at 35°C for 24 h and 48 h.

FIGURE 1. Results of Pulsed Field Gel Electrophoresis for 10 identical *Listeria monocytogenes* isolates from bulk tank milk and milk filters. Lanes 1, 7 and 13 are standard marker lanes (*Salmonella enterica* serotype Braenderup).



Esculin-positive colonies identified on Oxford medium were plated onto blood-agar plates and incubated for 24 h at 37°C. Catalase-positive, Gram-positive rods were further tested with the Christie, Atkins, Munch-Petersen test (CAMP), the API Coryne system (bioMérieux-Vitek Inc., Hazelwood, MO) and rhamnose sugar reaction. Gram-positive rods that were catalase-positive, beta-hemolytic, esculin-positive, rhamnose-positive, and CAMP-positive, and that had identification with API confidence levels greater than 0.9 were considered *L. monocytogenes (20)*. Non-hemolytic isolates with the same aforementioned characteristics were diagnosed at the genus level as *Listeria* spp.

Molecular strain typing of *L. monocytogenes* was performed for all isolates as described by the Centers for Disease Control and Prevention's Pulse-Net Protocol *(3)*. Digestion of DNA for each *L. monocytogenes* isolate was performed using both of the enzymes *AscI* and *Apa I* (New England BioLabs, Inc., Ipswich, MA). Digestion of the standard strain of *Salmonella enterica* serotype Braenderup (H9812) was performed with *Xba I* (New England BioLabs). Pulsed Field Gel Electrophoresis was performed using the CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) and gel images were obtained using the ChemiDoc system integrated with the software Quantity One (Bio-Rad Laboratories). To compare strains of *L. monocytogenes* isolates, PFGE patterns of chromosomal DNA restriction fragments were analyzed visually by two independent observers, according to the procedure of Tenover et al. *(18)*.

All samples were also tested for the presence of *L. monocytogenes* by use of the PEL system (3M, St. Paul, MN). Five mL of each sample (milk, water, composite milking machine swabs, or filtered silage, bedding, feces, and milk filters) were mixed with five mL of peptone water to prepare a dilution series (1:2, 1:4, and 1:8). A portion (three mL) of each diluted solution was plated on PEL plates and incubated at 37°C for 30 h. Plates were visually read based on the criteria described in the manufacturer's instruction manual and results were dichotomized into (1) *Listeria*-positive (presence of distinguishable intense red-violet colonies or indistinguishable colonies resulting in a pink-brown color throughout the plate for at least one of the dilutions plated) and (2) *Listeria*-negative (presence of distinguishable light pink or grey colonies or plates with unaltered color with no colonies for all dilutions plated). A single distinguishable red-violet colony was taken from three positive Petrifilms and cultured on Oxford and Blood agar to identify the bacterial genera.

Statistical analysis

Statistical analyses were performed using the Frequency Procedure of SAS *(17)*. Frequency distributions and cross-tabulations between discrete variables were used to produce summary statistics. The Chi-square test was used to compare the proportion of *L. monocytogenes*–positive samples between the first and second weekly sampling periods (September to November). When BTM and milk filters were collected on the same day (a subset of the data including 56 paired samples), the

TABLE 3. Distribution of microbiological results by sample type

Sample type	N	*Listeria monocytogenes* N (%)	*Listeria* spp. N (%)	No growth N (%)	Other bacteria N (%)
Bulk tank milk	81	7 (8.6)	0 (0.0)	41 (50.6)	33 (40.7)
Milk filter	59	19 (32.2)	9 (15.3)	13 (22.0)	19 (30.5)
Milking machine	67	0 (0.0)	1 (1.5)	32 (47.8)	34 (50.8)
Silage	12	0 (0.0)	2 (16.7)	7 (58.3)	3 (25.0)
Water	33	2 (6.1)	15 (45.5)	11 (33.3)	5 (15.2)
Feces	28	0 (0.0)	4 (14.3)	0 (0.0)	24 (85.7)
Bedding	28	0 (0.0)	0 (0.0)	0 (0.0)	28 (100.0)
In-line milk	18	1 (5.6)	0 (0.0)	8 (44.4)	9 (50.0)
Mammary gland	40	0 (0.0)	0 (0.0)	37 (92.5)	3 (7.5)
Total	366	29 (7.9)	31 (8.5)	149 (40.7)	157 (42.9)

McNemar's test was used to compare the proportions of BTM samples and milk filter samples from which *L. monocytogenes* was isolated.

