Molecular Subtyping and Tracking of *Listeria monocytogenes* in Latin-Style Fresh-Cheese Processing Plants

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ABSTRACT

Latin-style fresh cheeses, which have been linked to at least 2 human listeriosis outbreaks in the United States, are considered to be high-risk foods for *Listeria monocytogenes* contamination. We evaluated *L. monocytogenes* contamination patterns in 3 Latin-style fresh-cheese processing plants to gain a better understanding of *L. monocytogenes* contamination sources in the manufacture of these cheeses. Over a 6-mo period, 246 environmental samples were collected and analyzed for *L. monocytogenes* using both the Food and Drug Administration (FDA) method and the Biosynth *L. monocytogenes* detection system (LMDS). Finished cheese samples from the same plants (n = 111) were also analyzed by the FDA method, which was modified to include *L. monocytogenes* plating medium (LMPM) and the *L. monocytogenes* confirmatory plating medium (LMCM) used in the LMDS method. *Listeria monocytogenes* was detected in 6.3% of cheese and 11.0% of environmental samples. Crates, drains, and floor samples showed the highest contamination rates, with 55.6, 30.0, and 20.6% *L. monocytogenes* positive samples, respectively. Finished products and food contact surfaces were positive in only one plant. The FDA method showed a higher sensitivity than the LMDS method for detection of *L. monocytogenes* from environmental samples. The addition of LMPM and LMCM media did not further enhance the performance of the FDA method for *L. monocytogenes* detection from finished products. Molecular subtyping (PCR-based allelic analysis of the virulence genes *actA* and *hly* and automated ribotyping) was used to track contamination patterns. Ribotype DUP-1044A, which had previously been linked to a 1998 multistate human listeriosis outbreak in the United States, was the most commonly identified subtype (20/36 isolates) and was isolated from 2 plants. This ribotype was persistent and widespread in one factory, where it was also responsible for the contamination of finished products.

We hypothesize that this ribotype may represent a clonal group with a specific ability to persist in food processing environments. While previous listeriosis outbreaks were linked to Latin-style fresh cheeses made from unpasteurized milk, the presence of this organism in pasteurized cheese products illustrates that persistent environmental contamination also represents an important source of finished product contamination.

(Key words: *Listeria monocytogenes*, Latin-style fresh cheese, molecular subtyping)

Abbreviation key: BCM = biosynth chromogenic medium, BHI = brain heart infusion, CDC = Centers for Disease Control and Prevention, EB = enrichment broth, LMCM = *Listeria monocytogenes* confirmation media, LMDS = *L. monocytogenes* detection system, LMPEB = *L. monocytogenes* pre-enrichment broth, LMPM = *L. monocytogenes* plating medium, PBBA = purple broth base agar, PFGE = pulsed-field gel electrophoresis, PI-PLC = phosphatidylinositol phospholipase C, RAPD = random amplification of polymorphic DNA, RTE = ready-to-eat.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that causes serious invasive disease in humans. The Centers for Disease Control and Prevention (CDC) estimate that *L. monocytogenes* causes about 2500 cases and 500 deaths annually in the United States (Mead et al., 1999). A variety of different food products, including dairy products, have been implicated in human listeriosis cases and outbreaks (Fleming et al., 1985; Azadian et al., 1989; Bille, 1990; McLauchlin et al., 1990; Farber and Peterkin, 1991; Dalton et al., 1997). In particular, Latin-style fresh cheeses were responsible for a large human listeriosis outbreak in California in 1985 (Linan et al., 1988) and more recently, for an outbreak in North Carolina (Anonymous, 2001). Beyond these outbreaks, *L. monocytogenes* also has been isolated from Latin-style fresh cheeses from many different countries (Destro et al., 1991; Genigeorgis et al., 1991; El Marrakchi et al., 1993; Silva et al., 1998; Saltijeral et al., 1999). The term, "Latin-style fresh cheese" represents a heter-

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ogenous group of white, unripened soft cheeses, typically bearing between 1.0 to 3.0% salt (Torres and Chandan, 1981). The Latin-style cheese industry is a rapidly expanding segment of the US dairy industry; production of these products increased from 67 million lbs. in 1996 to 96 million lbs. in 2000 (USDA-NASS, 2002). This sector of the cheese industry is largely comprised of small- and medium-sized operations. For many of these operations, the owners and employees have had little previous commercial food processing experience. Control of *L. monocytogenes* in the manufacture of Latin-style fresh cheeses represents a critical need in the US dairy industry.

Product contamination with *L. monocytogenes* results in serious economic consequences for food processors, including costly product recalls and lawsuits. Dairy products represented the food category most frequently associated with recalls due to microbial contamination reported to the FDA during fiscal years 1994 to 1998 (Wong et al., 2000). During this period, dairy product recalls accounted for 304 (22%) of all food recalls, with *L. monocytogenes* isolated from 64.5% of these products. Since pasteurization efficiently kills *L. monocytogenes*, contamination of finished dairy products, as well as of other ready-to-eat (RTE) food products, appears most commonly to originate from postprocessing environmental sources. As *L. monocytogenes* is regularly isolated from food and dairy processing environments (Cox et al., 1989; Klausner and Donnelly, 1991; Jacquet et al., 1993; Pritchard et al., 1995; Sutherland and Porritt, 1996), a better understanding of the spread and survival of *L. monocytogenes* in food processing environments and of specific contributions of environmental sources to finished product contamination are needed to develop improved *Listeria* control strategies.

