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Molecular Typing To Trace *Listeria monocytogenes* Isolated from Cold-Smoked Fish to a Contamination Source in a Processing Plant

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ABSTRACT

In this study, *Listeria monocytogenes* contamination in a cold-smoked fish processing plant in Osaka, Japan, was examined from 2002 to 2004. A total of 430 samples were collected and divided into five categories: raw fish, materials during processing, processing equipment, environment, and finished products. A total of 59 finished products were examined throughout this study. *L. monocytogenes* was isolated from four of these samples during summer and autumn but was not found during winter or spring. During the warmer seasons, *L. monocytogenes* was more prevalent on processing equipment, especially slicing machines (8 of 54 samples in summer and autumn versus 1 of 50 samples in winter and spring). *L. monocytogenes* was not detected on whole skins removed from 23 frozen raw fish. *L. monocytogenes* strains isolated from 56 samples were characterized by serotyping, pulsed-field gel electrophoresis, and three PCR-based methods. Seventy-seven *L. monocytogenes* strains were recognized as contaminants of the samples: 2 distinguishable strains were identified in each of 13 samples, 3 strains were identified in 2 samples, 5 strains were identified in 1 sample, and the other 40 strains were identified in 40 samples. Combining the results from these techniques, 77 strains were classified into 13 different types. Three of these types prevailed throughout the plant, and two of the three were also isolated from final products. The DNA subtype found in the product was also found on the slicing machines. Our findings suggest that the slicing machines at this plant were the source of the product contamination. Implementing an appropriate cleaning regime for the slicing machines was effective in preventing contamination.

Listeria monocytogenes is the causative agent of foodborne listeriosis in humans. Disease syndromes include meningitis and septicemia, and the infection has a high lethal rate. In the United States, listeriosis is a leading cause of death from foodborne illness *(15).* Japan has an average of 83 cases of listeriosis per year and an estimated incidence of 0.65 cases per million residents, which is lower than the values reported in France, the United States, and the United Kingdom (5.4, 4.8, and 1.6 to 2.5 cases per million residents, respectively) *(19).* Nevertheless, the level of contamination in retail food products in Japan is roughly equivalent to that reported in some European countries and the United States, where large outbreaks have occurred *(11, 17–20).* Japan might therefore face a similar risk of foodborne listeriosis outbreaks *(19).*

L. monocytogenes is ubiquitous and can be isolated from many foods, especially ready-to-eat products. Several countries have regulations that prohibit the presence of *L. monocytogenes* in ready-to-eat foods. Ready-to-eat seafood (such as cold-smoked fish) has been linked to sporadic cases of listeriosis, including gastroenteritis. Epidemiological evidence suggests that listeriosis has resulted from the consumption of smoked mussels *(5),* gravad trout *(9),* and smoked trout *(16).* In Japan, a wide range of ready-to-eat seafood products are consumed in great quantities.

In a previous study, we investigated *L. monocytogenes* contamination in commercially available ready-to-eat seafood products in Osaka during 1999 and 2000 *(17). L. monocytogenes* was isolated from 13% (12 of 95) of the tested products. Of the positive samples, 9 (75%) of 12 were from cold-smoked fish obtained during the summer. Molecular typing of the isolates suggested that the products were contaminated with persistent strains that were unique to their respective manufacturers.

Numerous studies have indicated that *L. monocytogenes* can contaminate smoked fish processing plants *(1, 6, 8, 10, 22).* By contrast, few published studies have documented *L. monocytogenes* contamination of ready-to-eat seafood processing plants in Japan, although the bacterium does occur naturally in raw fish material *(18, 24).*

To manufacture products without *L. monocytogenes,* it is essential to identify the possible sources of contamination. Strain characterizations based on pulsed-field gel electrophoresis (PFGE) and on PCR-based typing methods such as arbitrarily primed (AP) PCR, repetitive extragenic palindromic elements (REP) PCR, and enterobacterial repetitive intergenic consensus sequences (ERIC) PCR have been used successfully to type *L. monocytogenes* in epidemiological and contamination investigations *(1, 6, 7, 12, 17,*

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TABLE 1. *Sample categories and number of samples positive for* Listeria monocytogenes

