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Elucidation of *Listeria monocytogenes* Contamination Routes in Cold-Smoked Salmon Processing Plants Detected by DNA-Based Typing Methods

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The contamination routes of *Listeria monocytogenes* in cold-smoked salmon processing plants were investigated by analyzing 3,585 samples from products (produced in 1995, 1996, 1998, and 1999) and processing environments (samples obtained in 1998 and 1999) of two Danish smokehouses. The level of product contamination in plant I varied from 31 to 85%, and no *L. monocytogenes* was found on raw fish (30 fish were sampled). In plant II, the levels of both raw fish and product contamination varied from 0 to 25% (16 of 185 raw fish samples and 59 of 1,000 product samples were positive for *L. monocytogenes*). A total of 429 strains of *L. monocytogenes* were subsequently compared by random amplified polymorphic DNA (RAPD) profiling, and 55 different RAPD types were found. The RAPD types detected on the products were identical to types found on the processing equipment and in the processing environment, suggesting that contamination of the final product (cold-smoked salmon) in both plants (but primarily in plant I) was due to contamination during processing rather than to contamination from raw fish. However, the possibility that raw fish was an important source of contamination of the processing equipment and environment could not be excluded. Contamination of the product occurred in specific areas (the brining and slicing areas). In plant I, the same RAPD type (RAPD type 12) was found over a 4-year period, indicating that an established in-house flora persisted and was not eliminated by routine hygienic procedures. In plant II, where the prevalence of *L. monocytogenes* was much lower, no RAPD type persisted over long periods of time, and several different *L. monocytogenes* RAPD types were isolated. This indicates that persistent strains may be avoided by rigorous cleaning and sanitation; however, due to the ubiquitous nature of the organism, sporadic contamination occurred. A subset of strains was also typed by using pulsed-field gel electrophoresis and amplified fragment length polymorphism profiling, and these methods confirmed the type division obtained by RAPD profiling.

Listeria monocytogenes is a gram-positive, food-borne pathogen that is widely distributed in the environment and occurs naturally in many raw foods. *L. monocytogenes* is psychro- and halotolerant (33) and consequently can grow in many lightly salted and chilled food products, which often have extended shelf lives (3, 11). Products which do not receive any heat treatment by the consumer, so-called ready-to-eat products like cheese, meat, and fish delicatessen products, may contain high levels of *L. monocytogenes* when they are eaten. Ingestion of high numbers of *L. monocytogenes* cells is a significant health threat for people in risk groups such as immunocompromised and elderly groups. In these groups, the rate of mortality from listeriosis is high, typically 20 to 30% (13). Also, infection by *L. monocytogenes* in pregnant woman may cause abortion, stillbirth, or delivery of an acutely ill baby (11). A diverse range of foods has been associated with both outbreaks and sporadic cases of listeriosis (25).

Ready-to-eat fish products like cold-smoked fish have been linked to sporadic cases of listeriosis, and more recently, epidemiological evidence has suggested that listeriosis has been caused by smoked mussels (4), gravad trout (9), and smoked trout (26). As such products support growth of *L. monocytogenes* (20), it is crucial to reduce the prevalence and level of this organism to an absolute minimum. An important prerequisite for control of *L. monocytogenes* is knowledge concerning its niches during food production. Cold-smoked salmon has become a major fish delicatessen commodity, and as the source of *L. monocytogenes* contamination during cold-smoked salmon production is not known, we focused on elucidating the contamination routes of this bacterium in cold-smoked salmon processing environments.

L. monocytogenes occurs naturally in fish raw materials, and Farber (10) reported the presence of *L. monocytogenes* in salmon from the United States, Chile, Norway, and Canada. The prevalence of *L. monocytogenes* in raw fish is quite low, ranging from 0 to 1% (2, 21) to 10% (20), but a higher prevalence can probably occur in fish from bodies of water that receive heavy runoff from land (18). *L. monocytogenes* may be detected in a large proportion of freshly produced cold-smoked fish; typically, it is detected in 10 to 40% of samples (2, 22). Great plant-to-plant variation is seen; some production plants are virtually free of *L. monocytogenes*, and others have a prevalence close to 100% (20, 22).

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TABLE 1. Numbers of samples, numbers of *L. monocytogenes*-positive samples, and distribution of RAPD types for *L. monocytogenes* isolates from processing plant I

Date	Sampling site	Total no. of samples	No. of *L. monocytogenes*-positive samples	No. of isolates with the following RAPD types as determined with HLWL85:							
				RAPD type 2	RAPD type 6	RAPD type 7	RAPD type 12	RAPD type 13	RAPD type 15	RAPD type 110	RAPD type x
1995	Product	20	17			1	16				
1996	Product	20	13				13				
November 1998	Raw fish	18	0								
	Raw fish area	239	55	5	36	4	1	1	6		2
	Smoking area	8	0								
	Slicing area 1	150	80	3	1		63	1	10		2
	Slicing area 2	147	39				37		1	1	
	Product	40	15	7			7		1		
March 1999	Raw fish	12	0								
	Raw fish area	105	17	3		5			6		3
	Smoking area	2	0								
	Slicing area 1	75	9				6		3		
	Slicing area 2	100	3				2		1		
	Product	48	15			6	2		7		

A number of studies have shown that *L. monocytogenes* is able to reside in food processing plants, including poultry production plants (24, 29, 40), meat processing plants (15, 28), ice cream plants (27), shrimp peeling plants (5), and plants in which gravad (2) and smoked trout (30) are produced. While some studies have pointed to raw materials as the most likely sources of product contamination with *L. monocytogenes* (7, 15), other studies have found that the major source of contamination is the processing environment and equipment (2, 21, 30, 40).