Listeria monocytogenes isolation and subtyping represent 2 critical steps in characterizing environmental contamination patterns. Classical culture-based methods for isolating *L. monocytogenes* are time consuming, typically requiring 5 to 7 d. Recently, differential media based on detection of phosphatidylinositol phospholipase C enzyme (PI-PLC) activity have been described and evaluated for isolation of *L. monocytogenes* from food and environmental samples (Restaino et al., 1999; Karpšková et al., 2000; Hoffman and Wiedmann, 2001). The PI-PLC is an enzyme that is only produced by *L. monocytogenes* and *Listeria ivanovii*. *Listeria ivanovii* is uncommon in foods and food-processing environments (Arimi et al., 1997) and can easily be differentiated from *L. monocytogenes* using biochemical tests. The Biosynth *L. monocytogenes* detection system (LMDS) contains fluorogenic and chromogenic PI-PLC substrates in both the selective enrichment and plating media, which can enable more rapid screening and dif-

ferentiation of *L. monocytogenes*. The Biosynth-LMDS method should thus allow for more rapid and sensitive detection of *L. monocytogenes* (Restaino et al., 1999) as compared with the FDA method, which includes the selective media Oxford and PALCAM, which are not differential for *L. monocytogenes*. Molecular typing methods, such as pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and ribotyping, provide appropriate tools for tracking bacterial contamination sources throughout food systems (Autio et al., 1999; Johansson et al., 1999; Miettinen et al., 1999; Norton et al., 2001; Hoffman et al., 2003) and also are increasingly used by public health laboratories to detect and track human listeriosis outbreaks (Jacquet et al., 1995; Dalton et al., 1997).

The major goal of this research was to explore the use of different detection strategies and molecular subtyping methods to characterize *L. monocytogenes* contamination patterns in Latin-style fresh-cheese processing plants. Our results show that persistent environmental contamination in the processing plant represents a major source of finished product contamination. The persistent presence of *L. monocytogenes* in these operations demonstrates the importance of designing and implementing effective strategies for eliminating environmental *L. monocytogenes* strains in Latin-style fresh cheese and other dairy-processing plants.

MATERIALS AND METHODS

Processing Plant and Sample Collection

A total of 357 samples were collected from 3 Latin-style fresh-cheese processing plants located in New York City during 4 visits to each facility over a 6-month period (June to December 2000). All 3 plants purchase pasteurized milk for cheese manufacture. Cheeses are manufactured by heat treating the pasteurized milk, adding rennet and starter culture and/or acidulant (e.g., lactic or acetic acid) to obtain a curd, draining the whey, pressing the curd, followed by milling, salting, molding, and packaging. The plants were selected to represent small to relatively large-scale manufacturers (up to 18,000 gallons of pasteurized milk per day). Plant A is a large manufacturer, processing between 12,000 to 18,000 gallons per day, plant B manufactures 75 gallons twice a week in a very small processing area, and plant C, a medium-size factory, processes 1200 gallons per day.

Samples collected included 111 finished products (fresh cheeses) and 246 environmental samples (119 food contact surfaces, 40 floor drains, 34 floors, 38 walls, and 15 miscellaneous sampling sites). Food contact surfaces tested included equipment surfaces (coagulation

vat, pressing machine, pasteurizer valve, grinder, mixer), stirring tools, a stainless steel container, tables (stainless steel and polytetrafluorethylene), a plastic strainer, a plastic barrel, padding, a plastic connecting tube (used to connect a milk stainless steel pipeline from the holding tank to the pasteurizer and from the pasteurizer to the coagulation vat). Whenever possible, environmental samples were collected from the same sites on each visit. Environmental samples were collected using a commercial environmental sponge sampling kit (Bacti Sponge Kit, Hardy Diagnostics, Santa Maria, CA) and a neutralizing buffer (Hardy Diagnostics) to neutralize the chlorine-based sanitizers and quaternary ammonium compounds used in the plants. Cheeses were collected as finished packaged products. All samples were kept refrigerated and were analyzed within 48 h.

Listeria monocytogenes Detection

Finished products were analyzed by the FDA method (Hitchins, 1998), which was modified to also include plating of the enrichments on *L. monocytogenes* plating medium (**LMPM**, Biosynth Biochemica & Synthetica [BCM], Naperville, IL) and *L. monocytogenes* confirmation media (**LMCM**, Biosynth), as described by Restaino et al. (1999). Briefly, 25-g samples of cheese were aseptically added to 225 mL of enrichment broth (**EB**) (Hitchins, 1998) and homogenized in a Stomacher (Seward Ltd., London, UK). The homogenized samples were incubated at 30°C, and selective agents were added after 4 h of incubation, as described by Hitchins (1998). After 24 and 48 h of incubation, aliquots from EB were streaked onto Oxford (Difco Laboratories, Detroit, MI), PALCAM (Oxoid Ltd., Basingstoke, Hampshire, England), and LMPM. Typical *Listeria*-like colonies from Oxford and PALCAM were purified on brain heart infusion agar (**BHI**, Difco) and screened by PCR, as described below, to identify *L. monocytogenes* isolates. Typical PI-PLC positive *Listeria*-like colonies from LMPM were streaked onto LMCM and onto purple broth base agar (**PBBA**, Difco) containing 1.0% rhamnose and scored according to the manufacturer's directions and as previously described (Restaino et al., 1999). Isolates confirmed as *L. monocytogenes* were verified as *L. monocytogenes* using the PCR assay described below.

Environmental sponge samples were analyzed using both the FDA method (Hitchins, 1998) and the *L. monocytogenes* detection system (**LMDS**, Biosynth) (Restaino et al., 1999). The sponges were homogenized manually in their bags and aseptically cut in half; one half was added to 50 mL of EB and the second half was added to 50 mL of *L. monocytogenes* preenrichment broth (**LMPEB**, Biosynth). The sponges were homoge-

nized for 30 s in a Stomacher blender and analyzed following the previously published protocols for the FDA method (Hitchins, 1998) and LMDS (Restaino et al., 1999).