Sample time	Sample categories	Total no. of samples	No. (%) of positive samples
Spring: May 2003, March 2004, and May 2004	Raw fish	0	0
	Materials during processing	25	2 (8.0)
	Processing equipment [slicing machine only][a]	34 [27]	2 (5.9) [0]
	Environment	6	4 (66.7)
	Product	14	0
Summer: August 2002, June 2003, July 2003, and August 2003	Raw fish	1	0
	Materials during processing	47	5 (10.6)
	Processing equipment [slicing machine only]	26 [21]	4 (15.4) [3 (14.3)]
	Environment	21	11 (52.4)
	Product	19	2 (10.5)
Autumn: September 2002, November 2002, September 2003, and October 2003	Raw fish	16	0
	Materials during processing	38	3 (7.9)
	Processing equipment [slicing machine only]	35 [33]	5 (14.3) [5 (15.2)]
	Environment	27	4 (14.8)
	Product	12	2 (16.7)
Winter: December 2002, December 2003, January 2004, and February 2004	Raw fish	30	0
	Materials during processing	23	1 (4.3)
	Processing equipment [slicing machine only]	29 [23]	3 (10.3) [1 (4.3)]
	Environment	13	8 (61.5)
	Product	14	0
All 15 visits	Raw fish	47	0
	Materials during processing	133	11 (8.3)
	Processing equipment [slicing machine only]	124 [104]	14 (11.3) [9 (8.7)]
	Environment	67	27 (40.3)
	Product	59	4 (6.8)
Total		430	56 (13.0)

[a] Processing equipment includes slicing machines. Among the different types of processing equipment, the prevalence of *L. monocytogenes* in the slicing machines was relatively high.

22, 23). In the current study, PFGE and PCR-based typing methods were applied simultaneously to trace *L. monocytogenes* isolates to the source of contamination in cold-smoked fish processing plant A, the products of which were shown to be highly contaminated in our previous investigation *(17).*

MATERIALS AND METHODS

Processing plant and product manufacture. Processing plant A used gutted farm-raised Chilean trout and wild-caught blueback salmon from Canada as raw materials for the production of cold-smoked fish. All fish were imported frozen. In the raw-fish processing areas, the heads were cut off manually before the fish were filleted and washed using commercial machines. The fillets were arranged flat on a plastic mesh tray and brined with a covering layer of salt, powdered onion and carrot, and spices in the brining room for 3 days at 4 to 5°C. After drying for 10 h at 10°C in the drying room, they were cold smoked for 8 h at 7 to 9°C in the smoking room. The skin was removed mechanically, and the smoked fillets were sliced using commercial slicing machines, packed into plastic packs, and refrigerated for storage.

Sampling procedure. The cold-smoked fish processing plant was inspected 15 times as follows: during August, September, November, and December 2002; from May to October and in December 2003; and from January to March and in May 2004. A total of 430 samples were collected (Table 1). Samples were transported to the laboratory with ice packs in insulated boxes and were analyzed within 3 h of arrival. All of the sampling sites and machines in the production environment were swabbed with sterile cotton swabs (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) moistened with 0.1% (wt/vol) peptone water (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan). Samples (50 to 200 g) of final products, sliced but unpacked products, unfinished products, and fish trimmings were collected in sterile disposable cups or bags. Liquid samples were taken from the floor surfaces, drain water, water in the washing machine, and footbaths used for disinfection. Samples included 46 skins from both sides of 23 frozen raw fish. One sample also was taken from the boxes used to transport frozen raw fish. We also took 124 samples from the processing equipment (filleting, washing, skinning, and slicing machines). An additional 133 samples were taken from surfaces that came into contact with fish, unfinished products, and trimmings (gills, lower jaw, and skin) from raw fish, which might have been contaminated

TABLE 2. *Sample categories and types of* Listeria monocytogenes *strains*

Sample category	Total no. of samples	No. (%) of positive samples	Types found among strains
Product	59	4 (6.8)	
Sliced salmon	19	3	1, 5
Final product	40	1	1, 11
Raw fish	47	0 (0)	
Materials during processing	133	11 (8.3)	
Skins before slicing	6	4	1, 2, 5, 11
Trimmings and swab from raw salmon	31	4	1, 2, 3
Skins from brining trout	7	1	2, 11
Drying trout	2	1	2
Drying shelf	6	1	11
Others (fish contact surfaces, unfinished products, etc.)	81	0	
Processing equipment	124	14 (11.3)	
Washing conveyor after brining	7	5	2, 8, 9
Product trimmings from slicing machines	16	4	1, 2, 10, 11
Slicing machine 2	28	3	5, 11
Slicing machine 1	44	1	1
Slicing machine 3	16	1	2
Others (filleting machines, skinning machines, etc.)	13	0	
Environment	67	27 (40.3)	
Floor (swab), brining room	15	12	1, 2, 6, 7, 9, 11
Floor (surface water), raw fish processing area	14	9	1, 2, 3, 4, 5, 9, 12, 13
Floor (swab), drying room	10	3	1, 11
Floor (swab), thawing room	2	1	1
Floor (surface water), in front of the brining room	2	1	1, 9
Floor (swab), smoking room	5	1	1
Others (walls, drains, footbaths, etc.)	19	0	
Total	430	56 (13.0)	

