Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*

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ABSTRACT

The objective of this study was to assess the presence of a *Listeria monocytogenes*-containing biofilm in milking equipment as a potential source of bulk tank milk contamination on a dairy farm where milk contamination had been previously documented. Samples were collected from milking equipment and milking parlor premises on 4 occasions and analyzed for the presence of *L. monocytogenes*. Pulsed-field gel electrophoresis (PFGE) typing was conducted on *L. monocytogenes* isolates from the milking equipment, parlor and storage room floors, bulk tank milk, and in-line milk filters. Pieces from milk meters and rubber liners were obtained to visually assess the presence of a biofilm using scanning electron microscopy. A total of 6 (15%), 4 (25%), and 1 (6%) samples were culture-positive for *L. monocytogenes* in the first, second, and third sample collection, respectively. Two samples were *L. monocytogenes hly* PCR-positive but were culture-negative in the fourth sample collection. Combined *AscI* and *ApaI* restriction analysis yielded 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment, parlor, bulk tank milk, and milk filters. A predominant and persistent PFGE type (PFGE type T) was observed among these *L. monocytogenes* isolates (9/15 isolates). Scanning electron microscopy of samples from the bottom cover of 2 milk meters showed the presence of individual and clusters of bacteria, mainly associated with surface scratches. The presence of a bacterial biofilm was observed on the bottom covers of the 2 milk meters. Prevention of the establishment of biofilms in milking equipment is a crucial step in fulfilling the requirement of safe, high-quality milk.

Key words: biofilm, raw milk, milking equipment, *Listeria monocytogenes*

INTRODUCTION

The safety of milk is an important attribute for consumers of milk and dairy products. Milk pasteurization safeguards consumers from many potential foodborne hazards in milk and milk products. Despite the pasteurization process, the quality and safety of raw milk are important in reducing the risk of foodborne diseases associated with milk because raw milk is the starting point of the milk production-consumption chain.

Dairy products contaminated with *Listeria monocytogenes* have been responsible for human listeriosis outbreaks (Dalton et al., 1997; Centers for Disease Control and Prevention, 2008). The total number of listeriosis cases reported in the United States was 808 and 656 in 2007 and 2008, respectively (Centers for Disease Control and Prevention, 2009). The serious consequences of listeriosis, such as a septicemic form of the illness in elderly and immunocompromised people, and abortion in pregnant women or death of their newborn, constitute a serious threat to public health.

The presence of biofilms has been well documented in the food industry (Carpentier and Cerf, 1993) and these biofilms are a potential source of bacterial contamination. *Listeria monocytogenes* has the potential to form biofilms on materials such as stainless steel (Norwood and Gilmour, 1999; Beresford et al., 2001), rubber, or plastic (Beresford et al., 2001), and these materials are frequently found in milk handling equipment, milk lines, or milk tanks. The ability of *L. monocytogenes* to form biofilms (Harvey et al., 2007) may contribute to its persistence in food processing plants (Thimothe et al., 2004).

Previous biofilm studies have primarily been conducted in processing plants and little is known about the presence of biofilms on dairy farms. The presence of *Listeria*-containing biofilms in milking equipment has not yet been reported. However, previous work suggested biofilms in the milking equipment as a possible source of persistent *L. monocytogenes* contamination

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of bulk tank milk (**BTM**) (Latorre et al., 2009). This previous study demonstrated the frequent presence of *L. monocytogenes* in BTM and in in-line milk filter samples collected from a single farm between 2004 and 2007. Subsequent samplings indicated that *L. monocytogenes* was still frequently isolated from BTM and in-line milk filter samples collected until the end of the present study (March 2008; data not shown). The objective of this study was to assess the presence of *L. monocytogenes*-containing biofilm in milking equipment as a potential source of BTM contamination on a dairy farm.

MATERIALS AND METHODS

Study Farm

The study was conducted on a single 330-cow dairy farm in New York State (Latorre et al., 2009; Pradhan et al., 2009). The farm has an average milk production of approximately 9,071 kg of milk/d and the milk is transported daily to a milk processing plant and subsequently pasteurized. The cows are milked 3 times/d at an interval of approximately 8 h in a double 8 herring-bone milking parlor. Plastic (polysulfone) milk meters were installed in May 2005. Rubber liners were replaced every 2 wk (approximately every 928 milkings).