PCR-Based Identification of *Listeria monocytogenes*

Four *Listeria*-suspect colonies from each selective agar (Oxford, PALCAM, and/or LMCM) were screened using a *L. monocytogenes* specific PCR assay targeting the *L. monocytogenes* listeriolysin O gene, *hly*, as previously described (Norton et al., 2000).

Virulence Gene Allele Characterization

One *L. monocytogenes* isolate from each selective plating medium was selected for further characterization. Thus, for samples that yielded positive results on all 3 selective media (Oxford, PALCAM, and/or LMCM), 3 isolates were further characterized. Virulence gene allele typing of *actA* and *hly* by PCR-RFLP was performed as previously described (Wiedmann et al., 1997). Briefly, *hly* was characterized by PCR-RFLP using the restriction endonucleases *HhaI* and *HpaII*. One of 8 possible allelic types was assigned based on the resulting restriction pattern. Following amplification of *actA*, one of 2 possible allelic types (either type "3" or "4") was assigned based on a PCR product size difference, which corresponds with the presence or absence of a 105 nucleotide region encoding a proline-rich repeat structure (Wiedmann et al., 1997).

Ribotyping

For each sample positive for *L. monocytogenes*, one isolate of each different *actA* and *hly* polymorphic type was selected for ribotype analysis. Ribotyping was performed using the restriction endonuclease *EcoRI* and the RiboPrinter Microbial Characterization System (Qualicon, Inc., Wilmington, DE) as described previously (Bruce, 1996). Images were acquired with a charge-coupled device camera and processed using the Riboprinter's custom software. This software normalizes fragment pattern data for band intensity and relative band position (Bruce, 1996). Ribotype patterns were automatically assigned a DuPont ID (e.g., DUP-1039) by the Riboprinter, which was confirmed by visual inspection. If visual inspection found a given DuPont ID included more than one distinct ribotype pattern, which generally differed by position of only a single weak band, then each pattern was designated with an additional alphabetized letter (e.g., DUP-1039A and DUP-1039B).

Table 1. Occurrence of *Listeria monocytogenes* in Latin-style fresh-cheese processing plants.

Plant	Visit	Finished products ¹		Environmental samples		
		No. of samples	No. of <i>Listeria monocytogenes</i> positive samples	No. of samples	No. of <i>L. monocytogenes</i> positive samples	No. of <i>Listeria</i> spp. positive samples ²
A	1	10	0	27	2	5
	2	18	0	23	1	5
	3	18	0	23	1	3
	4	18	0	24	0	5
	Subtotal	64	0 (0.0%)	97	4 (4.1%)	18 (18.5%)
B	1	5	0	17	3	1
	2	6	0	13	2	0
	3	6	0	13	1	2
	4	6	0	12	2	0
	Subtotal	23	0 (0.0%)	55	8 (14.5%)	3 (5.4%)
C	1	6	1	23	5	3
	2	6	3	22	6	1
	3	6	3	25	2	1
	4	6	0	24	2	1
	Subtotal	24	7 (29.2%)	94	15 (15.9%)	6 (6.4%)
	Total	111	7 (6.3%)	246	27 (11.0%)	27 (11.0%)

¹If finished product samples were negative for *L. monocytogenes*, they were also negative for *Listeria* spp.

²Number of samples negative for *L. monocytogenes*, but positive for *Listeria* spp.

RESULTS

Occurrence of *Listeria* spp. and *L. monocytogenes* in Cheese and Environmental Samples

Environmental and cheese samples were collected from 3 processing plants over a total of 4 visits to each plant. *Listeria monocytogenes* was detected in 6.3% of finished product samples and in 11.0% of environmental samples (Table 1). Only finished products from one plant (plant C) were positive for *L. monocytogenes*. Environmental samples from plant A showed a lower frequency of *L. monocytogenes* contamination (4.1%) as compared with plants B and C, which showed contamination frequencies of 14.5 and 15.9%, respectively (Table 1). All 3 plants showed similar prevalences of *Listeria* spp. in environmental samples (20.0 to 22.7%). Among the environmental samples, *L. monocytogenes* was most commonly isolated from drains (30.0%) and floors (20.6%) (Table 2). Only 2 food contact surface

samples (a plastic connecting tube at the exit of the pasteurizer, which was used to transfer milk to the coagulation vat, and a polytetrafluorethylene table; Table 2), both collected in plant C, were positive for *L. monocytogenes*. Crates were contaminated with *L. monocytogenes* in all 3 plants (Table 3).

Molecular Subtyping

A total of 80 *L. monocytogenes*, representing up to 3 isolates from each positive sample (one isolate from each selective plating media), were initially subtyped using allelic analysis of the virulence genes *actA* and *hly*. Both *actA* types 3 and 4 were identified among the isolates. Although 8 different *hlyA* types exist (Wiedmann et al., 1997), only types 1 and 2 were identified among the isolates collected in this study. With 2 exceptions, multiple isolates from the same site always displayed the same *hly* and *actA* types. Specifically, sponge samples collected from crates in plant B at visits 2 and 4 each contained 2 different *actA* and *hly* subtypes (Table 3). Based on these initial subtyping results, 36 representative isolates (one from each positive sample, unless multiple subtypes were observed based on *hly* and *actA* allelic analysis) were selected for further subtyping by automated *EcoRI* ribotyping. Ribotyping discriminated 8 distinct patterns among the 36 isolates (Table 3). Ribotype DUP-1044A/*hly* type 1, *actA* type 4 represented the most commonly isolated subtype (55.6%). Two ribotypes (DUP-1044A and DUP-1062C) were identified among the isolates from plant A, whereas 5 ribotypes (DUP-1042A, DUP-1042C, DUP-1049A, DUP-1062C, and DUP-1052) and 3 ribotypes

Table 2. Occurrence of *Listeria monocytogenes* in environmental samples collected in 3 Latin-style soft cheese processing plants.