in the plant; these samples were kept separate from those taken from the raw fish themselves. Sixty-seven samples were taken from the processing environment (e.g., floors, walls, and drains), and 59 samples were taken from finished products (19 from the sliced fish before packing and 40 from the final products). We also took duplicate swab samples from single sites, for which each swab was soaked in 10 ml of 0.1% peptone water.

Bacteriological analysis. *L. monocytogenes* strains were isolated using the enrichment procedure reported previously *(17)* with minor modifications. A 50-g aliquot of each sample was pummeled with a stomacher with 50 g of 0.1% peptone water. A 50-g portion of the stomacher fluid was then added to 450 g of *Listeria* enrichment broth (LEB; Difco, Becton Dickinson, Sparks, Md.). Next, 10 ml of the liquid samples or of the 0.1% peptone water used to soak swab samples was mixed with 100 ml of LEB (the swabs were transferred into the broth). The LEB was then incubated for 7 days at 30°C. Isolation was performed on enhanced hemolysis agar (EHA) *(2)* and CHROMagar *Listeria* (CL; CHROMagar Microbiology, Paris, France), which were incubated at 37°C for 48 h. Colonies that showed hemolysis and fluorescence under UV light on EHA and typical blue colonies forming a white halo on CL were isolated for further identification as described previously *(17).* At least 10 presumptive colonies were picked from each sample. The strains that were identified as *L. monocytogenes* were further evaluated for serotype. The identity of *L. monocytogenes* isolates was confirmed using a PCR assay targeting the 16S rRNA and listeriolysin O gene, as described by Border et al. *(3),* and by detecting the five chromosomal genes (*hlyA, plcA, plcB, mpl,* and *prfA*), as described previously *(17).* For enumeration of *L. monocytogenes* cells in positive samples, the most probable number (MPN) method was used with LEB, as described previously *(17).*

***L. monocytogenes* strain typing.** When different serotypes of strains were isolated from one sample, one or two strains of each serotype were used for molecular typing. These isolates were typed using PFGE and the three PCR-based methods, AP-PCR, REP-PCR, and ERIC-PCR, as described previously *(17).* PFGE analysis was modified so that 100 U of the *Apa*I restriction enzyme (TaKaRa Bio Inc., Kyoto, Japan) was used to prevent partial digestion rather than 50 U as in the original method *(17).* PFGE profiles that differed by three or fewer bands were regarded as indistinguishable.

Statistical analysis. The chi-square independence test and Fisher's exact probability test were used to compare the seasonal incidence of *L. monocytogenes.* Calculations were performed using Excel software combined with Statcel add-in software (Oms Publishing, Saitama, Japan).

RESULTS

The 430 samples were divided into five categories for analysis as follows: raw fish ($n = 47$), materials during processing ($n = 133$), processing equipment ($n = 124$), environment ($n = 67$), and finished products ($n = 59$). Of these samples, 56 were positive for *L. monocytogenes* (Ta-