The accuracy of the PEL system to detect the presence of *L. monocytogenes* on all samples collected was assessed by estimating its sensitivity (Se) and specificity (Sp) *(13)*. The post-test likelihoods positive predictive value (PPV), and negative predictive value (NPV) were also calculated. Traditional microbiological test results were considered the reference method to which the PEL system was compared. This assessment was also performed separately for subsets of the data consisting of only environmental (n = 168) or only milk-based samples (n = 198; milk filters, BTM, in-line milk, and quarter milk samples). To assess differences between the appearance of Petrifilm positive results for environmental and milk-based samples, a subset of all Petrifilms with positive results (n = 308) was created. The Chi-square test was used to compare the proportion of positive Petrifilms that appeared as distinguishable intense red-violet colonies (as opposed to indistinguishable colonies resulting in a pink-brown color throughout the plate) between environmental and milk-based samples. For all analyses, statistical significance was considered a level of $P < 0.05$.

RESULTS

A total of 366 samples were collected during the study (Table 2). The number of BTM, samples and milk filters collected was not consistent across study periods or follow-up periods (Table 2) because farm personnel did not collect all samples according to the suggested sampling frequency. Likewise, there was a discrepancy between the number of environmental samples collected in the first and second phases of the study because some of the planned water (n = 3), bedding (n = 2), and fecal (n = 2) samples could not be collected on the first visit day.

The distribution of microbiological results for all samples was: *L. monocytogenes*: 7.9% (n = 29); *Listeria* spp.: 8.5% (n = 31); other bacteria: 43% (n = 157); and no growth of bacteria: 40.7% (n = 149). The percent of *L. monocytogenes*-positive samples was not different between the first (13.8%; n = 15) and second (7.4%; n = 14) weekly sampling periods ($P = 0.07$). *L. monocytogenes* was not isolated from any sample during the follow-up periods.

Listeria monocytogenes was most frequently isolated from milk filters. In all samples collected during the first and second phases of the study (n = 299), *L. monocytogenes* was isolated from 66% of milk filters (19 of 29), 16% of BTM (7 of 44), and 6% of water samples (2 of 33). All milk samples (n = 9 groups) were negative when in-line milk sampling was initially performed. However, *L. monocytogenes* was isolated from both the milk filter and BTM on this day. On the second in-line sampling day, *L. monocytogenes* was isolated from milk obtained when the hospital group was milked. On this day, the milk filter was also positive for *L. monocytogenes* (a BTM sample was not collected). During the subsequent weekly farm visit, individual quarter milk samples were obtained from cows that had been present in the hospital group on the day that *L. monocytogenes* was isolated from the in-line milk sample. A total of

TABLE 4. Test characteristics of the Petrifilm Environmental *Listeria* system to detect *Listeria monocytogenes* from study samples, with traditional microbiology as the reference method

		Test Characteristic[c] (%)			
Dataset	**N**	**Se**	**Sp**	**PPV**	**NPV**
All samples	366	75.9	61.7	14.6	96.7
Environmental[a] samples	168	100.0	48.8	2.3	100.0
Milk samples[b]	198	74.1	74.3	31.3	94.8

[a]Water, silage, bedding, milking machine, and fecal samples

[b]Milk filters, bulk tank milk, in-line milk, and quarter (mammary glands) milk samples

[c]Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value

10 cows (40 quarters) were sampled, but five additional cows had been culled from the herd by the time sampling was performed. No quarter milk samples were positive for *L. monocytogenes* (Table 3). Except for one isolate from a water trough, all *L. monocytogenes* isolates were identical and of the same molecular type (Fig. 1). Based on its slightly different band pattern (deletion of DNA from a fragment), the water isolate was classified as a substrain closely related to the other strains.