Type of surface sampled	No. of samples tested	No. of positive samples (%)
Food contact surfaces	119	2 ¹ (1.7%)
Floor drains	40	12 (30.0%)
Floors	34	7 (20.6%)
Walls	38	0
Miscellaneous	15	6 ² (40.0%)
TOTAL	246	27 (11.0%)

¹Positive samples were a plastic connecting tube and a polytetrafluorethylene table (plant C).

²Positive samples were crates and plastic milk packages.

Table 3. *Listeria monocytogenes* contamination patterns and subtyping results for 3 Latin-style soft cheese processing plants.

Factory	Visit	Sites (sample code)	<i>actA</i> type	<i>hlyA</i> type	Lineage	Ribotype (DUP)	RiboPrinter pattern
A	1	Drain 10, processing area 2	4	1	I	1044A	
		Floor, processing area 2	4	1	I	1044A	
	2	Drain, cooler room	4	2	II	1062C	
	3	Crate	4	2	II	1062C	
B	1	Drain, processing area	4	1	I	1042C	
		Floor, processing area	4	1	I	1042C	
		Crate	3	1	I	1042A	
	2	Drain, processing area	4	1	I	1042C	
		Crate ¹	3	1	I	1042A	
			4	2	II	1049A	
	3	Drain, processing area	4	1	I	1042C	
	4	Crate ¹	3	1	I	1042A	
			4	2	II	1062C	
		Milk package	3	1	I	1052	
C	1	Cheese	4	1	I	1044A	
		Drain 1, processing area 1	4	1	I	1044A	
		Drain 2, processing area 2	4	1	I	1044A	
		Drain 3, processing area 3	4	1	I	1044A	
		Floor 2, processing area 2	3	2	II	1045B	
		Floor 4, cooler room	4	1	I	1044A	
	2	Cheese	4	1	I	1044A	
		Cheese	4	1	I	1044A	
		Cheese	4	1	I	1044A	
		Plastic connecting tube, processing area 2	4	1	I	1044A	
		Drain 1, processing area 2	4	1	I	1044A	
		Drain 2, processing area 2	3	2	II	1045B	
		Drain 3, processing area 3	4	1	I	1044A	
	3	Floor 3, processing area 3	4	1	I	1044A	
		Floor 4, cooler room	4	1	I	1044A	
		Cheese	4	1	I	1044A	
		Cheese	4	1	I	1044A	
		Cheese	4	1	I	1044A	
		Floor 4, cooler room	4	1	I	1044A	
		Crate	4	2	II	1039C	
	4	Polytetrafluorethylene table, processing area 3	4	1	I	1044A	
		Drain 2, processing area 2	3	2	II	1045B	

¹ Two subtypes as determined by allelic analysis of *hly* and *actA* were isolated from these samples; subtype characteristics for both strains are shown.

Table 4. Comparison of Biosynth chromogenic medium-*Listeria monocytogenes* detection system (LMDS) and FDA methods for detection of *L. monocytogenes* from 246 environmental samples.

Isolation methods	No. of positive samples ¹	No. of true negative samples	No. of false positives	No. of false negatives	Sensitivity (%) ²	Specificity (%) ³
LMDS	13	219	0	14	48.2	100
FDA	23	219	0	4	85.2	100

¹ The total number of positive samples identified (27) is the sum of positives detected only by LMDS plus those detected only by the FDA method, plus those detected by both.

²% Sensitivity = $100 \times [\text{true positives} / (\text{true positives} + \text{false negatives})]$.

³% Specificity = $100 \times [\text{true negatives} / (\text{true negatives} + \text{false positives})]$.

(DUP-1044A, DUP-1045B, and DUP-1039C) were identified in plants B and C, respectively (Table 3).

Evaluation of FDA Method and BCM-LMDS

A total of 111 cheese samples were analyzed for *L. monocytogenes* using a modified FDA method, which included plating on Oxford, PALCAM, and LMPM. Both Oxford and PALCAM allowed *L. monocytogenes* isolation from 7 cheese samples, whereas LMPM allowed *L. monocytogenes* isolation from only 5 of the same samples. Some *L. monocytogenes* do not produce PI-PLC (Notermans et al., 1991) and, therefore, may produce false negative results on LMPM. However, isolates that had been obtained from samples that yielded positive results on Oxford and PALCAM, but negative results on LMPM, did display PI-PLC production when restreaked on LMPM.

A total of 246 environmental sponge samples were analyzed using both the FDA method and the LMDS method. Twenty-seven samples were positive for *L. monocytogenes* by one or both methods. The FDA method yielded 23 positive samples but failed to allow detection of *L. monocytogenes* in 4 samples that were positive by LMDS. The use of LMDS allowed isolation of *L. monocytogenes* from 13 samples, but did not detect *L. monocytogenes* in 14 samples that were positive by the FDA method (Table 4). The sensitivities of the FDA method and BCM LMDS were thus 85.2 and 48.2%, respectively (Table 4). All isolates that showed reactions typical for *L. monocytogenes* on LMPM and LMCM were confirmed as *L. monocytogenes* by a *hlyA* PCR assay. For the 23 environmental samples that were positive using the FDA method, *L. monocytogenes* was detected in 91.3% (21/23) of the samples after 24 h of enrichment; 20 and 15 samples yielded *L. monocytogenes* on PALCAM and Oxford, respectively. For 2 samples, *L. monocytogenes* was only isolated after 48 h of enrichment.