Early studies of contamination routes depended solely on isolating and counting the organism at different places along the processing line (7), whereas recent studies have been greatly facilitated by the use of molecular typing methods with high discriminatory power. These methods have included pulsed-field gel electrophoresis, (PFGE) (2, 5, 27, 29) and randomly amplified polymorphic DNA, (RAPD) profile analyses (5, 24, 39). The amplified fragment length polymorphism (AFLP) technique, which is a very powerful molecular typing technique (19), has not been used previously to trace contamination routes of *L. monocytogenes* but has been used successfully to fingerprint *Pseudomonas* isolates from a poultry processing plant (14).

The purpose of the present study was to elucidate the *L. monocytogenes* contamination routes during the production and processing of Danish cold-smoked salmon. *L. monocytogenes* was isolated from cold-smoked salmon from two Danish smokehouses in 1995 and 1996, and the processing environments of these plants, as well as the final products, were sampled in 1998 and 1999. A total of 429 strains of *L. monocytogenes* were subsequently grouped by RAPD profiling. Concerns about the reproducibility and stability of this method have been raised (41); however, careful standardization allowed us to reproducibly obtain a high level of discrimination with this method (12). To confirm clonal separation, a subset of strains (mainly the persistent types) was also characterized by PFGE and AFLP profiling.

MATERIALS AND METHODS

Processing plants and product manufacturing. The raw material was farm (ocean)-raised salmon (*Salmo salar*) from Norway and the Faroe Islands. The fish were gutted; they were then transported to the smokehouses stored in ice, or occasionally they were stored and transported frozen (less than 10% of the fish). Fish stored in ice were used 1 to 5 days after slaughter. The ambient temperatures in both plants were between 10 and 17°C.

In the raw fish processing areas of plant I, head cutting, filleting, brining (injection of saturated brine), and removal of the skin were done by commercial machines in one flow. The fillets were held in a saturated salt solution from a few minutes to 2 h before cold smoking (16 h at 22°C). The amount of brine and salting was adjusted to obtain an NaCl level of approximately 4% (water phase salt) in the final product. The smoked fillets were quick frozen and stored for a few days at −18°C. The fish were sliced and packed in a building separate from the building in which raw fish were handled and smoking took place. The fish were manually trimmed before slicing. Each of the two slicing areas contained four commercial slicing machines (Mass) coupled in pairs to two production lines. The sliced smoked fish were separated into 100-, 150-, or 200-g portions before vacuum packing.

In the raw fish processing area of plant II the head of each salmon was cut off by hand, and the fish were washed in a commercial fish washer. The fish were mechanically filleted, brined (saturated brine) with commercial injection machines, and manually trimmed. The brined fillets were ripened for 18 h at 0°C with a cover layer of salt. The amount of brine and salting was adjusted to obtain an NaCl level of approximately 4% (water phase salt) in the final product. After cold-smoking (16 h at 22°C), the skin was mechanically removed, and the fillets were quick frozen and stored for a few days at −18°C. The fillets were sliced with three commercial slicing machines (Mass) in the slicing area of plant II and were manually separated into 50- 100- or 200-g portions before vacuum packing. The different processing operations took place in different rooms with a continuous process flow, and there was negative airflow in the slicing area. Thus, care was taken to separate the raw material from the processed material. A strict procedure regarding personal hygiene was followed. Disinfecting foot baths were installed at the entrance to each area, and all employees wore gloves which were changed every 1.5 h at each break. The slicing machines were cleaned and disinfected with ethanol twice a day.

A complete cleaning and disinfecting procedure was carried out in both plants at the end of each production day, fulfilling legal requirements, and once a week a decalcification procedure was performed. All removable parts of the machines and conveyer belts were cleaned and disinfected separately. Cleaning was performed by using low pressure and foam cleaners. In plant I primarily sodium hypochlorite was used as the sanitizing agent, whereas in plant II peracetic acid was used.

Sampling procedure. A total of 944 and 869 samples were collected during two visits at processing plants I and II, respectively (Tables 1 and 2). The plants were visited at times when there was high throughput (before Christmas) and at times when there was less activity (during the spring). The same sampling strategy was

TABLE 2. Numbers of samples, numbers of *L. monocytogenes*-positive samples, and distribution of RAPD types for *L. monocytogenes* isolates from processing plant II

Date	Sampling site	Total no. of samples	No. of *L. monocytogenes*-positive samples	No. of isolates with the following RAPD types as determined with HLWL85:	
				RAPD type 2	RAPD type x
February 1995	Product	10	0		
November 1996	Product	10	0		
October 1998	Raw fish	15	1	1	
	Raw fish area	197	29	25[a]	4
	Smoking area	60	5	5	
	Slicing area	211	9	7	2
	Product	45	8	8	
March 1999	Raw fish	14	0		
	Raw fish area	151	5	2	3
	Smoking area	6	0		
	Slicing area	135	0		
	Product	35	1		1

[a] Fourteen of the strains were isolated from the brine solution or from surfaces of the brine injector.

used at both plants. Samples were taken at control points, which were contact surfaces during different production steps, at the start and at the end of production for 2 to 3 days. Cleaning control samples for equipment surfaces were taken once during each visit after disinfection and just before the start of production. Samples were also obtained before and after interval cleaning of slicing machines in processing plant II. The sampling areas varied depending on the sampling location. The smallest areas were screw heads (area approximately 1 cm^2); the largest areas were surfaces of conveyor belts (area up to 1 m^2). Other product samples from processing plants I and II were also analyzed (Tables 1 and 2). During each visit a number of whole raw fish (*S. salar*) were selected, and samples of these fish were taken from the raw fish during production; samples of the final products were also taken (Tables 3 and 4). In addition, we took swab samples from surfaces (equipment surfaces, conveyor belts, gloves, etc.) that came into contact with these fish and of the processing environment (walls, tables, below machines, trucks, drains, etc.) when the fish were processed. At plant I, 18 and 12 fish were monitored during the two visits, and at plant II 15 and 14 fish were monitored (Tables 3 and 4).