Routine washes of the milking machine and milk line were carried out after each milking using the following protocol: 1) prerinse cycle with water, 2) wash cycle using a cleaning product with potassium hydroxide, polyphosphates, and sodium hypochlorite as active ingredients, and 3) acid wash cycle using a clean-in-place (**CIP**) acid cleaner (phosphoric and sulfuric acids as active ingredients). The milking equipment was sanitized with a sodium hypochlorite solution immediately before every milking. Milk tank washes were carried out every 24 h using the same CIP protocol described above. Time and temperatures of the pipeline and bulk tank washes were monitored and recorded by Milk-Guard (Dairy Check Inc., Ontario, Canada).

Weekly tests to monitor SCC, SPC, and preliminary incubation count (**PIC**) in BTM samples were conducted from February 2004 until April 2008. The analysis of BTM samples was performed as described by Jayarao et al. (2004). A DeLaval cell counter (DeLaval International AB, Tumba, Sweden) was used for SCC determination.

Sample Collection

Sponge-swab samples from the inner surface of milking equipment and the parlor environment were collected on 4 occasions using BactiSponge kits (Hardy Diagnostics, Santa Maria, CA) as described previously

(Thimothe et al., 2004; Latorre et al., 2009). For the first sample collection, 40 sampling sites were selected based on the criteria described previously (Latorre et al., 2009). Samples were obtained in May 2007 from the sites described in Table 1. Based on the results obtained in the first sample collection, in January 2008 swab samples were collected from all milk meters and all individual rubber liners (second collection; Table 1). Based on the results obtained in previous sample collections, a resampling of all milk meters was carried out in February 2008 (third collection; Table 1). Two sets of swab samples were obtained in March 2008 from the bowl of 2 milk meters (fourth collection). One set of sponges was used for culture of *L. monocytogenes* and the other was used for nonselective enrichment with brain heart infusion broth (**BHI**; BD Diagnostics, Sparks, MD) for further analysis by PCR (Table 1).

All samples were transported on ice overnight to the laboratory. A summary of sample sources, number of samples, and collection dates is presented in Table 1.

Laboratory Procedures

Listeria monocytogenes Analysis. *Listeria monocytogenes* analysis was conducted on all sponge samples that were collected (Table 1). For this, 20 mL of 1% buffered peptone water (BD Diagnostics) was added to the Whirl-Pak bags (included in BactiSponge kits; Hardy Diagnostics) containing the sampling sponge. The Whirl-Pak was put into a stomacher bag (GSI Creos Corporation, Tokyo, Japan) and pummeled 3 or 4 times for 30 s in an automatic bag mixer (BagMixer Interscience Laboratories Inc., Weymouth, MA). Then, 5 mL of the extract was added to 5 mL of double-strength modified *Listeria* enrichment broth (BD Diagnostics). Enrichments were incubated at 37°C for 48 h. Enriched broth (10 µL) was streaked onto modified Oxford agar plates (Difco Laboratories, Detroit, MI), incubated at 37°C, and read at 24 and 48 h for presumptive *Listeria* colonies as described previously (Van Kessel et al., 2004). Presumptive *Listeria* colonies were further analyzed as described by Van Kessel et al. (2004) and Latorre et al. (2009).

Pulsed-Field Gel Electrophoresis Typing. Pulsed-field gel electrophoresis (**PFGE**) typing was conducted on 1 *L. monocytogenes* isolate from each positive sample obtained from the milking equipment (n = 9), floors in the parlor (n = 1), and storage room (n = 1). Pulsed-field gel electrophoresis typing was also done on *L. monocytogenes* isolates from select BTM and milk filter samples, which were collected weekly as part of another ongoing study (data not shown).