DISCUSSION

Listeria monocytogenes contamination of RTE foods appears to occur predominantly through postprocessing

contamination from food-processing environments (Tompkin, 2002). Therefore, environmental testing is a critical component of *L. monocytogenes* control programs in the dairy and food-processing industries (Tompkin, 2002). Data on the efficacy of environmental testing methods and on environmental *L. monocytogenes* and *Listeria* spp. contamination patterns in different food processing systems are crucial for development of science-based regulations to enable better control of this food-borne pathogen at its sources. Fresh Latin-style cheeses have been responsible for at least 2 human listeriosis outbreaks in the United States (Linnan et al., 1988; Anonymous, 2001). We selected 3 Latin-style fresh-cheese processing plants representing different scales of operation to probe environmental *L. monocytogenes* contamination patterns and to explore the use of different detection and subtyping methods to monitor environmental contamination patterns.

L. monocytogenes and *Listeria* Contamination Patterns

Listeria monocytogenes was isolated from 6.3% of finished product samples and from 11.0% of environmental samples; isolation frequencies varied considerably from plant to plant (Table 1). In 1991, Genigeorgis et al. reported a 2% *L. monocytogenes* prevalence in Latin-style fresh cheeses collected in California. In 2003, Gombas et al. reported a 0.07% prevalence in cheeses tested in Northern California and a 0.28% prevalence in those tested in Maryland. *L. monocytogenes* prevalence in similar products tested in other countries ranged from 3.3 to 41.2% (Destro et al., 1991; El Marakchi et al., 1993; Furlanetto et al., 1996; Silva et al., 1998; Saltijeral et al., 1999). Whereas cheeses manufactured from raw milk have been reported to bear a higher prevalence of *L. monocytogenes* than cheeses made from pasteurized milk (Genigeorgis et al., 1991; Silva et al., 1998; Saltijeral et al., 1999), our data demonstrate that *L. monocytogenes* can also be found in products manufactured from pasteurized milk.

Listeria monocytogenes was found in up to 15.9% of environmental samples collected from the 3 processing plants studied; highest contamination rates were found from floor drains and floors as well as from crates. These findings are consistent with previous reports, which have also found that drain and floor samples showed the highest prevalence of *L. monocytogenes* among samples collected from dairy processing plants (Klausner and Donnelly, 1991; Pritchard et al., 1995; Sutherland and Porritt, 1996). Our observation that crates from all 3 plants were at least sporadically positive for *L. monocytogenes* also further confirms that these appliances represent an important potential source of *L. monocytogenes* contamination in dairy processing plants (Sutherland and Porritt, 1996). Crates had multiple possible functions within each plant, thus providing multiple opportunities for the possible spread of *L. monocytogenes* from point to point within a plant. To illustrate, crates were used to transport pasteurized milk packages from cooler rooms to the processing areas; to transport finished products from processing areas to cooler rooms; and to store finished products in the cooler rooms. Whereas *L. monocytogenes* has previously been isolated from dairy industry food contact surfaces (Jacquet et al., 1993; Pritchard et al., 1995; Sutherland and Porritt, 1996), we isolated *L. monocytogenes* from only 2 food contact surfaces (a connecting plastic tube coming from the pasteurizer and a polytetrafluorethylene table) in one of the 3 plants included in our study.

When comparing the 3 processing plants tested in this study, the largest plant (plant A), which had recently been constructed, showed a low incidence of *L. monocytogenes* contamination, comparable to environmental contamination prevalence data for other well-managed RTE food-processing plants. These data indicate that manufacturing of Latin-style fresh cheeses under appropriate conditions can produce products of comparable safety to other RTE food products. Plant B represents the smallest establishment included in this study. Whereas this plant showed a high frequency of *L. monocytogenes*-positive environmental samples, which are likely associated with the poor design and layout of the facility, no finished product samples were found positive for *L. monocytogenes*. In this factory, the presence of *L. monocytogenes* on the outside of packages containing incoming pasteurized milk revealed a potential route of environmental contamination. Plant C was the only plant in this study that had both *L. monocytogenes* contamination of many environmental sites (Table 3) as well as contamination of finished products, most likely due to postpasteurization contamination. The plant C plastic food contact surfaces that were positive for *L. monocytogenes* were manufactured using materials that are difficult to sanitize (Krysinski et al.,

1992) and that appear to facilitate biofilm formation (Sinde and Carballo, 2000). These findings highlight the importance of using appropriate materials (e.g., stainless steel) and sanitary design for the manufacture of food-processing equipment.

Tracking of *L. monocytogenes* Contamination Patterns by Molecular Subtyping

Molecular subtyping (allelic analysis of *actA* and *hly* and automated ribotyping) was used to further elucidate *L. monocytogenes* contamination patterns. Initial subtyping using allelic analysis of *actA* and *hly* was used to prescreen isolates when multiple isolates from different enrichment procedures were available for a given sample. Whereas a combination of *actA* and *hly* allelic types provides less sensitive subtype differentiation as compared with more expensive methods, such as PFGE or automated ribotyping (Wiedmann, 2002), we reasoned that these methods provide a cost-effective alternative for identification of at least some samples that contain multiple *L. monocytogenes* subtypes. In this study, we identified 2 samples that contained multiple subtypes (Table 3). This finding is consistent with previous studies, which also described the presence of multiple subtypes within a single sample (Ryser et al., 1996). Clearly, it is possible to underestimate the true subtype diversity present in a given sample when only a single or limited number of isolates are tested from a sample. Unfortunately, cost considerations currently make it impractical to subtype large numbers of isolates from each positive sample using PFGE or ribotyping, but less expensive screening methods, such as the allele analyses used in this study, may help address this issue.