The proportions of *L. monocytogenes*-positive samples were different ($P < 0.01$) for milk filters and for BTM samples collected on the same day (n = 56 pairs). Eleven percent (n = 6) of BTM samples were positive for *L. monocytogenes* whereas 32% (n = 18) of the milk filters were positive. Of the 18 *L. monocytogenes*-positive milk filters, in only four (22%) cases were the samples also BTM positive. In contrast, four (67%) of the six positive BTM samples were also associated with positive milk filter samples.

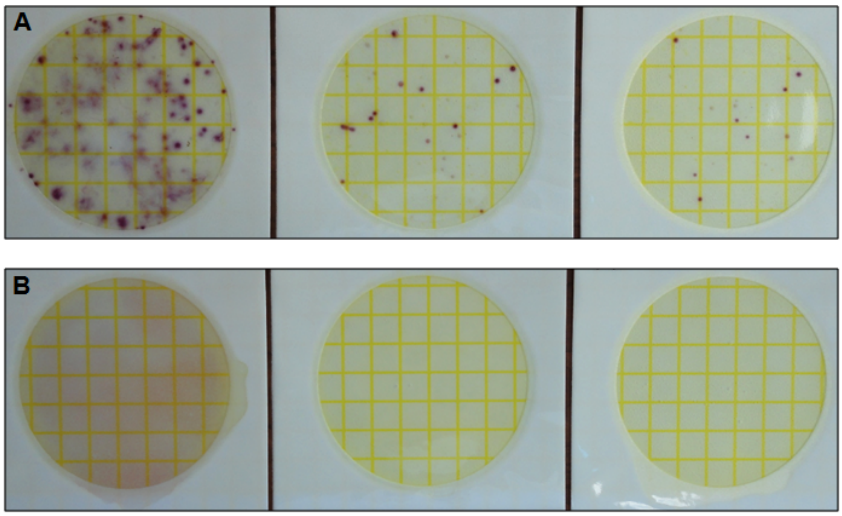
When results of the PEL system were dichotomized into positive or negative for the presence of *Listeria*, 41% (n = 151) of the 366 study samples were classified as positive and 59% (n = 215) as negative. The PEL system was highly sensitive (Se = 100%) for detecting *L. monocytogenes* in environmental samples (Table 4). However, the Sp was low (48.8%) and there was a large proportion of false-positive results (51.2%). As compared to results for environmental samples, Se was lower (0.74%) and Sp higher (74.3%) for the PEL system for detecting *L. monocytogenes* in milk samples. The probability of a positive Petrifilm result correctly representing a truly *L. monocytogenes*-positive sample (based on traditional microbiology) was low for either environmental or milk samples, but was lowest for environmental samples (PPV = 2.3%). In contrast, the probability of a negative Petrifilm test result representing a truly negative sample was 94.8% for milk and 100% for environmental samples (Table 4).

Of all 1,098 Petrifilms analyzed (n = 366 samples × 3 dilutions per sample), 790 (72%) were negative, 201 (18%) had distinguishable intense red-violet colonies, and 107 (10%) had indistinguishable colonies that resulted in a pink-brown color throughout the plate. For the subset of the data consisting of only Petrifilm with positive results (n = 308), there was a significant difference in the expression of positive results for environmental or milk-based samples (Fig. 2). Most results (77%) of positive environmental samples (n = 213) appeared as distinguishable intense red-violet colonies, whereas only 23% of the positive milk samples (n = 95) had the same appearance ($P < 0.01$). Most (61%) positive results of milk samples were expressed as a pink-brown color throughout the plates (Fig. 2).