The PFGE typing of *L. monocytogenes* isolates was done following the Centers for Disease Control and Pre-

Table 1. Summary of sample sources, number of samples obtained, and date of sample collections to milking parlor room and milking equipment¹

Sample source	May 2007	January 2008	February 2008	March 2008
Drains in parlor room	2	NC ²	NC	NC
Floor in parlor pit	3	NC	NC	NC
Milk pipelines	7	NC	NC	NC
Teat cup rubber liners	4	64	NC	NC
Milk meters before washing	8	NC	NC	NC
Milk meters after washing	8	16	16	2 ³
Milk tank outlet	1	NC	NC	NC
Floor under milk tank	1	NC	NC	NC
Milk pump surface	1	NC	NC	NC
Vacuum pump surface	1	NC	NC	NC
Floor in washing room	1	NC	NC	NC
Floor in storage area	1	NC	NC	NC
Drains in washing room	2	NC	NC	NC
Total	40	80	16	2

¹All samples collected were cultured to assess the presence of *Listeria monocytogenes*.

²NC = no samples were collected.

³Milk meters that tested *Listeria* spp.- or *Listeria monocytogenes*-positive on the third sample collection. These milk meters were selected for further analysis by scanning electron microscopy and *hly*-PCR.

vention (2004) Pulsenet Protocol with modifications, as described previously (Latorre et al., 2009). Analysis of DNA band patterns of *AscI* and *Apal* digestions was carried out as described by Latorre et al. (2009).

Scanning Electron Microscopy Analysis. In March 2008, pieces from milk meters and rubber liners were obtained to assess the presence of a biofilm using scanning electron microscopy (SEM). Selection of the units 3-left and 8-left (numbers indicate the number assigned to each of the milking units tested; left and right indicate the position of the units in the parlor) was based on the presence of *L. monocytogenes* and *Listeria* spp., respectively, in samples collected on the third sample collection. Immediately after the routine washing cycle was finished, the top and bottom cover of the selected milk meters as well as 3 rubber liners (from unit 3-left) were aseptically removed. Samples were individually placed in zip-lock bags, labeled, and immediately transported on ice to the laboratory for processing (transport time of approximately 3.5 h).

The covers of milk meters were aseptically cut in half using a junior hacksaw (Task Force, Mineola, NY) with steam-sterilized 15-cm hacksaw blades (Task Force). After cutting, the internal surface of the pieces was stained using an aqueous solution of 0.1% Alcian blue 8GX (Sigma-Aldrich, St. Louis, MO; Rayner et al., 2004). Then, the samples were washed 3 times with sterile distilled water and immediately transported for additional cutting using a Bridgeport 39196 milling machine (Bridgeport Inc., Bridgeport, CT). For this purpose, a steam-sterilized 25-mm hole saw (Lenox, East Longmeadow, MA) was used. Samples (25 mm × 25 mm) were aseptically obtained from each cover of the milk meters (top and bottom) and placed into a sterile Petri dish.

Rubber liners were cut into pieces that would fit into 25 mm × 25 mm SEM specimen mounts using sterile surgical scissors. The liner pieces were stained with an aqueous solution of 0.1% Alcian blue 8GX, as described above, and placed into sterile Petri dishes.

Milk meter and liner parts were transported to the Cornell Integrated Microscopy Center (Ithaca, NY) for SEM. The parts were immediately put in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) and stored overnight at 4°C. Samples were then washed 3 times in 0.1 M sodium cacodylate buffer (pH 7.0) at 4°C, placed in a 1% buffered solution of osmium tetroxide at 4°C, and held overnight. The samples were washed 3 times in water at 4°C and then gradually dehydrated in a graded series of 10, 30, and 50% ethanol (10 min at each step). Following the dehydration step, the samples were soaked in 2% uranyl acetate in 70% ethanol for 20 min. Dehydration was continued in 90% ethanol followed by 3 changes of 100% ethanol. The samples were then critical point dried in a Bal-Tec Critical Point dryer (model 030, Bal-Tec Inc., Brookline, NH), attached to a specimen support, and coated with a gold and palladium target using a Bal-tec SCD sputter coater (model 050; Bozzola and Dee Russell, 1999). Samples were then viewed using a Hitachi S4500 scanning electron microscope using 3 KV (Hitachi High Technologies America Inc., Electron Microscope Division, Pleasanton, CA).

PCR Analysis. For nonselective enrichment, 20 mL of BHI was added to each of the sponges. The sponges were manually pummeled and 5 mL of the extract was put into 5 mL of double-strength BHI (in triplicate for each sponge) and incubated at 37°C for 48 h. After incubation, 1.5 mL of each of the enrichments was centrifuged and the pellet was removed, put in a Microbank

vial (Pro-Lab Diagnostics, Austin, TX), and stored at -80°C until PCR analysis.