Analysis of subtyping data revealed persistent contamination with ribotype DUP-1044A in plant C. This subtype was widespread in this plant and was isolated during each of 4 visits. Although it is difficult to establish directionality of the spread of contamination, the presence of ribotype DUP-1044A in finished products as well as in environmental sites (drains, floors) and food contact surfaces (plastic connecting tube, processing table) indicates probable postprocessing contamination of finished products from environmental sources. Jacquet et al. (1993) previously described similar contamination patterns in a cheese manufacturing plant. This group recovered *L. monocytogenes* of the same serovar and phagovar from cheese manufactured with pasteurized milk as well as from processing equipment and shelves, suggesting product contamination during the ripening process. In addition to ribotype DUP-1044A, a second ribotype, DUP-1045B, also persisted in plant C (Table 3). Our findings add to a growing body of evidence implicating persistent *L. monocyto-*

genes subtypes in food processing plants as a major concern. Sites harboring persistent strains may facilitate the spread of *L. monocytogenes* to downstream points within the processing environment, and hence may ultimately serve as sources of finished-product contamination (Loncarevic et al., 1998; Autio et al., 1999; Miettinen et al., 1999; Norton et al., 2001; Hoffman et al., 2003; Lundén et al., 2003). Interestingly, ribotype DUP-1044A/*actA* type 4/*hly* type 1 strains had been previously associated with a 1998 multistate human listeriosis outbreak in the United States, which was linked to contaminated hot dogs and deli meats (Sauders et al., 2003). It had been postulated that persistent contamination of the processing plant environment after a construction event contributed to this outbreak. We thus hypothesize that DUP-1044A may represent a strain or clonal group that is specifically capable of persisting in food processing environments. In further support of this hypothesis, ribotype DUP-1044A also had been isolated on multiple occasions in 2 of 3 smoked-fish processing plants tested by Norton et al. (2001).

In plant A, we identified the same ribotype (DUP-1062C) in a cooler room drain and, on a subsequent visit, in a crate used for transport and storage of finished products in the cooler room. This finding may indicate the crate as a vehicle of cross contamination. In this plant, ribotype DUP-1044A was isolated from both a floor and a drain sample collected during the same visit. No ribotypes persisted over more than 2 visits or at multiple sites in plant A, suggesting that persistent *L. monocytogenes* contamination was successfully prevented in this plant. Plant B showed the highest ribotype diversity, with a total of 5 different ribotypes isolated from this plant. Two different ribotypes persisted in different environmental sites in this plant. Ribotype DUP-1042A was isolated from crates on 3 separate visits, and DUP-1042C was isolated from drain and floor samples on 3 separate visits. These results further demonstrate the role of molecular subtyping data in environmental monitoring programs to differentiate transient from persistent contamination and to allow development of appropriate control and intervention strategies.

***L. monocytogenes* Detection Methods**

Detection of *L. monocytogenes* using culture-based methods is time consuming and can be challenging, particularly if *L. monocytogenes* is present in a background of other *Listeria* spp. Other *Listeria* spp. may overgrow *L. monocytogenes* during enrichment (Beumer et al., 1996) and most selective and differential *Listeria* plating media do not differentiate *L. monocytogenes*

from nonpathogenic *Listeria* spp. Thus, specific detection of *L. monocytogenes* relies on phenotypic and biochemical testing of selected *Listeria*-like colonies collected from plating media. Usually only a few isolates (<5) are selected for screening to determine the presence of *L. monocytogenes*; samples with a high *Listeria* spp. to *L. monocytogenes* ratio may thus be scored incorrectly as *L. monocytogenes* negative. The increasing need for economical testing strategies with rapid turnaround times has led to the common use of *Listeria* spp. as an indicator for conditions that may enable the presence of *L. monocytogenes* in the food industry. Our results indicate that environmental testing for *Listeria* spp. and *L. monocytogenes* are likely to yield different results. For example, all 3 Latin-style fresh-cheese processing plants showed between 20 and 22% prevalence of *Listeria* spp. in environmental samples, which could lead to the hypothesis that similar hygiene and *Listeria* control programs were in place for all 3 plants. However, one plant had a significantly lower *L. monocytogenes* prevalence (4.1%) as compared with the 2 other plants (14.5 and 15.9%). Our results thus indicate that the presence of *Listeria* spp. may not be a good predictor of *L. monocytogenes* contamination, and that *L. monocytogenes*-specific testing of environmental samples may be a more appropriate strategy for monitoring the effectiveness of *L. monocytogenes* control strategies.

Recently developed and evaluated selective and differential media for *L. monocytogenes* (Restaino et al., 1999) may provide a rapid and less labor-intensive alternative to *Listeria* spp. testing of environmental samples. LMDS represents a complete detection system, including preenrichment and enrichment broths and a differential and selective media, which can distinguish *L. monocytogenes* and *L. ivanovii* from other *Listeria* spp. based on PI-PLC production. LMCM (which is also included in LMDS) and PBBA subsequently can distinguish *L. monocytogenes* from *L. ivanovii*. We compared the performance of LMDS to the FDA methods for detection of *L. monocytogenes* from 246 environmental samples. The FDA method showed a sensitivity of 85.2%, as compared with 48.2% for the LMDS. The LMDS preenrichment broth used in our study was an early production batch manufactured before the decision had been made to include the selective agents nalidixic acid or cycloheximide in the medium (L. Restaino, personal communication, 2000). We suspect that, as a consequence, the *L. monocytogenes* present in the samples that were falsely negative with the LMDS method may have been overgrown by other bacteria. The absence of the selective agents in the batch of media used in our study might explain why our results differ from the findings of Restaino et al. (1999), who showed that LMDS (containing a selective agent) was more sensitive

than the USDA method for detecting *L. monocytogenes* from environmental samples collected in meat processing facilities. Additionally, we also evaluated the inclusion of LMPM as a third plating medium in the FDA method for detection of *L. monocytogenes* from 111 cheese samples. *Listeria monocytogenes* was not isolated on LMPM for 2 of 7 samples that yielded *L. monocytogenes* from the FDA-recommended plating media, Oxford and PALCAM. In contrast, in a study using the same 3 plating media to detect *L. monocytogenes* in 2000 food and environmental samples, Jinne-man et al. (2003) reported that the use of LMPM, combined with Oxford or PALCAM media, allowed for improved detection of *L. monocytogenes* over the use of Oxford and PALCAM alone.