DISCUSSION

Results of this study strongly suggest that the contamination of BTM with *L. monocytogenes* originated from a common source. This can be supported by the lack of isolation of this pathogen from environmental samples and yet the isolation of similar strains from milk filters and BTM. It is likely that the source of BTM contamination was either a cow shedding *L. monocytogenes* because of intramammary infection or a localized incubation site within the milking machine. It has been clearly demonstrated that cows can develop chronic intramammary infections caused by *L. monocytogenes (21)*. Such infections may be unnoticed for long periods of time because if a cow is in a subclinical state, its milk appears normal. The longitudinal shedding pattern of infected mammary glands (as measured using the number of colony forming units of bacteria per mL of milk) can also vary substantially over time *(21)*. Thus, depending on the number of bacteria shed in milk on a particular day, the number of cows infected in the herd, and the amount of milk commingled in the bulk tank, an inconsistent pattern of detectable and undetectable levels

FIGURE 2. Results of the Petrifilm Enviromental *Listeria* (PEL) plates for environmental and milk samples. Panel A: Serial dilution (1:2, 1:4, 1:8 from left to right) of a water sample (collected from a trough) whose results were positive. *Listeria monocytogenes* was isolated from the same sample by use of the reference method (broth-agar conventional microbiology). The plates contain both distinct red-violet colonies (considered *Listeria* colonies) and light pink colonies (other bacterial genera). Panel B: Serial dilutions of a bulk tank milk sample that was positive using both methods (conventional microbiology and the PEL system). Most positive milk-based samples appeared as a pink color-throughout the plate, as in the first plate of the series, and transitioned from positive to negative within the series without the appearance of distinct colonies in intermediary dilution steps.



of *L. monocytogenes* in BTM (as observed in this study) may occur. A similar pattern was reported by Latorre et al. *(11)* for BTM collected longitudinally on a single farm.

When in-line sampling was performed, only milk from the hospital group was positive for *L. monocytogenes*. Because this was the last group milked, it was not clear whether a cow was shedding bacteria on that day, or whether contamination occurred from an incubation site within the milking machine, after approximately seven hours of milking. Even though milk culture results were negative for 10 cows that were in the hospital group on the day of in-line milk sampling, five cows had been culled from the herd by the time quarter milk samples were collected; thus, the possibility of a cow being the source of *L. monocytogenes* in the BTM could not be confirmed.

Another possible origin of BTM contamination with *L. monocytogenes* was the inner surface of the milking machine that is constantly exposed to milk. It has been demonstrated that *L. monocytogenes* is capable of forming biofilms on milking equipment (especially in areas of difficult access such as gaskets, dead-ends, and joints), where it may be protected and more resistant to killing by chemicals and by the temperature of sanitizing solutions *(22)*. Latorre et al. (*11*) conducted a longitudinal study on a single farm and isolated *L. monocytogenes* from 20% of 172 BTM samples. The authors concluded that the milking machine was the most likely source of contamination, because strains of *L. monocytogenes* found on the milking machine surfaces were similar to each other and different from strains found in other environmental samples (e.g., feces, bedding and water). In that study, *L. monocytogenes* was isolated from BTM somewhat consistently throughout the course of the study (over three years), whereas in the present study, contamination of BTM ceased upon completion of the second weekly sampling period. It may be hypothesized that contamination originating from the milking machine would have been persistent, because no changes in equipment or cleaning routines were observed during the course of the study. In addition, *L. monocytogenes* was not isolated from any milking machine samples such as liners, or from milk residues on gaskets and other strategic parts that could be a source of this pathogen.

It was notable that the proportion of *L. monocytogenes*-positive samples was about three times greater for milk filters than for BTM. This finding agrees with previous research and suggests that the milk filter is an important point of concentration of this bacterial species. Latorre et al. *(11)* reported that 68% of milk filters were *L. monocytogenes*-positive, as compared to 20% of BTM samples. Likewise, results of the National Animal Health Monitoring System survey *(7)* indicate that 28% of farms had *Listeria*-positive (any *Listeria* species) milk filters, whereas 9% of farms had positive BTM samples. The use of management practices already adopted on many dairies, such as monitoring the cleanliness of milk filters after milking and replacing milk filters during milking, may deserve further evaluation for minimizing BTM contamination with *L. monocytogenes*.