All sampling sites in the production environment, machines, and aprons of employees were swabbed with sterile cotton swabs or cellulose sampling sponges (Bio-spo CS-100; Solar Biologicals Inc., Ogdensburg, N.Y.) moistened with 0.1% peptone–0.85% saline. Sampling of surface areas after disinfection was done with sterile sponges premoistened with neutralizing buffer (Bio-spo BS-10NB; Solar Biologicals Inc.). After sampling the swabs and sponges were soaked in 20 and 40 ml of *Listeria* selective medium (UVM 1) [see below], respectively, and kept at 10 to 15°C during transport to the laboratory. Samples of raw fish and samples after every processing step were collected by scraping the surface with a sterile jagged knife, and each of these samples was mixed with 20 ml of UVM 1. Gloves of the fish handlers were placed in sterile plastic bags containing 40 ml of UVM 1; we made sure that only the outer surfaces of the gloves were in contact with the liquid. Twenty milliliters of saturated brine was mixed with 100 ml of brain heart infusion (CM225; Oxoid Ltd., Basingtake, Hampshire, England) to dilute the salt. After incubation at 30°C for 24 h, 1 ml was mixed with 20 ml of UVM 1. Samples of the final product that had been stored at 5°C for 1 and 21 days were examined by combining cross sections from two packages into one 25-g sample that was suspended in 225 ml of UVM 1.

As part of its quality assurance program, processing plant II has a standard sampling regime for *L. monocytogenes*. A total of 1,712 samples were collected by the processor as part of routine checks during a 10-month period (Table 5). Twenty-five-gram samples were cut off the bellies of raw fish and 25-g samples were also collected from products and suspended in 225 ml of UVM 1. Samples taken from the production environment during production and after cleaning and disinfection were swabbed with sterile cotton swabs and soaked in UVM 1.

Bacteriological analyses. *L. monocytogenes* strains were detected (and isolated) by a two-step enrichment procedure. Samples were mixed with modified University of Vermont broth 1 (Oxoid *Listeria* enrichment broth base [CM863] with Oxoid *Listeria* selective supplement UVM 1 [SR 142]) and incubated for 24 h at 30°C. One milliliter was transferred to 10 ml of modified University of Vermont broth 2 (Oxoid *Listeria* enrichment broth base [CM863] with Oxoid *Listeria* selective supplement UVM 2 [SR 143]) and incubated for 24 h at 30°C. If visible growth occurred with UVM 2, a loopful was plated on Oxford selective agar (*Listeria* selective agar base [CM856] with *Listeria* selective supplement Oxford [SR 140]; Oxoid) and incubated at 37°C for 48 h. Three presumptive *Listeria* colonies per plate were identified as *L. monocytogenes* colonies, by beta-hemolysis, positive Gram and catalase tests, motility (as determined by phase-contrast microscopy), and the ability to ferment rhamnose and methyl mannoside but not xylose. Isolates collected at processing plant II (Table 5) were verified by the laboratory of the processor as *L. monocytogenes* isolates by using the Accuprobe *L. monocytogenes* culture identification test (Gen-Probe Inc., San Diego, Calif.). Thirty strains from products from processing plant I isolated in November 1995 or April 1996 (12) were also included.

RAPD typing. RAPD analysis was performed as described previously (12). Briefly, DNA was prepared with Dynabeads DYNAL DIRECT system 1 (Dynal A/S & Nordic, Oslo, Norway). Two microliters of the DNA preparation was transferred to a PCR tube containing a PCR mixture (Ready-To-Go RAPD analysis beads [Amersham Pharmacia Biotech, Inc., Piscataway, N.J.]) dissolved in 23 μl of Milli Q water along with 4 μM primer HLWL85 (5′-ACAACTGCTC; DNA Technology, Aarhus, Denmark). The PCR was performed with a thermocycler (model 9600; Perkin-Elmer, Norwalk, Conn.) by using 45 cycles of 1 min

TABLE 3. Incidence of RAPD types for *L. monocytogenes* strains from samples obtained at different production stages for 18 fish monitored during production in plant I in November 1998

Sampling site	Sample source	Total no. of samples	No. of positive samples	No. of isolates with the following RAPD types:			
				RAPD type 2	RAPD type 6	RAPD type 12	RAPD type 15
Raw fish area	Raw fish	18	0				
	Contact surfaces[a]	20	5		4		1
	Production environment[b]	52	5	3	2		1
Smoking area	Fish after smoking	4	0				
Slicing area 1							
Slicer 1	Contact surfaces[a]	9	5	3		2	
	Final product	12	7	6		1	
Slicer 2	Contact surfaces[a]	7	3			2	1
	Final product	12	8	1		6	1
	Production environment[b]	20	5		1	3	1
Slicing area 2, slicer 6							
	Contact surfaces[a]	14	7			7	
	Final product	12	0				
	Production environment[b]	24	6			6	

[a] Surfaces which were in contact with the unfinished product (equipment surfaces, conveyor belts, gloves of employees, etc.).
[b] Production environment (walls, tables, undersides of machines, trucks, drains, etc.).

at 95°C, 2 min at 35°C, and 1 min at 72°C, followed by 10 min at 72°C. Amplification products were visualized after electrophoresis in a 2% agarose gel by staining with ethidium bromide. A 100-bp ladder (Amersham Pharmacia Biotech Inc.) was included three times in each agarose gel as a standard. RAPD reaction mixtures without bacterial DNA and DNA preparations from *L. monocytogenes* strains La22 and La150 (isolated from cold-smoked salmon) and H4239 (isolated from a human case in Denmark in 1998) were used as negative and positive controls, respectively

PFGE typing. PFGE was performed as described previously (29), with a few modifications. The restriction enzyme used was *Apa*I (Boehringer, Mannheim, Germany). The restriction fragments were separated by using the polygonal contour-clamped homogeneous electric field system (CHEF-DR III; Bio-Rad, Richmond, Calif.). The initial pulse time was 1 s, and the final time was 15 s. The running time was 20 h.

