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Aarnisalo, K.

http://hdl.handle.net/1975/769

Typing of *Listeria monocytogenes* Isolates Originating from the Food Processing Industry with Automated Ribotyping and Pulsed-Field Gel Electrophoresis

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ABSTRACT

A total of 486 *Listeria monocytogenes* isolates originating from 17 Finnish food processing plants (representing meat, poultry, fish, and dairy production) were collected and typed by automated ribotyping using *Eco*RI as the restriction enzyme. The isolates were divided into 16 different ribotypes (RTs). Some of these isolates (121), representing all *Eco*RI types and 16 food plants, were subjected to ribotyping with the *Pvu*II enzyme, to pulsed-field gel electrophoresis (PFGE) typing with *Asc*I and *Sma*I restriction enzymes, and to serotyping with O-antigen antisera. Nineteen ribotypes were generated with *Pvu*II, 42 macrorestriction patterns were generated with *Asc*I and 24 with *Sma*I, and three serotypes were generated with antisera. When the results were combined, the overall number of RTs was 23, and that of the PFGE types was 46. Thus, the overall discrimination power of PFGE was higher (discrimination index [DI] 0.966) than that of ribotyping (DI 0.906). The most common serotype (90.1% of the isolates) was 1/2, and isolates of serotype 4 (3.3%) were rare. There was no connection between food sectors and RTs or PFGE types, but PFGE indicated the single plants (78.3% of the types) better than ribotyping (56.5%). On the basis of its automation and on the availability of identification databases, automated ribotyping had some advantages over PFGE. Overall, automated ribotyping can be considered a practical and rapid tool when *Listeria* contamination is suspected and when screening a large number of isolates is necessary, e.g., when tracing contamination sources. However, in cases of outbreaks, the identical patterns must be confirmed by PFGE, which is a more discriminatory method.

Listeria monocytogenes is a ubiquitous pathogenic bacterium that may cause listeriosis in individuals belonging to risk groups such as neonate, elderly, and immunocompromised persons and pregnant women. Since the 1980s, *L. monocytogenes* has been thought to be transmitted to humans mainly via different foods *(14, 37)*. In recent years, there has been increased concern with *L. monocytogenes* contaminations because of the changing lifestyle and age structure of the population. In particular, this concern has been focused on ready-to-eat foods, which are not heated before consumption and which are most often involved in outbreaks *(14, 37)*. Furthermore, there is an increased consumer demand for chilled foods containing less salt and other preservatives. On the other hand, shopkeepers demand longer shelf lives for retail foodstuffs. These issues are strongly reflected in the production methods and hygiene management of food processes.

More than 10 different molecular typing methods are applied for *L. monocytogenes (17)*, of which pulsed-field gel electrophoresis (PFGE) is considered the best *(11, 13, 19, 31)*. However, it is a time-consuming and laborious method for practical use. Serotyping, the traditional and still routinely used typing method in cases of outbreaks, has a relatively poor discrimination power, and some industrial

isolates may be untypeable with standard typing antisera *(17)*. Hitherto, the only fully automated typing method available was ribotyping *(9)*. Ribotyping is a form of Southern hybridization analysis in which isolates are characterized for restriction fragment length polymorphisms associated with ribosomal operons. Manual *(7, 18, 23, 24, 26, 34)* and automated *(1, 2, 4, 15, 33, 35, 38, 41)* ribotyping methods have been used in the typing of *L. monocytogenes* in several studies. Recently, the efficiency of the RiboPrinter System was compared to that of PFGE by Hollis et al. *(20)* using a variety of clinical gram-positive and gram-negative bacteria. However, no *Listeria* isolates were included in their study.

The aim of this study was to compare the suitability of automated ribotyping using the restriction enzymes *Eco*RI and *Pvu*II to PFGE using the restriction enzymes *Asc*I and *Sma*I in distinguishing *L. monocytogenes* isolates from different food sectors and plants. The occurrence of different ribotypes (RTs) and PFGE types in the meat, poultry, fish, and dairy industries was also investigated. The presence of serotype 4 isolates was screened for by O-antigen antisera.