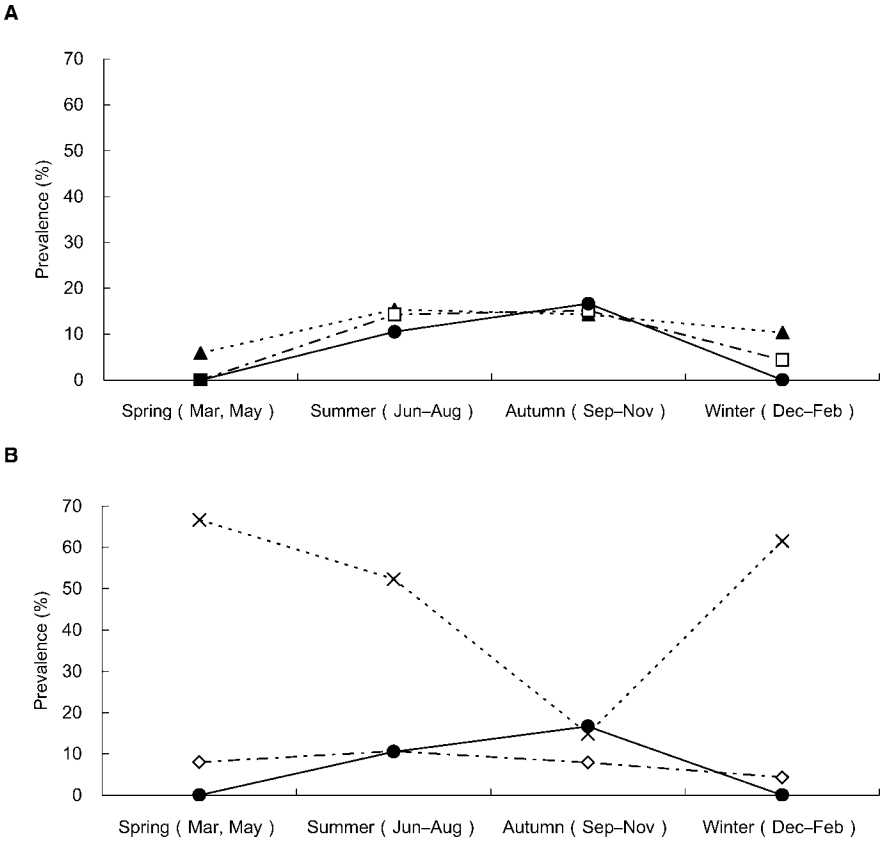

FIGURE 1. *Seasonal prevalence of* Listeria monocytogenes *in each sample category. The sample size of each category is indicated in Table 1. (A) Prevalence in all processing equipment (▲), in slicing machines only (□), and in finished products (●). Among the different types of processing equipment, the prevalence of* L. monocytogenes *in the slicing machines was relatively high. (B) Prevalence in the environment (×), in materials during processing (◇), and in finished products (●).* L. monocytogenes *was not detected on raw fish samples.*

ble 2). The highest MPN values for *L. monocytogenes* were >220/ml in floor surface-water samples from the raw-fish processing area in front of the brining room. The MPN values for *L. monocytogenes* were <0.12 to 1.72/g for both finished products and materials during processing. In the swab samples, the MPN values were <12 to 36/100 cm^2 (data not shown).

The prevalence of *L. monocytogenes* in each sample category was compared for the four seasons: spring (March to May), summer (June to August), autumn (September to November), and winter (December to February) (Table 1). *L. monocytogenes* was isolated from the finished products during summer and autumn but not during spring or winter (Table 1 and Fig. 1). The prevalence of *L. monocytogenes* was also higher in the processing equipment, especially in the slicing machine, during the warmer seasons (8 of 54 samples in summer and autumn versus 1 of 50 samples in winter and spring; $P < 0.05$; Table 1). *L. monocytogenes* was not detected in the whole skins that were removed from frozen raw fish that were still in the original boxes as received from the suppliers.

The incidence of *L. monocytogenes* on fish trimmings

TABLE 3. *Types of* Listeria monocytogenes *isolates*

Type no.	Serotype	PFGE		AP-PCR[a]	REP-PCR	ERIC-PCR	Total no. of isolates
		*Asc*I	*Apa*I				
1	1/2a	C	C	III, iii	3	3	21
2	1/2a	G	G	VII, iii	7	7	20
3	1/2a	J	J	VIII, ix	9	7	2
4	1/2a	G	G	VII, iii	10	7	1
5	1/2b	D	D	IV, iv	4	4	9
6	1/2b	F	F	VI, vi	6	6	1
7	1/2b	I	I	VI, vii	6	6	1
8	1/2b	G	G	VII, iii	7	7	1
9	3a	G	G	VII, iii	7	7	6
10	3a	H	H	VII, viii	8	7	1
11	3b	F	F	VI, vi	6	6	12
12	UT[b]	G	G	VII, iii	7	7	1
13	UT	F	F	VI, vi	6	6	1

[a] The large and small Roman numerals indicate the types obtained using the primers PJ118 and PJ108, respectively.
[b] UT, untypeable.