AFLP analysis. All AFLP procedures were performed as described by Kokotovic et al. (23); however, the enzyme combination was changed, and consequently the primers were also changed. Genomic DNA were extracted from strains of *L. monocytogenes* by using an Easy-DNA kit (Invitrogen, De Schelp, The Netherlands) according to the manufacturer's instructions. Bacterial DNA (100 to 500 ng) was digested with 10 U of *Bam*HI and 10 U of *Eco*RI at 37°C for 3 h in *Eco*RI buffer (New England Biolabs, Beverly, Mass.). Double-stranded adapters were prepared by mixing equimolar amounts of corresponding oligonucleotides (Table 6) and were incubated for 10 min at 65°C and cooled for 15 min at room temperature. Adapters were ligated to the restriction fragments by using a 20-μl (total volume) reaction mixture containing 5 μl of the digested DNA, 2.6 pmol of the BKO-RC adapter, 26 pmol of the BKO-FC adapter, 1 U of T4 DNA ligase, 2 μl of 10× ligase buffer (Amersham Pharmacia, Cleveland, Ohio), and 8 μl of TAC buffer (38). Ligation was carried out overnight at room temperature. Two microliters of 10-fold-diluted ligation product was transferred to a PCR tube containing 48 μl of a PCR mixture containing (final concentrations) 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, each of the four deoxynucleoside triphosphates (Perkin-Elmer) at a concentration of 200 μM, 130 ng of primer BamHI0 (DNA Technology), 130 ng of primer EcoRIO-F (labelled at the 5′ end with 6-carboxyfluorescein; Oswell DNA Services, Ltd., Southampton, United Kingdom), and 0.3 U of *Taq* polymerase (Perkin-Elmer). Fragments were amplified with a thermocycler (Biometra T3 thermocycler) by using 3 min at 94°C, followed by 23 cycles of 60 s at 94°C, 60 s at 54°C, and 90 s at 72°C and then 10 min at 72°C. The amplification products (1-μl portions of PCR products and 0.25-μl portions of internal-lane size standards labelled with ROX, [GeneScan-500 ROX size standard; Perkin-Elmer Applied Biosystems, Warrington, England]) were analyzed by electrophoresis in 5% denaturing polyacrylamide gels for 3.5 h using an ABI 377 automated sequencer (Perkin-Elmer). The AFLP patterns were collected with the GeneScan software (Perkin-Elmer Applied Biosystems). DNA preparations obtained from *L. monocytogenes* strains La22 and La150 (isolated from cold-smoked salmon) and H4239 (isolated from a human case in Denmark in 1998) were included in each gel to control reproducibility.

Numerical pattern analysis. Photographs of RAPD patterns were scanned with a Pharmacia DeskTop scanner (Pharmacia Biotech) and transferred the PC Windows software package GelCompar (version 4.1; Applied Maths, Kortrijk, Belgium) as tiff files. AFLP patterns were transferred as densitometric values to GelCompar (version 4.1), and gels were normalized by using the internal size standard ROX-500 (Perkin-Elmer Biosystems), which was added to each lane. RAPD gels were normalized by using a 100-bp ladder (Pharmacia Biotech) that was included three times in each agarose gel as a standard. The data analyzed were transferred to the Bionumerics software (Applied Maths). Photographs of PFGE patterns were scanned (Vista scan; Umas Data Systems, Inc.) and transferred to Bionumerics as tiff files. Normalization was done with a Low Range PFG marker (New England Biolabs) that was included in every sixth lane as a standard. Grouping was performed by using the Dice coefficient and cluster analysis by the unweighted pair group method using arithmetic averages. One band difference was used to differentiate between types of RAPD, PFGE, and AFLP patterns. When at least two patterns were allocated to the same type, the type was given a number designation. When a pattern was obtained for only one strain, the type was designated x. Thus, x indicates a multitude of different types, each represented by only one strain. The band tolerances (maximum tolerance expressed as a percentage of the curve to match bands) for the RAPD and PFGE patterns were 3 and 1% of the band tolerance for the AFLP patterns.

RESULTS

Prevalence of *L. monocytogenes*. A total of 3,585 samples were tested. Of these, 60 samples were obtained in 1995 and 1996 (22), while 944 and 869 samples were obtained in 1998 and 1999 from two visits to each of the two cold-smoked salmon processing plants, respectively. During a 10-month routine inspection 1,712 samples were taken by the processor at plant II.

In November 1998, 602 samples were taken from plant I, and 189 of these samples (approximately 31%) were positive for *L. monocytogenes* (Table 1). For the second visit in March 1999, of 342 samples (approximately 13%) were positive for *L. monocytogenes*. At plant II 52 of 528 samples (10%) were *L. monocytogenes* positive in October 1998 (Table 2), whereas 6 of 341 samples (2%) were positive in March 1999. During these four visits, a total of 59 raw fish were sampled, and 1 was found to contain *L. monocytogenes*.

At plant II, a more frequent sampling regime was undertaken by the processor (Table 5). During the period from June 1998 to March 1999, a total of 1,712 samples were analyzed, and 123 of the samples were positive for *L. monocytogenes*. A total of 108 isolates were obtained from these samples. The prevalence varied from 1 to 25% in the final product and from 0 to 25% in raw fish.