For lysate preparation, enrichments stored at -80°C were put in 5 mL of BHI broth and incubated overnight at 37°C . Bacterial lysates were prepared as described by Furrer et al. (1991) with minor modifications (M. Wiedmann, Cornell University, Ithaca, NY; personal communication). Briefly, overnight cultures were vortexed and 250 μL was removed and centrifuged for 10 min at $15,000 \times g$. The pellet was resuspended in 95 μL of $1\times$ PCR buffer (Roche Diagnostics, Indianapolis, IN). Four microliters of lysozyme (50 mg/mL) was added. After incubation, 1 μL of proteinase K (20 mg/mL) was added and suspensions were incubated in a heating block for 60 min at 58°C , followed by a final incubation step of 8 min at 95°C . Lysates were centrifuged for 1 min at $15,000 \times g$ and stored at -20°C until analysis.

Each PCR reaction contained 10.25 μL of nuclease-free water, 12.50 μL of Go-Taq Green (Promega, Madison, WI), 0.125 μL of *hly* primers F (5' TCC GCA AAA GAT GAA GTT C'3) and R (5' ACT CCT GGT GTT TCT CGA TT'3) (Jothikumar et al., 2003), and 2 μL of the lysate template. *Listeria monocytogenes* FSL K2-017 (information available at www.pathogen-tracker.net) was used as a positive control. *Escherichia coli* ATCC 25922, BHI, and nuclease-free water were used as negative controls. Polymerase chain reaction conditions as described by Jothikumar et al. (2003) were used with the following modifications: initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. Electrophoresis conditions, gel stain and destain, and image capture were done according to standard procedures.

Data Analysis

For statistical analysis and graphics of SPC and PIC data, all bacterial counts reported as $>1,000,000$ cfu/mL were truncated at 1,000,000 cfu/mL. Statistical analysis and graph of SCC were carried out using the data as reported by the cell counter. Statistical analysis of SCC, SPC, and PIC data was done using the software JMP 7.0 (SAS Institute Inc., Cary, NC). Graphs were generated using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA).

RESULTS

BTM Quality Parameters

A total of 193 BTM samples were analyzed between February 2004 and April 2008 to assess SCC, whereas

196 samples were analyzed to assess the total number of aerobic bacteria and PIC. The geometric means of SCC, SPC, and PIC were 233,301 cells/mL, 5,109 cfu/mL, and 18,778 cfu/mL, respectively. The trends in SCC, SPC, and PIC from January 2004 to April 2008 are shown in Figure 1.

Assessment of the Presence of *Listeria monocytogenes* in Parlor and Milking Machines

In the first sampling (May 2007), *L. monocytogenes* was isolated from 2 out of 10 samples from the milk house environment. These positive samples corresponded to floors in the parlor pit and storage area. One sample collected from the bulk tank outlet was positive for *L. monocytogenes*, as was 1 set of rubber liners (i.e., 4 liners in 1 cluster). In the second sample collection (January 2008), 5 individual rubber liners (from 4 milking units) were positive for *Listeria* spp. but none of the rubber liners were positive for *L. monocytogenes*. A summary of the milk meters culture results for the 4 sample collections is presented in Table 2.

PFGE Typing

Combined AscI and ApaI restriction analysis showed 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment (9 isolates), floors in the parlor and storage room (2 isolates), BTM (2 isolates), and in-line milk filters (2 isolates). Pulsed-field gel electrophoresis banding patterns using AscI for 12 of these isolates are shown in Figure 2 and a dendrogram based on the combined AscI and ApaI digestion profiles for all isolates is shown in Figure 3.