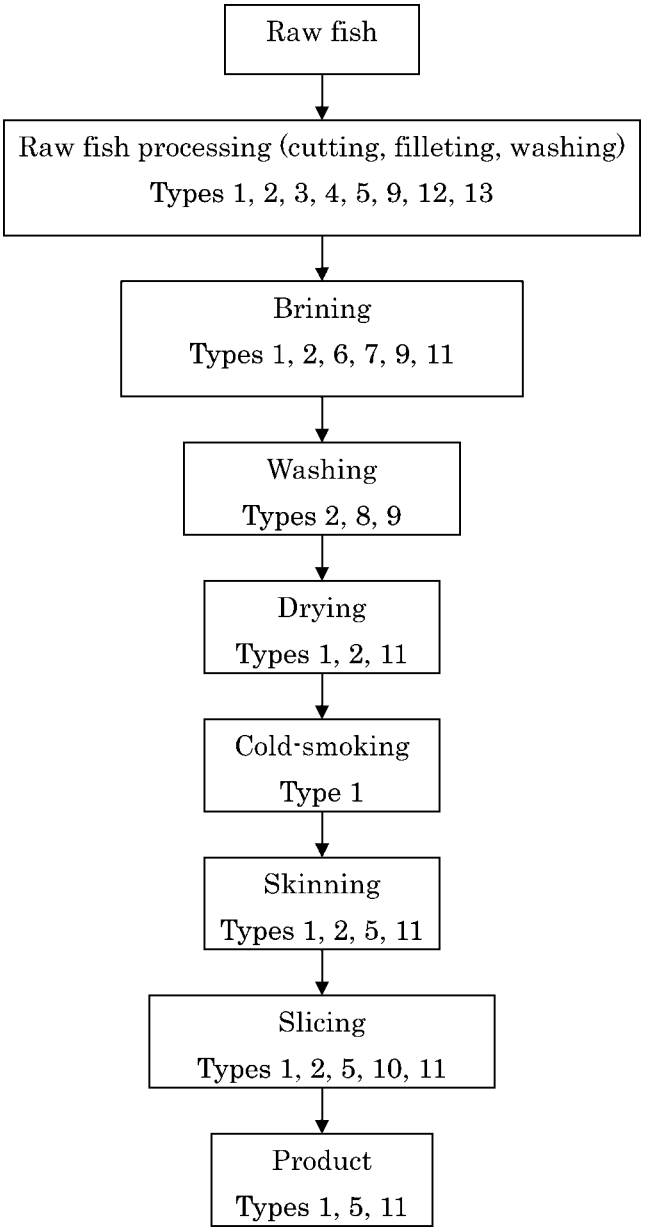

FIGURE 2. *Types of* Listeria monocytogenes *isolates found at each processing step as determined by serotyping, PFGE (*Asc*I and* Apa*I), and three PCR-based typing methods (Table 2).*

remaining in the slicing machine was relatively high at 25% (4 of 16 samples; Table 2). The slicing area at the plant was separated from the outside by a single door. During the warmer seasons, the temperature in this room became high enough to support listerial growth after the daily operations had finished. Various *L. monocytogenes* isolates from this plant were inoculated (360 to 690 CFU/g) into the fish trimmings and incubated at 30°C. The *L. monocytogenes* counts after 24 h were between 6.3 and 7.6 log CFU/g (data not shown).

A total of 77 *L. monocytogenes* isolates were identified from 56 positive samples: 2 serologically distinguishable strains were identified in each of 13 samples, 3 strains were identified in 2 samples, 5 strains were identified in 1 sample, and the other 40 strains were identified in 40 samples. These strains were classified into 13 different types by combining the results of the serotyping (1/2a, 1/2b, 3a, 3b, and untypeable), PFGE of *Asc*I and *Apa*I (seven patterns with both enzymes), AP-PCR (seven patterns with two random primers), REP-PCR (seven patterns), and ERIC-PCR (four patterns) (Table 3). Of the 77 isolates, 53 strains (68.8%) belonged to three dominant types, 1, 2, and 11 (Table 3), which prevailed in all sections of the plant. Types 1 and 11 were also isolated from the final products (Table 2 and Fig. 2). The seasonal changes in the prevalences of type 1, 2, and 11 isolates are shown in Figure 3. The prevalence of the type 1 and 11 isolates was higher among the samples from both the final products and the processing equipment during summer and autumn. Type 2 isolates were not detected in the products, even though this type was the most prevalent type at the plant. The incidence of type 2 was higher in the winter, whereas types 1 and 11 were more common during the warmer seasons (Fig. 3).

DISCUSSION

In our previous report *(17)*, we suggested that individual processing plants might harbor persistent strains of *L. monocytogenes.* We visited the plant of manufacturer A, whose commercially available products were found to be highly contaminated in our previous study *(17).* Our investigation recovered *L. monocytogenes*–positive samples from numerous sites within this plant.