RAPD profiling of *L. monocytogenes* isolated at plants I and II. One *L. monocytogenes* isolate from each of 429 of the 444 positive samples was analyzed by the RAPD technique performed with primer HLWL85. The 429 strains were divided into 55 different RAPD profiles, and 41 of these profiles included only one strain (designated type x in Tables 1 to 3, 5, and 7), and RAPD type 12 included as many as 166 strains. The RAPD profiles of five strains isolated from processing plant I and five strains isolated from processing plant II are shown in Fig. 1.

During the visits to plant I in November 1998 and March 1999, RAPD type 12 dominated, and 118 of 233 strains were this type. This type was also dominant in products from plant I sampled in 1995 and 1996; 29 of the 30 strains examined were RAPD type 12 (Table 1). RAPD types 2, 6, 7, and 15 were typically isolated from the raw fish processing area of plant I (Table 1). RAPD type 2 was the dominant type in plant II during the visits in 1998 and 1999, representing 48 of 58 strains (Table 2).

TABLE 4. Incidence of *L. monocytogenes* RAPD types from samples obtained at different production stages for 15 fish monitored during production from plant II in October 1998

Sampling site	Sample source	Total no. of samples	No. of positive samples	No. of isolates with the following RAPD types:	
				RAPD type 2	RAPD type x
Raw fish area	Raw fish	15	1	1	
	Contact surfaces[a]	18	3	3	
	Production environment[b]	114	18	17	1
Smoking area	Fish after drying	6	3	3	
	Fish after smoking	11	1	1	
Slicing area	Production environment[b]	68	1	1	
	Contact surfaces[a]	11	3	3	
	Final product	45	8	8	

[a] Surfaces which were in contact with the unfinished product (equipment surfaces, conveyor belts, gloves of employees, etc.).
[b] Production environment (walls, tables, machines, trucks, drains, etc.).

TABLE 5. Numbers of samples, numbers of *L. monocytogenes*-positive samples, and distribution of RAPD types for *L. monocytogenes* isolates from processing plant II[a]

Date	Sampling site	Total no. of samples	No. of positive samples	No. of isolates with the following RAPD type as determined with HLWL85											
				RAPD type 2	RAPD type 7	RAPD type 12	RAPD type 103	RAPD type 105	RAPD type 106	RAPD type 107	RAPD type 108	RAPD type 109	RAPD type 110	RAPD type 127	RAPD type x
June 1998	Raw fish	8	2												2
	Processing surfaces	20	3						1		1			1	
	Processing surfaces after cleaning	0	0												
	Final product	105	6						1	3					
July 1998	Raw fish	1	0												2
	Processing surfaces	0	0												
	Processing surfaces after cleaning	0	0												
	Final product	24	6	2				3							1
August 1998	Raw fish	10	0												
	Processing surfaces	30	7												
	Processing surfaces after cleaning	50	0								1			2	4
	Final product	138	7	1				5			1				
October 1998	Raw fish	27	4[b]											1	
	Processing surfaces	25	1			1							1		
	Processing surfaces after cleaning	0	0												
	Final product	143	5		1		2								
November 1998	Raw fish	41	1												2
	Processing surfaces	48	9[b]	1											1
	Processing surfaces after cleaning	66	0												
	Final product	137	2			1		1							1
December 1998	Raw fish	21	4[b]		1										
	Processing surfaces	8	0												
	Processing surfaces after cleaning	82	2		1										1
	Final product	91	10	2	2								1		
January 1999	Raw fish	13	2[b]										3		3
	Processing surfaces	14	1		1										1
	Processing surfaces after cleaning	16	5	2	1	1									
	Final product	69	6	1	4							1			
February 1999	Raw fish	8	1			1									1
	Processing surfaces	42	19	2	6	7						1			3
	Processing surfaces after cleaning	45	9	3	2	1						3			
	Final product	118	7[b]			5									
March 1999	Raw fish	27	1												1
	Processing surfaces	94	1		1										1
	Processing surfaces after cleaning	116	1			1									
	Final product	75	1			1									

[a] Data for samples taken by the processor during a 10-month period as part of routine checking.
[b] The RAPD types of some strains were not determined.

(i) Contamination routes in plant I. In processing plant I, RAPD types 2, 7, 12, and 15 were found in final products that were produced from salmon that were free of *L. monocytogenes* (in November 1998 and March 1999). These RAPD types were also found in many samples obtained from the processing environment, whereas the raw fish area harbored different RAPD types. RAPD type 6 (except for one strain) was found only in the raw fish processing area of plant I was not found in the slicing area or in the products. RAPD type 7 was found only in the raw fish processing area and in the products. When 18 raw fish (all with no detectable *L. monocytogenes*) were monitored throughout processing (Table 3), they were contaminated with RAPD type 2, 6, and 15 strains in the raw fish processing area. No *L. monocytogenes* could be detected after smoking, but RAPD type 12 was isolated from slicing machines when the fish were sliced and subsequently from smoked salmon produced from the 18 fish (Table 3). Six of seven *L. monocytogenes* strains in products from slicing machine 1 were RAPD type 2 (Table 3). This type was also isolated from three contact surfaces related to slicing machine 1. These surfaces included specific components of slicing machine 1 that were in direct contact with the unfinished product and gloves from the worker who had handled the product. RAPD type 2 was not found elsewhere in the slicing areas but was isolated in the raw fish area from three samples obtained from the filleting machine on the same day that the 18 fish were processed (Table 3). RAPD type 15 was found in a product sample sliced by slicing machine 2, and this type was found on different machines and equipment in both the raw fish and slicing areas.