Pulsed-field gel electrophoresis type T was observed in 2 *L. monocytogenes* isolates obtained from BTM and milk filter samples that were collected approximately 1 wk before the first sample collection (May 2007) from the milking parlor and milking equipment. The same PFGE type T was subsequently found in a BTM sample received in the laboratory 1 d before the sampling of the parlor, as well as in the bulk tank outlet and in one of the milk meters sampled on the first sample collection (milk meter 2-right). In addition, *L. monocytogenes* PFGE type T was isolated from 3 milk meters in the second sample collection (January 2008; milk meters 3-left, 4-left, and 3-right) and from a milk meter at the third sampling (February 2008; milk meter 3-left). The PFGE type U, closely related (approximately 96.5% similarity) to PFGE type T (Tenover et al., 1995), was isolated from a milk meter in January 2008 as well (milk meter 8-left). Closely related *L. monocytogenes* PFGE types D and E (92.5% similar to each other) were isolated from one of the milk meters (milk meter

Table 2. Summary of milk meters (MM) culture results for *Listeria* spp. and *Listeria monocytogenes*

Description ¹	May 2007	January 2008	February 2008	March 2008
MM 1-left	<i>Listeria</i> spp. ²	<i>Listeria</i> spp.	— ³	NC ⁴
MM 2-left	NC	<i>Listeria</i> spp.	—	NC
MM 3-left	—	<i>L. monocytogenes</i>	<i>L. monocytogenes</i>	— +
MM 4-left	NC	<i>L. monocytogenes</i>	—	NC
MM 5-left	<i>L. monocytogenes</i>	<i>Listeria</i> spp.	—	NC
MM 6-left	NC	<i>Listeria</i> spp.	—	NC
MM 7-left	—	<i>Listeria</i> spp.	—	NC
MM 8-left	NC	<i>L. monocytogenes</i>	<i>Listeria</i> spp.	— +
MM 1-right	NC	<i>Listeria</i> spp.	—	NC
MM 2-right	<i>L. monocytogenes</i>	<i>Listeria</i> spp.	—	NC
MM 3-right	NC	<i>L. monocytogenes</i>	—	NC
MM 4-right	—	<i>Listeria</i> spp.	—	NC
MM 5-right	NC	<i>Listeria</i> spp.	—	NC
MM 6-right	<i>Listeria</i> spp.	<i>Listeria</i> spp.	—	NC
MM 7-right	NC	—	—	NC
MM 8-right	—	—	—	NC

¹Numbers from 1 to 8 indicate the number assigned to each of the milking units tested. Left and right indicate the position of the units in the parlor.

²Including *Listeria monocytogenes*.

³— = culture negative for *Listeria* spp.; + = *hly*-PCR positive for *Listeria monocytogenes*.

⁴NC = no samples were collected.

5-left) and in a rubber liner analyzed on May 2007 (Figure 3). A PFGE type D was also isolated from a milk filter sample that was collected 1 wk after this sampling. Pulsed-field gel electrophoresis types X and

Y were isolated from floor samples in the parlor and storage room during the first sample collection from parlor and milking equipment. The similarity between these PFGE types was only 76%, and types X and Y

Figure 1. Bulk tank SCC, SPC, and preliminary incubation counts (PIC) over a period of 4.25 yr (February 2004–April 2008).

Figure 2. *Listeria monocytogenes* pulsed-field gel electrophoresis types and subtypes among isolates obtained between May 2007 and February 2008 using restriction endonuclease *AscI*. The first and second rows on the bottom indicate the sample identification and source: bulk tank milk (BTM), bulk tank outlet (BTO), rubber liner (LN), milk meter (MM) with corresponding milking unit number and position in the parlor (R = right, L = left), floor in the parlor room (FPR), and floor in the storage room (FSR). Sampling date (month-day) and year are shown on the third and fourth (bottom) rows, respectively. Lanes 1, 8, and 15 contain the standard *Salmonella enterica* serotype Braenderup (SB). Pulsed-field gel electrophoresis profiles of *L. monocytogenes* isolates L1-027, L1-020, L1-018, L1-028, and L1-029 were described previously by Latorre et al. (2009).

were remarkably different (approximately 57.5% similarity) from the other *L. monocytogenes* PFGE types observed in this study.

SEM

Deep scratches in the inner surface of the milk meters were readily observed with no magnification when cutting the parts for SEM analysis. Scanning electron microscopy of a sample from the bottom cover of milk meter 3-left showed the presence of numerous bacteria, mainly associated with surface scratches (Figure 4). Bacteria were also observed on surface scratches on the bottom cover of milk meter 8-left, but in fewer number than on milk meter 3-left.