Regardless of the source of contamination, it is important to note that results of this and previous studies *(11, 21)* demonstrate that farms can develop persistent sources of BTM contamination with *L. monocytogenes*. Therefore, longitudinal screening of BTM or milk filters could be valuable for programs developed to improve the safety of milk. Identification of such farms could not only minimize the risk of listeriosis for consumers of unpasteurized dairy products but also prevent colonization of milk processing facilities and further cross-

contamination of pasteurized dairy products.

As a secondary objective, diagnostic test characteristics of the PEL system were assessed, with traditional microbiology as the reference method. While the PEL system correctly identified all *L. monocytogenes* truly positive environmental samples and 75% of the milk-based samples, there were a large percentage of false-positive test results (51% for environmental and 26% for milk-based samples). False positives were expected because the PEL system was developed to identify *Listeria* spp. rather than *L. monocytogenes* alone. In addition, other bacterial genera such as *Bacillus* and *Streptococcus* also produced colonies on PEL that were identical to the intense red-violet *Listeria* colonies.

In certain circumstances, identification of *L. monocytogenes* on dairy products or in dairy processing facilities can result in serious actions taken by regulatory agencies, such as massive product recalls or revocation of the license to commercialize dairy products. Similarly, when the aim of the testing program is longitudinal monitoring of *L. monocytogenes* or investigation of contamination problems on individual dairies, false-positive test results would dramatically overestimate the true prevalence or incidence of this pathogen on samples tested. One solution that could be used to confirm the presence of *L. monocytogenes* in the samples studied is confirmation of PEL-positive samples with traditional or molecular microbiological methods. In the present dataset, 52 and 32% of the environmental and milk samples, respectively, would need to be confirmed by use of a reference test.

Although no false-negative results were observed for environmental samples, 26% of milk-based samples were classified as such. The consequences of false-negative PEL results can also be serious, especially when milk or dairy products are being tested. In this case, truly contaminated samples would be erroneously diagnosed as *L. monocytogenes*-negative, which could increase the risk of human infection.

In contrast to the low PPV observed, results of this study suggest that a negative result obtained with Petrifilm has a high probability of correctly representing a *L. monocytogenes*-negative environmental or milk-based sample. If the prevalence of *L. monocytogenes* is low, this characteristic could be valuable because large number of samples could be screened rapidly and at a low cost. However, the NPV could decrease substantially as the prevalence of *L. monocytogenes* increases.

Another important characteristic of the PEL system was the expression of positive results for different types of samples. Positive results were mostly expressed as a pink-brown cover throughout the plate for milk-based samples (milk filters and milk) whereas most positive results of environmental samples were expressed as distinguishable red-violet colonies. Manufacturer's instructions do not include the use of PEL for samples that contain milk, and it is possible that milk components interfere with the development of typical *Listeria* colonies on the plates. It could be argued that a pink-brown cover on positive plates can be a result of too many overlapping colonies (as described in the Petrifilm instructions manual); however, it was noticed that several milk-based samples shifted from a pink-brown cover to negative (absence of any colonies) between consecutive dilutions without exhibiting distinguishable colonies in intermediary dilution steps. Further research is necessary for evaluating the use of the PEL system for detecting *L. monocytogenes* in samples that contain milk.

CONCLUSIONS

Results of this study strongly suggest that the contamination of BTM with *L. monocytogenes* originated from a common source. Although the source of contamination could not be precisely determined, results indicate that farms can develop persistent sources of contamination. Longitudinal screening programs would be warranted to identify such farms and ensure the safety of milk. The most useful characteristic of the PEL system in detecting *L. monocytogenes* for environmental and milk-based farm samples was its high sensitivity. Limitations such as low specificity and high proportion of false-positive results need to be considered when using the PEL system for on-farm screening of *L. monocytogenes*. Further testing of PEL-positive samples may be necessary to confirm this pathogen in environmental or milk samples.