(ii) Contamination routes in plant II. *L. monocytogenes* RAPD type 2 was the dominant type during the visits to plant II in October 1998 and March 1999, and only few sporadic

TABLE 6. Adapter and primer oligonucleotides used for AFLP DNA typing of *L. monocytogenes* from cold-smoked salmon processing plants

Oligonucleotide	Sequence(s)
AFLP adapters	
BKO-FC adapter[a]	5′AATTCCAAGAGCTCTCCAGTAC 3′, 3′GGTTCTCGAGAGGTCATGAT 5′
BKO-RC adapter[b]	5′CGGACTAGAGTACACTGTC 3′, 3′CTGATCTCATGTGACAGCTAG 5′
AFLP primers	
Pr-BamHIO	5′GAGTACACTGTCGATCC 3′
Pr-EcoRIO-F	5′GGAGAGCTCTTGGAATTC 3′

[a] MFE adaptor (23).
[b] BGL adaptor (23).

non-type 2 strains were obtained (Table 2). Following 15 specific fish through plant II in October 1998, we found only one sample of raw fish contaminated with *L. monocytogenes*, whereas 8 of 45 samples of the final product harbored this type (Table 4). Several RAPD type 2 *L. monocytogenes* strains were found in the raw fish area and on contact surfaces where processing occurred. In particular, samples from the brine injector needles and the recirculated brine solution and some other samples from the brine injector harbored this type. RAPD type 2 could also be found in dried and smoked fillets and in the final product.

Change in RAPD types over time at plant II. During the routine sampling regimen conducted by the processor at plant II from June 1998 to March 1999, several different RAPD types were detected (Table 5). RAPD types 2, 7, and 12 were all found in the final products and in the processing environment (Table 5). Thus, despite recurring isolation of RAPD type 2 during visits in October 1998 and March 1999, other RAPD types were present in plant II. Specifically, RAPD type 2, which was detected during intensive sampling along the processing lines (Table 3), was not found by the processor on other days in October 1998 and March 1999. RAPD types 7 and 12 were found in the raw fish. Seventeen strains (designated RAPD type X) with distinct, different RAPD profiles were found in raw fish or in the final products.

Comparison of typing methods. Ninety-five and 30 randomly selected strains were also typed with the PFGE and AFLP techniques, respectively (Table 7). A subset of 30 strains typed by the RAPD, PFGE, and AFLP techniques is shown in Fig. 2. PFGE and AFLP fingerprinting resulted in the same groups as RAPD typing, with only two exceptions. Strain V190a was identified as RAPD type 6, and the RAPD pattern of this strain was similar to the RAPD patterns of strains V5a, V110a, and V607a; however, PFGE and AFLP data separated V190a from the other three strains (Table 7). Similarly, the RAPD pattern of strain 2V920b was identical to the RAPD patterns of strains identified as RAPD type 12, whereas the PFGE and AFLP patterns separated strain 2V920b from strains La22, V203a, V417a, V455a, V477a, V517b, and 2V575a (PFGE type 36 and AFLP type 12) (Table 7).

TABLE 7. Comparison of RAPD, PFGE, and AFLP analysis results for selected strains of *L. monocytogenes* isolated from cold-smoked salmon and processing environments

Date	Strain	Origin		RAPD type	PFGE type	AFLP type
		Processing plant	Site			
November 1996	R77a	II	Rubber plate before brine injector	2	2	2
	R108b	II	Brine injector needles	2	2	2
	R207a	II	Product after drying	2	2	2
	R416a	II	Slicing machine 2	x[a]	x	x
	R479a	II	Final product	2	2	2
March 1999	2R167a	II	Footstool	2	2	2
October 1995	La22	I	Final product	12	36	12
October 1998	V62a	I	Dusch at maincut	2	2	2
	V608a	I	Stainless steel surface near brine injector	2	2	2
	V5a	I	Baader	6	6	6
	V110a	I	Recycling NaCl solution used for brining	6	6	6
	V607a	I	Plastic surface near brine injector	6	6	6
	V190a	I	Slide board at maincut	6	x	x
	V511a	I	Slicing machine 1, inside surface	7	42	7
	V203a	I	Slicing machine 2, inside surface	12	36	12
	V417a	I	Slicing machine 5, inside surface	12	36	12
	V455a	I	Slicing machine 2, trim board	12	36	12
	V477a	I	Slicing machine 5, inside surface	12	36	12
	V517a	I	Final product from slicing machine 2	12	36	12
	V408a	I	Slicing machine 2, inside surface	15	15	15
	V410a	I	Line 1 conveyor belt	15	15	15
	V104a	I	Sprinkler near head cutter	15	15	15
	V129a	I	Knife used for triming	15	15	15
	V201a	I	Slicing machine 2, inside surface	15	15	15
	V518a	I	Final product from slicing machine 2	15	15	15
March 1999	2V508a	I	Head cutter	2	2	2
	2V575a	I	Slicing machine 2, inside surface	12	36	12
	2V920b	I	Final product from slicing machine 2	12	x	x
	2V903b	I	Final product	15	15	15
	2V556b	I	Slicing machine 2, outside surface	15	15	15

[a] Type x indicates a type that was found only once.

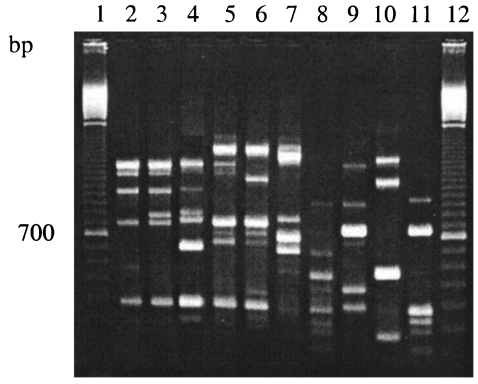

FIG. 1. RAPD patterns of 10 isolates of *L. monocytogenes* from samples of cold-smoked salmon from processing plants I and II generated with primer HLWL85. Lanes 1 and 12, 100-bp ladder (Pharmacia); lanes 2 to 6, strains from plant I (RAPD types 2, 6, 12, 13, and 15, respectively); lanes 7 to 11, strains from plant II (RAPD types 105, 107, 108, 110, and 127, respectively).