A bacterial biofilm was observed on the bottom cover of the 2 milk meters analyzed (Figure 5 and 6). Only a few bacterial cells were observed in the top cover of milk meters 3-left and 8-left. Scanning electron microscopy of a rubber liner sample showed the presence of cracks and of areas containing patches of a foreign

material on the surface. No bacteria were observed in the microscopy fields of the sample that was analyzed.

PCR

The *hly* gene was detected in the BHI enrichments of the 2 sponge samples obtained from the bowl component of milk meters 3-left and 8-left. The 3 enrichments from milk meter 3-left were *hly* PCR-positive. For milk meter 8-left, 2 of 3 BHI enrichment replicates were *hly*-positive.

Analysis of CIP Temperatures for Milk Pipelines and Milk Tank

The pipeline and milk tank washing cycle temperatures collected and stored by the MilkGuard equipment during an approximately 1.7-mo period (between September and October 2007) were evaluated. The recorded peak temperature of drainage water in the routine pipeline wash cycles never exceeded 53°C (approximately)

Figure 3. Dendrogram of *Listeria monocytogenes* isolates obtained from bulk tank milk, milking equipment, and floors in the parlor and storage rooms. In the dendrogram, milk meter numbers 1, 2, 3, 4, 5, and 6 correspond to milk meters 2-right, 5-left, 3-left, 4-left, 8-left, and 3-right, respectively. The dendrogram is based on the combined analysis of the AscI and ApaI digestion profiles using a cutoff of 100%. Pulsed-field gel electrophoresis (PFGE) types assigned by automated cluster analysis (ACA) of the combined AscI and ApaI restriction digest profiles are shown at the right of the dendrogram. Pulsed-field gel electrophoresis profiles of *L. monocytogenes* isolates L1-027, L1-020, L1-018, L1-028, and L1-029 were described previously by Latorre et al. (2009). QMP = Quality Milk Production identification.

during this 1.7-mo monitoring period. For milk tank washes, the recorded temperatures never went above 47°C (approximately) during the monitoring period.

DISCUSSION

Previously reported research on the attachment of bacteria to dairy equipment surfaces involved in vitro studies (Speers et al., 1984) or insertion of pieces of pipeline (Austin and Bergeron, 1995) or gaskets (Czechovski, 1990; Austin and Bergeron, 1995) into the milk line. To our knowledge, this is the first study that evaluated the presence of biofilms in milking equipment from samples removed directly from pieces of milking equipment that had been in use on an operating farm for almost 3 yr. In this study, despite the established CIP procedures, a bacterial biofilm as well as numerous bacteria were found attached to the surface of the milk meters as evidenced by the SEM micrographs. Bacteria were especially prominent in scratches on the inner surfaces of the plastic milk meters.

According to information provided by the producer, the milk meters were cleaned using an abrasive device

(a wire brush) in 2006. Scratches on the surface of the milk meters could have facilitated the attachment and colonization of bacteria because roughness could limit the effectiveness of the CIP procedures. In a study of cleaning protocols on stainless steel surfaces, Wirtanen et al. (1995) demonstrated that smooth surfaces are easier to clean than rough surfaces. Additionally, the accumulation of milk residues on the surfaces of milking equipment may contribute to subsequent bacterial proliferation (Murphy and Boor, 2000).

The visualization of bacteria attached to the plastic surface by means of an apparent exopolymeric matrix (Carpentier and Cerf, 1993) indicated the presence of a biofilm in 2 milk meters. Rod-shaped bacteria were observed in SEM micrographs of the biofilm. The morphology and size were similar to *L. monocytogenes* on in vitro biofilms (see Figure 1 in Chavant et al., 2004). Using the presented methods, however, it was not possible to definitively identify *L. monocytogenes* among the bacteria present in the observed biofilms.

Listeria monocytogenes was isolated from samples obtained from milk meters, a teat cup liner, and bulk tank outlet. The PFGE type T persisted in milking

Figure 4. Scanning electron microscopy image of scratches on the surface of the bottom cover of milk meter 3-left (scale: 1,000 nm). Arrows indicate the presence of bacteria associated with these scratches in the plastic material.

equipment over a 9-mo period, suggesting the presence of a persistent source of *L. monocytogenes*, consistent with our biofilm hypothesis. In addition, the large heterogeneity of PFGE types among *L. monocytogenes* isolates from fecal and environmental samples compared with the more limited heterogeneity in PFGE types in isolates from BTM, milk filter, milking equipment, and bulk tank outlet samples on this study farm (Latorre et al., 2009) also support the biofilm hypothesis.