Among the five sample categories examined, the prevalence of *L. monocytogenes* varied seasonally only on the processing equipment (including the slicing machines) and

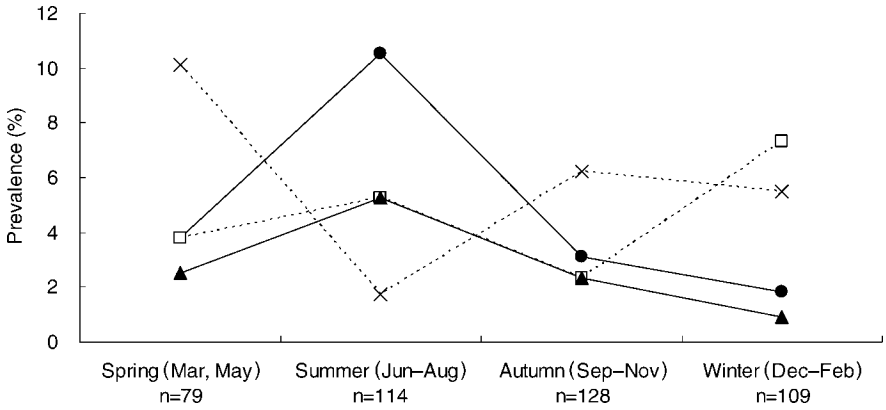

FIGURE 3. *Seasonal prevalence (%) of types of* Listeria monocytogenes *isolates: type 1 (●), type 2 (□), type 11 (▲), and other types (×).*

in the final products (Fig. 1). The subtypes of the strains isolated from the final products were identical to those of the isolates from the processing equipment (Fig. 2). The incidence of *L. monocytogenes* on fish trimmings remaining in the slicing machines was high. The level of contamination in the final products decreased from 85% (6 of 7 samples) *(17)* to 2.5% (1 of 40 samples; Table 2) after implementation of the recommendation made during our first visit that the slicing machine be disassembled and washed to ensure sanitary conditions. This circumstantial evidence suggests that the final products were probably contaminated during the slicing process. Vogel et al. *(22)* reported that the processing lines for cold-smoked salmon consist of complex equipment, such as slicing machines, that can be difficult to clean. *L. monocytogenes* strains that were adapted to this niche may have proliferated during the summer and contaminated the finished products. Thorough washing and cleaning of the slicing machines should therefore help to prevent contamination of the final products.

Throughout this study, the MPNs of *L. monocytogenes* detected from positive samples were low, except in the surface water from the floor. We could not identify the niche of *L. monocytogenes* growth in this plant on a numerical basis. Therefore, we applied molecular typing methods to *L. monocytogenes* isolates from the plant to clarify the distribution of each type and to deduce the contamination routes to the final products.

Three *L. monocytogenes* strains (types 1, 2, and 11) were frequently isolated during our investigation. These were widely distributed throughout the plant, with types 1 and 2 the most dominant (representing 21 and 20 of the 77 strains, respectively). Numerous previous studies have indicated that *L. monocytogenes* resides in cold-smoked fish processing plants in the form of persistent strains *(6, 10, 13, 21, 22).* The type 1 and 2 strains in the current plant appeared to be persistent strains. Type 1 strains had continued to be detected in final products made in this plant since our previous study in 1999 *(17).*

L. monocytogenes is capable of adhering to stainless steel and forming biofilms *(14).* Borucki et al. *(4)* reported that the persistent strains of *L. monocytogenes* produce large quantities of biofilms. The dominant strains isolated in our present study might form biofilms and persist for long periods of time within the plant, particularly in the slicing machines. These strains proliferated in the summer seasons and contaminated the products. We therefore monitored the seasonal prevalence of *L. monocytogenes.* Further studies will be required to assess the ability of these isolates to attach to material surfaces and form biofilms.

We investigated manufacturing plant A from 1999 onward after we previously found its products to be highly contaminated *(17).* We were unable to identify the niche in which huge numbers of *L. monocytogenes* organisms had prospered. However, a combined analysis of the seasonal prevalence of this bacterium and molecular typing of the isolates in the plant suggested that the product contamination was associated with the slicing machines. Implementation of an effective washing and cleaning regime for the slicing machines resulted in a marked decrease in the incidence of *L. monocytogenes* contamination of the finished products.

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