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REFERENCES

1. Antognoli, M. C., J. E. Lombard, B. A. Wagner, B. J. McCluskey, J. S. v. Kessel, and J. S. Karns. 2009. Risk factors associated with the presence of viable *Listeria monocytogenes* in bulk tank milk from U.S. dairies. *Zoonoses Public Health* 56:77–83.
2. Center for Science in the Public Interest. 2008. Outbreak alert: closing the gaps in our federal food safety net. Available at: http://www.cspinet.org/new/pdf/outbreak_alert_2008_report_final.pdf. Accessed March 10, 2010.
3. Centers for Disease Control and Prevention. 2009. One-day (24–28 h) standardized laboratory protocol for molecular subtyping of *Listeria monocytogenes* by Pulsed Field Gel Electrophoresis (PFGE). Available at:

http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/5.3_2009_PNetStandProtLMonocytogenes.pdf. Accessed January 20, 2011.
4. Centers for Disease Control and Prevention. 2011. Estimates of foodborne illness in the United States. Available at: http://www.cdc.gov/foodborneburden/index.html#. Accessed May 15, 2011.
5. Centers for Disease Control and Prevention. 2011. Listeriosis: Technical information. Available at: http://www.cdc.gov/nczved/divisions/dfbmd/diseases/listeriosis/technical.html. Accessed May 15, 2011.
6. Food and Drug Administration. 2003. *Listeria monocytogenes* risk assessment: I. Introduction. Available at: http://www.fda.gov/Food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm184052.htm. Accessed May 5, 2011.
7. Food and Drug Administration, 2007. Dairy 2007: *Salmonella*, *Listeria*, and *Campylobacter* on U.S. dairy operations, 1996–2007. Available at: http://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_ir_Food_safety.pdf. Accessed May 5, 2011.
8. 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.
9. Hassan, L., H. O. Mohammad, P. L. McDonough, and R. N. Gonzalez. 2000. A cross-sectional study on the prevalence of *Listeria monocytogenes* and *Salmonella* in New York dairy herds. *J. Dairy Sci.* 83:2441–2447.
10. 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.
11. Latorre, A. A., J. A. S. v. 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.
12. Mandell, G. L., J. E. Bennett, and R. Dolin (ed.). 2004. Mandell, Douglas and Bennett's principles and practice of infectious diseases. 6th ed. Churchill Livingstone, Philadelphia, PA.
13. Pagano, M., and K. Gauvreau. 2000. Principles of biostatistics. 2nd ed. Duxbury Press, Belmont, CA.
14. Radostitis, O. M., C. C. Gay, K. W. Hinchcliff, and P. D. Constable (ed.). 2007. Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats, 10th ed. W. B. Saunders, Edinburgh.
15. Ryser, E. T. 2001. Public health concerns, p. 397–545. *In* E. H. Smith and J. L. Steele (ed.). *Applied Dairy Microbiology.* 2nd ed. Marcel Dekker, Inc., New York.
16. 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.
17. SAS Institute. 2009. SAS/STAT 9.2 User's Guide. 2nd ed. SAS Institute Inc., Cary, NC.
18. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233–2239.
19. van Kessel, J. S., J. S. Karns, L. Gorski, B. J. McCluskey, and M. L. Perdue. 2004. Prevalence of salmonellae, *Listeria monocytogenes*, and fecal coliforms in bulk tank milk on U.S. dairies. *J. Dairy Sci.* 87: 2822–2830.
20. Winn, W. C., S. D. Allen, W. M. Janda, E. W. Koneman, G. W. Procop, P. C. Schreckenberger, and G. L. Woods. 2006. Aerobic and facultative gram-positive bacilli, p. 765–857. *In* Koneman's color atlas and textbook of diagnostic microbiology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
21. Winter, P., F. Schilcher, Z. Bago, D. Schoder, M. Egerbacher, W. Baumgartner, and M. Wagner. 2004. Clinical and histopathological aspects of naturally occurring mastitis caused by *Listeria monocytogenes* in cattle and ewes. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* 51:176–179.
22. Wong, A. C. L. 1998. Biofilms in food processing environments. *J. Dairy Sci.* 81:2765–2770.
23. Zadoks, R. N., B. E. Gillespie, H. W. Barkema, O. C. Sampimon, S. P. Oliver, and Y. H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. *Epidemiol. Infect.* 130:335–349.