In our study, although the presence of *L. monocytogenes* or bacterial biofilms could not be directly microscopically assessed on bulk tank surfaces, an *L. monocytogenes* isolate obtained from a bulk tank outlet sample showed the same PFGE type T that was found in milk meters and BTM.

The continuous sloughing of cells from a biofilm could explain the presence of 3 persistent *L. monocytogenes* PFGE types in samples of BTM and in-line milk filters collected over a period of 22 mo (Latorre et al., 2009).

During 1.7 mo of monitoring, the bulk tank washing temperatures were below the temperatures recommended by the manufacturer (73°C). Inappropriate temperatures during the cleaning cycle of the bulk tank may make the removal of milk residues difficult (Na-

tional Mastitis Council, 2004). Accumulation of organic material debris in the milking machines may create appropriate conditions for bacterial growth (Murphy and Boor, 2000) and could also help in the attachment of bacteria by creating a conditioning film (Zottola and Sasahara, 1994). High SPC and PIC counts in BTM on this farm could be explained by deficiencies in washing of the milking equipment (Murphy and Boor, 2000; Jayarao et al., 2004). This problem may have been further compounded by the presence of scratches on the surface of milk meters.

The presence of biofilms in dairy equipment may be a relevant finding for many dairy farms because CIP temperatures that are lower than the temperatures recommended by the equipment manufacturers are frequently observed (Elmoslemany et al., 2009). In addition, the wear of materials in the milking equipment that causes the appearance of cracks and crevices (Czechovski, 1990), or bacterial contamination during milking, could contribute to the presence of bacteria in milk (Murphy and Boor, 2000). And if these organisms present in milk find favorable conditions, they could eventually form a biofilm in the milking system. In the case of *L. monocytogenes*, the presence of water,

nutrients, and cold temperatures found in bulk tanks provide favorable conditions for the organism not only to survive but to replicate. *Listeria monocytogenes* cells could then attach to the stainless steel (Norwood and Gilmour, 1999) and establish as a biofilm, causing subsequent continuous contamination of milk.

Raw milk from this particular farm is hauled to a milk processing plant for pasteurization and so does not present a risk to consumers. Nevertheless, raw milk

contaminated with *L. monocytogenes* could pose a human health hazard, especially among consumers of raw milk. *Listeria monocytogenes* has been found several times during the past few years in milk from farms that sell certified raw milk in New York State (<http://www.agmkt.state.ny.us/AD/alertList.asp>). Additionally, studies have shown that consumption of raw milk is not uncommon among dairy farm personnel (Jayarao et al., 2006), which could put them at risk for listeriosis

Figure 5. A) Scanning electron microscopy image of a biofilm on the surface of the bottom cover of milk meter 3-left (scale: 2,000 nm). Arrows show the presence of exopolymeric matrix. Panels B and C show 2 different areas of this biofilm that are magnified. B) Close-up of different types of bacteria in the biofilm (scale: 1,000 nm). C) Arrows show the presence of exopolymeric matrix anchoring the bacteria to the surface of the milk meter (scale: 1,000 nm).

Figure 6. Scanning electron microscopy image of the bottom cover of milk meter 8-left showing a cluster of bacteria covered by exopolymeric matrix and attached to the surface of the milk meter.

if the raw milk at the farm is contaminated with *L. monocytogenes*.

Listeria present in raw milk could also pose a risk of contamination for a milk processing plant (Waak et al., 2002). The presence of *L. monocytogenes* in a processing plant could lead to postprocessing contamination, which also draws attention to the need to reduce the level of contamination of milk that will eventually be transported to a milk processing plant.

Prevention of biofilm establishment in milking equipment is a crucial step in fulfilling the requirement of safe, high-quality milk. Hygiene in the milking routine, correct implementation of milking equipment cleaning protocols (following manufacturer recommendations on duration, chemicals, and temperature), and replacing plastic and other materials in milking equipment that are susceptible to wear on a regular basis would help to prevent the establishment of biofilms and subsequent contamination of the bulk milk.

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