CONCLUSIONS

Whereas at least one previous listeriosis outbreak has been linked to Latin-style fresh cheese manufactured from *L. monocytogenes*-contaminated unpasteurized milk (Linnan et al., 1988), our results show that persistent environmental contamination represents another important source of finished product contamination for these products. Comprehensive environmental monitoring programs for *L. monocytogenes*, including specific testing for *L. monocytogenes* along with application of molecular subtyping strategies, are required to detect persistent contamination and to monitor the effectiveness of control strategies, which require integration of an effective sanitation program with good manufacturing practices. Whereas *Listeria* spp. testing is often recommended as an indicator for conditions that may enable the presence of *L. monocytogenes*, our results indicate that the presence of *Listeria* spp. may not be a good predictor of *L. monocytogenes* contamination. Whereas additional studies will be required to better understand correlations between *Listeria* positive samples and the presence of *L. monocytogenes*, the further development and refinement of rapid, inexpensive, and easy to use detection methods (e.g., chromogenic media) that allow specific detection of *L. monocytogenes* in environmental samples will clearly improve our ability to control this foodborne pathogen in the dairy industry.

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REFERENCES

- Anonymous. 2001. Outbreak of listeriosis associated with homemade Mexican-style cheese—North Carolina, October 2000—January 2001. *MMWR*. 50:560–562.
- Arimi, S. M., E. T. Ryser, T. J. Pritchard, and C. W. Donnelly. 1997. Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. *J. Food Prot.* 60:811–816.
- Autio, T., S. Hielm, M. Miettinen, A. M. Sjöberg, K. Aarlisalo, J. Björkroth, and T. Mattila-Sandholm. 1999. Sources of *Listeria monocytogenes* contamination in a cold smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Appl. Environ. Microbiol.* 65:150–155.
- Azadian, B. S., G. T. Finnerty, and A. D. Pearson. 1989. Cheese-borne *Listeria* meningitis in immunocompetent patient. *Lancet* 1:322–323.
- Beumer, R. R., M. C. te Giffel, S. V. R. Anthonie, and L. J. Cox. 1996. The effect of acriflavin and nalidixic acid on the growth of *Listeria* spp. in enrichment media. *Food Microbiol.* 13:137–148.
- Bille, J. 1990. Epidemiology of human listeriosis in Europe with special reference to the Swiss outbreak. Pages 71–74 in *Foodborne Listeriosis*. A. J. Miller, J. L. Smith, G. A. Somkuti, ed. Elsevier, New York, NY.
- Bruce, J. 1996. Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol.* 50:77–81.
- Cox, L. J., T. Kleiss, J. L. Cordier, C. Cordellana, P. Konkel, C. Pedrazzini, R. Beumer, and A. Siebenga. 1989. *Listeria* spp. in food processing, non-food, and domestic environments. *Food Microbiol.* 6:49–61.
- Dalton, C. B., C. C. Austin, J. Sobel, P. S. Hayes, W. F. Bibb, L. M. Graves, B. Swaminathan, M. E. Proctor, and P. M. Griffin. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *N. Engl. J. Med.* 336:100–105.
- Destro, M. T., A. M. Serrano, and D. Y. Kabuki. 1991. Isolation of *Listeria* species from some Brazilian meat and dairy products. *Food Control* 2:110–112.
- El Marrakchi, A., A. Hamama, and F. El Othmani. 1993. Occurrence of *Listeria monocytogenes* in milk and dairy products produced or imported into Morocco. *J. Food Prot.* 56:256–259.
- Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55:476–511.
- Fleming, D. W., S. L. Cochi, K. L. Mac Donald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Hoimes, A. Audurier, C. V. Broome, and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* 312:404–407.
- Furlanetto, S. M., M. A. A. Santos, and C. Hara. 1996. Avaliação da eficiência de quatro meios de plaqueamento no seu isolamento. *Higiene Alimentar*. 10:30–34.
- Genigeorgis, C., J. H. Toledo, and F. J. Garayzabal. 1991. Selected microbiological and chemical characteristics of illegally produced and marketed soft Hispanic-style cheeses in California. *J. Food Prot.* 54:598–601.
- Gombas, D. E., Y. Chen, R. S. Clavero, and V. N. Scott. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. *J. Food Prot.* 66:559–569.
- Hitchins, A. D. 1998. *Listeria monocytogenes*. Pages 10-01–10-13 in *Food and Drug Administration. Bacteriological Analytical Manual*, 8th ed. Revision A. AOAC, Arlington, VA.
- Hoffman, A. D., and M. Wiedmann. 2001. Comparative evaluation of culture and BAX polymerase chain reaction-based detection methods for *Listeria* spp. and *Listeria monocytogenes* in environmental and raw fish samples. *J. Food Prot.* 64:1521–1526.
- Hoffman, A. D., K. L. Gall, D. M. Norton, and M. Wiedmann. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. *J. Food Prot.* 66:52–60.
- Jacquet, C., B. Catimel, R. Brosch, C. Buchrieser, P. Dehaumont, V. Goulet, A. Lepoutre, P. Veit, and J. Rocourt. 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Appl. Environ. Microbiol.* 61:2242–2246.