DISCUSSION

Prevalence of *L. monocytogenes*. The prevalence of *L. monocytogenes* in cold-smoked salmon has been shown to vary from 0 to 100% in the final product (22). In the present study, we found that the prevalence in a processing plant may vary significantly over time. The higher number of positive samples found during the first visits than during the second visits in March 1999 could reflect the fact that October and November are very busy periods with both day and evening shifts. Consequently, disinfection of the whole plant cannot take place between shifts, and this could explain the higher level of *L. monocytogenes* in November. In general, the prevalence in the raw fish was low, which has been reported in other studies (30).

RAPD profiling and *L. monocytogenes* contamination routes. In agreement with our earlier study (12), we found that different *L. monocytogenes* RAPD types were dominant in vacuum-packed cold-smoked salmon from different Danish smokehouses. The present study showed that the RAPD types found in the product from a particular plant are also associated with the specific processing environment; i.e., RAPD type 12 appeared to have colonized plant I, and RAPD type 2 was dominant in plant II. The presence of a few dominant clones or closely related strains in food processing plants has been reported for a Norwegian salmon smokehouse (30), poultry abattoirs (29), and pork slaughtering and cutting plants (15). It is not known if RAPD type 2 and 12 strains are the most common *L. monocytogenes* strains in Denmark or if they are strains with a special ability to adapt the processing environment. Rørvik et al. (30) determined by using multilocus enzyme electrophoresis that the most common electrophoretic type in Norway seemed to have colonized a smokehouse.

To reduce the number of *L. monocytogenes* cells, the source of contamination must be known. Products from plant I which were produced from salmon free of *L. monocytogenes* were contaminated with RAPD types identical to those found in the processing environment, specifically in the slicing area. Processes before slicing did not contribute to contamination with RAPD type 12. These results indicate that the slicing machines can spread a certain type and may be a reservoir for *L. monocytogenes* (Table 3). In plant II, the bringing process may have contributed to contamination of the product, as *L. monocytogenes* was isolated from brine and injection needles. Autio et al. (2) similarly found that the predominant *L. monocytogenes* pulsotypes in cold-smoked trout final products were associated with brining and slicing. In general, our results indicate that contamination occurs downstream along the processing line. Other studies dealing with different found processing operations have similarly concluded that the plant and processing environment rather than the raw material is the source of product contamination with *L. monocytogenes* (2, 15, 24, 27, 29, 30). However, this does not exclude the possibility that the raw fish or material is an important initial source for contaminating the processing equipment and environment. In plant II RAPD types 2, 7, and 12, which were all found in the final product, were also detected on the raw fish and in the raw fish handling area, indicating that raw fish may also have been a source of contamination. Eklund et al. (7) found that the primary source of contamination was the surfaces of frozen or fresh raw fish

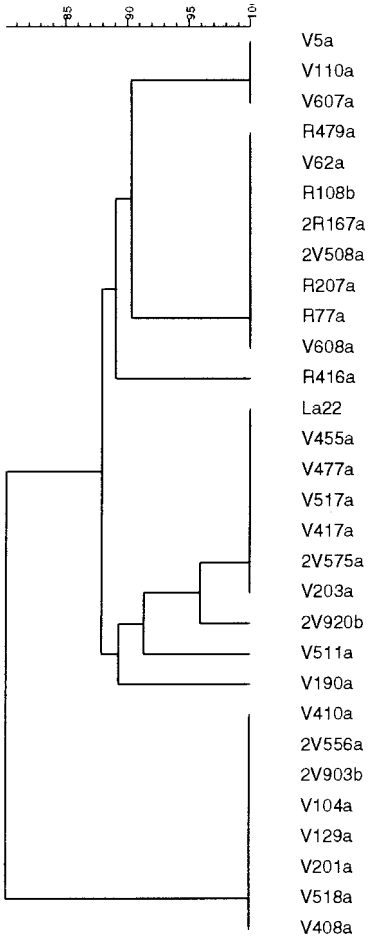

FIG. 2. Combined dendrogram for 30 *L. monocytogenes* isolates analyzed by RAPD, AFLP, and PFGE typing. The dendrogram was constructed with GelCompar and Bionumerics (Applied Maths) software by using the Dice correlation and cluster analysis by the unweighted pair group method using arithmetic averages. Percentages of similarity are shown above the dendrogram. The molecular sizes of RAPD, AFLP, and PFGE DNA ranged from 200 to 2,000 bp, from 40 to 500 bp, and from 50 to 400 kb, respectively. The origins of strains are shown in Table 7.

coming into a plant; however, this conclusion was based on prevalence studies, and comparisons of the strains at the DNA level were not conducted.

Certain RAPD types were found only in specific sections of the plants; in particular, some RAPD types were found only in the raw material area and not in the slicing section. Two processes, smoking and freezing, separate the raw material from the sliced material, and both processes are known to reduce bacterial levels (8, 35, 36). The shift in DNA types during processing could be explained by different abilities of different DNA types to withstand freezing or smoking. Differential susceptibility to freezing was suggested previously by Destro et al. (5), who found that only two PFGE profile groups were present on a frozen shrimp product even though nine different groups were isolated from shrimp in the processing area. Guyer and Jemmi (16) found that the cold-smoking process did not affect *L. monocytogenes*, although it has been reported that smoke compounds are inhibitory to *L. monocytogenes* (35, 36). Eklund et al. (7) observed a decrease in *L. monocytogenes* populations in surface-inoculated portions treated with smoke and an increase when the organism was injected into the injector of the flesh. Correspondingly, RAPD types 6 and 7 may have been located on the surfaces of the fillets and exposed to the smoking and drying process. To our knowledge, no studies have evaluated the possible differential susceptibility of *L. monocytogenes* genetic types to food processing factors.