- Jacquet, C., J. Rocourt, and A. Reynaud. 1993. Study of *Listeria monocytogenes* contamination in a dairy plant and characterization of the strains isolated. *Int. J. Food Microbiol.* 20:13–22.
- Jinneman, K. C., J. M. Hunt, C. A. Eklund, J. S. Wernberg, P. N. Sado, J. M. Johnson, R. S. Richter, S. T. Torres, E. Ayotte, S. J. Eliasberg, P. Istafanos, D. Bass, N. Kexel-Calabresa, W. Lin, and C. N. Barton. 2003. Evaluation and interlaboratory validation of a selective agar for phosphatidylinositol-specific phospholipase C activity using a chromogenic substrate to detect *Listeria monocytogenes* from foods. *J. Food Prot.* 66:441–445.
- Johansson, T., L. Rantala, L. Palmu, and T. Honkanen-Buzalki. 1999. Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish and in a production plant. *Int. J. Food Microbiol.* 47:111–119.
- Karpisková, R., M. Pejchalová, J. Mokrosová, J. Vytrasová, P. Smuhanová, and J. Ruprich. 2000. Application of a chromogenic medium and the PCR methods for the rapid confirmation of *Listeria monocytogenes* in foodstuffs. *J. Microbiol. Meth.* 41:267–271.
- Klausner, R. B., and C. Donnelly. 1991. Environmental sources of *Listeria* and *Yersinia* in Vermont dairy plants. *J. Food Prot.* 54:607–611.
- Krysinski, E. P., L. J. Brown, and T. J. Marchisello. 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *J. Food Prot.* 55:246–251.
- Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B. D. Plikaytis, S. L. Fannin, A. Kleks, and C. V. Broome. 1988. Epidemic listeriosis associated with Mexican-style cheese. *N. Engl. J. Med.* 319:823–828.
- Loncarevic, S., E. Bannerman, J. Bille, M. L. Danielsson-Tham, and W. Tham. 1998. Characterization of *Listeria* strains isolated from soft and semi-soft cheeses. *Food Microbiol.* 15:521–525.
- Lundén, J. M., T. J. Autio, A.-M. Sjöberg, and H. J. Korkeala. 2003. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *J. Food Prot.* 66:2062–2069.
- McLauchlin, J., M. H. Greenwood, and P. N. Pini. 1990. The occurrence of *Listeria monocytogenes* in cheese from a manufacturer associated with a case of listeriosis. *Int. J. Food Microbiol.* 10:255–262.
- Mead, P. S., L. Slutsker, V. Deitz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
- Miettinen, M. K., K. J. Björkroth, and H. J. Korkeala. 1999. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. *Int. J. Food Microbiol.* 46:187–192.
- Norton, D. M., M. A. McCamey, K. J. Boor, and M. Wiedmann. 2000. Application of the BAX for screening/genus *Listeria* polymerase chain reaction system for monitoring *Listeria* species in cold-smoked fish and in the smoked fish processing environment. *J. Food Prot.* 63:343–346.
- Norton, D. M., M. A. McCamey, K. L. Gall, J. M. Scarlett, K. J. Boor, and M. Wiedmann. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl. Environ. Microbiol.* 67:198–205.
- Notermans, S. H. W., J. Duffrenne, M. Leimeister-Wächter, E. Dömann, and T. Chakraborty. 1991. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. *Appl. Environ. Microbiol.* 57:2666–2670.
- Pritchard, T. J., K. J. Flanders, and C. W. Donnelly. 1995. Comparison of the incidence of *Listeria* on equipment versus environmental sites within dairy processing plants. *Int. J. Food Microbiol.* 26:375–384.
- Restaino, L., E. W. Frampton, R. M. Irbe, G. Schabert, and H. Spitz. 1999. Isolation and detection of *Listeria monocytogenes* using fluorogenic and chromogenic substrates for phosphatidylinositol-specific phospholipase C. *J. Food Prot.* 62:244–251.
- Ryser, E. T., S. M. Arimi, M. M.-C. Bunduki, and C. W. Donnelly. 1996. Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. *Appl. Environ. Microbiol.* 62:1781–1787.
- Saltijeral, J. A., V. B. Alvarez, and B. Garcia. 1999. Presence of *Listeria* in Mexican cheeses. *J. Food Safety* 19:241–247.
- Sauders, B. D., E. D. Fortes, D. L. Morse, N. Dumas, J. A. Kiehlbauch, Y. Schukken, J. R. Hibbs, and M. Wiedmann. 2003. Molecular subtyping to detect human listeriosis clusters. *Emerg. Infect. Dis.* 9:672–680.
- Silva, M. C. D., E. Hofer, and A. Tibana. 1998. Incidence of *Listeria monocytogenes* in cheese produced in Rio de Janeiro, Brazil. *J. Food Prot.* 61:354–356.
- Sinde, E., and J. Carballo. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: The influence of free energy and the effect of commercial sanitizers. *Food Microbiol.* 17:439–447.
- Sutherland, P., and R. Porritt. 1996. Dissemination and ecology of *Listeria monocytogenes* in Australian dairy factory environments. *Food Australia* 48:172,174–176,178.
- Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65:709–725.
- Torres, N., and R. C. Chandan. 1981. Latin American white cheese—A review. *J. Dairy Sci.* 64:552–557.
- USDA NASS. U.S. Department of Agriculture National Agricultural Statistics Service. 2002. Agricultural Statistics 2002, Chapter VIII. Dairy and Poultry Statistics (accessed at <http://www.usda.gov/nass/pubs/agr02/acro02.htm>).
- Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. Batt. 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect. Immun.* 65:2707–2716.
- Wiedmann, M. 2002. Molecular subtyping methods for *Listeria monocytogenes*. *J. AOAC* 85:524–531.
- Wong, S., D. Street, S. I. Delgado, and K. C. Klonts. 2000. Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration. *J. Food Prot.* 63:1113–1116.