Change in RAPD types over time at plant II. The routine check performed at plant II over time showed that several different *L. monocytogenes* RAPD types could be isolated. During this period, a variety of different products (smoked salmon, gravad salmon, gravad halibut, smoked tuna, etc.) were produced, and if the different kinds of raw fish were contaminated, this could explain the large variation in genetic types. The appearance of different *L. monocytogenes* RAPD types over time could also be a reflection of the lower prevalence. Thus, it is likely that rigorous cleaning and disinfecting in plant II (in which the prevalence was significantly lower than the prevalence in plant I) actually eliminate the organism at the end of production but that sporadic recontamination occurs due to the ubiquitous nature of *L. monocytogenes*. Based on the data available it was not possible to point to specific sources of contamination in plant II.

Comparison of plants I and II. The frequency of *L. monocytogenes* contamination was lower in plant II than in plant I. The buildings of plant II were specifically designed for production of smoked fish, and the facilities were in a good state of repair. In plant II different processing operations were located in different rooms with a continuous process flow. For example, raw material was received in one room and separated from the filleting machine, and fish waste material and products from the filleting process were transported on conveyor belts to a separate room. In comparison, these processes were done in the same hall in plant I. Consequently, improper traffic by trucks and use of wooden pallets from the outside in the processing environment took place. These could potentially be high-risk sources; however, samples obtained from trucks and wooden pallets did not substantiate this hypothesis. Likewise, sampling did not allow us to identify other potentially high-risk sources. In plant II more careful attention was paid to changing parts of machines, which were difficult to clean and sanitize. Also, attention was paid to removing smaller parts of different machines to allow separate and thorough cleaning. In plant II the slicing machines were cleaned twice during production, and this may have reduced the levels, as reported by Rørvik et al. (31) The processing lines for cold-smoked salmon in plants I and II consist of several very complex machines, such as filleting, skinning, brining, and slicing machines, which can be difficult to clean; thus, complete eradication of *L. monocytogenes* is difficult. Differences in the sanitizing procedures in the two plants were observed, but as other factors varied in the plants varied, the results did not allow us to determine if different sanitation regimes contributed to the lower prevalence of *L. monocytogenes* in plant II.

Comparison of typing methods. The use of both RAPD and PFGE for typing *L. monocytogenes* has previously been reported to identify similar groups (12, 15); however, using more than one method may increase the discriminatory ability (5), which was the case in this study, albeit for a very limited number of strains. Consequently, the two DNA typing approaches should be nearly equally suitable and can efficiently differentiate strains from well-defined habitats like cold-smoked salmon processing plants I and II investigated in this study. Compared to PFGE and AFLP typing, RAPD profiling is rapid and inexpensive, and we therefore chose RAPD typing with primer HLWL85 as the sole method for typing all the isolates of *L. monocytogenes*. AFLP fingerprinting for typing *L. monocytogenes* has been described previously (1), but the discriminatory power was compared only with that of serotyping. AFLP fingerprinting is universally applicable and has been used successfully for typing and classifying a number of bacterial strains (6, 14, 32). For the subset of 30 strains which we examined, the groups of strains determined by AFLP typing correlated completely with the groups resulting from PFGE typing. In our study, AFLP analysis was used for 30 *L. monocytogenes* strains isolated from well-defined habitats. A more thorough evaluation of the discriminatory power of AFLP typing of *L. monocytogenes* would require further testing of both environmental and clinical strains of *L. monocytogenes*.

Concluding remarks. RAPD types 2 and 12 were dominant among 429 *L. monocytogenes* isolates from Danish salmon smokehouses and cold-smoked salmon. RAPD type 12 was typical of the slicing environment and the products of plant I, whereas RAPD type 2 prevailed in all sections of plant II. RAPD typing of the isolates indicated that contamination with *L. monocytogenes* was mostly due to direct contact with contaminated processing equipment, and it was also possible to identify specific areas (bringing and slicing) at which contamination of the final product occurred. However, raw materials may also have contributed to contamination, particularly in one plant. Over time, different RAPD types appeared in plant II, probably as a result of a general relatively low prevalence of *L. monocytogenes*. In contrast, specific types of *L. monocytogenes* became established in the factory environment of plant I, and one RAPD type (RAPD type 12) was isolated from products from processing plant I over a 4-year period. This indicates that an established in-house flora was not eliminated by the hygiene procedures used. Several investigations have suggested that reservoirs of *L. monocytogenes* seem to be established in processing plants (15, 27, 29, 30). This view is supported by this study. Some strains may be adapted to specific

niches. Similarly, specific types have been found to persist for up to 7 years in a dairy industry plant by Unnestad et al. (37) and in an ice cream plant by Miettinen et al. (27). In several instances we isolated *L. monocytogenes* from cleaned and sanitized surfaces. *L. monocytogenes* is capable of adhering to food processing surfaces like stainless steel (17, 34), and cells in the adherent state may be more resistant to cleaning and disinfecting procedures than cells in the planktonic state (42). Such resistance may explain why the same *L. monocytogenes* DNA types can persist in a food processing plant for years (27).

The ways in which *L. monocytogenes* may be introduced into cold-smoked salmon processing plants are numerous due to the ubiquitous nature of *L. monocytogenes*, and raw fish could be an important source for contaminating the processing equipment and environment. Because *L. monocytogenes* will continue to be introduced into plant environments, control must be directed toward preventing establishment and growth of this organism in these environments. The control options must rely primarily on a proper cleaning and sanitation programs. However, production of products consistently free of the organism may be impossible.

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