MATERIALS AND METHODS

Bacterial isolates. A total of 486 *L. monocytogenes* isolates originating from 17 Finnish food processing plants representing five fish (206 isolates), eight meat (139 isolates), one poultry (103

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TABLE 1. *The* EcoRI *ribogroups generated from 486* Listeria monocytogenes *isolates, the* PvuII *ribogroups generated from 121 isolates, and the combination of the results (final ribotypes)*

EcoRI ribogroups (no. of patterns)	PvuII ribogroups (no. of patterns)	Final ribotypes (no. of isolates) $n = 121$
129-S-2 (23)	1013-S-1 (4)	a (4)
339-S-5 (7)	1013-S-5 (3)	b (1)
366-S-2 (15)	1013-S-6 (5)	c (5)
160-S-2 (106)	1014-S-3 (13)	d (11)
	1014-S-5 (5)	e (3)
518-S-8 (15)	1014-S-3 (13)	f (1)
	1014-S-5 (5)	g (1)
366-S-5 (4)	1021-S-5 (3)	h (1)
420-S-8 (3)	1016-S-1 (34)	i (1)
60-S-1 (81)	1016-S-1 (34)	j (12)
23-S-7 (287)	1016-S-1 (34)	k (20)
	1017-S-6 (4)	l (4)
	1017-S-8 (3)	m (1)
	1018-S-7 (3)	n (1)
	1019-S-2 (3)	o (1)
344-S-2 (7)	1019-S-5 (3)	p (1)
350-S-1 (29)	1019-S-6 (10)	q (8)
353-S-4 (12)	1020-S-6 (4)	r (4)
366-S-7 (4)	1021-S-2 (3)	s (3)
41-S-3 (164)	1021-S-6 (29)	t (18)
41-S-8 (160)	1024-S-8 (19)	u (18)
	1028-S-2 (3)	v (1)
732-S-1 (16)	1030-S-2 (3)	w (1)
Total 16	19	23

isolates), and three dairy plants (38 isolates) were collected from 1997 to 1999. The samples were taken from equipment, personnel, the environment (tables, tools, doors, drains, floors, etc.), and products. Isolation was carried out according to the Nordic Committee on Food Analysis method *(3)*. For further studies, a set of 121 isolates was selected from these isolates, representing all the *Eco*RI RTs generated and 16 of the food plants (coded A through P) surveyed. The isolates were maintained in 5% glycerol at −70°C.

Ribotyping. The isolates were ribotyped using the Ribo-Printer System (DuPont Qualicon, Inc., Wilmington, Del.) as described by Bruce *(9)*. The restriction enzymes used were *Eco*RI (Qualicon) and *Pvu*II (Qualicon). The automated system processed

the batches and generated a pattern for each sample and marker lane using proprietary algorithms. Each batch included six marker lanes and a total of 30 molecular markers, which the system used for selecting a ribogroup already existing in the database or for creating a new one and for calculating the similarities between different patterns (software Qualicon version 11.2 (c) 1999). A ribogroup is defined as a set of closely related patterns (threshold similarity 0.96) that are mathematically indistinguishable from one another by the system *(9)*. The ribogroup patterns represent composite patterns for all members (isolates) of the group analyzed with the same instrument. The isolates are called RTs, and they have the same code as the relevant ribogroup. To ensure reproducibility, the patterns of all new ribogroups were analyzed three times.

PFGE. In situ DNA isolation and PFGE were performed as described by Autio et al. *(5)*. The restriction enzymes used were *Asc*I and *Sma*I (New England Biolabs, Beverly, Mass.). As used by other investigators for interpreting PFGE banding patterns, a difference of one or more bands was used as a means to discriminate between two PFGE types of *L. monocytogenes* *(26, 30, 32, 36)*.

Serotyping. The serotyping was performed using commercial O-antigen *Listeria* antisera (Denka Seiken, Tokyo, Japan) according to the instructions of the manufacturer.

Discrimination index. The discrimination power of the typing methods was determined by calculating the discrimination index (DI) using the formula of Hunter and Gaston *(21)*.

$$DI = 1 - \frac{1}{N(N - 1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of isolates in the sample population, s is the total number of types described, and n_j is the number of isolates belonging to the jth type.

RESULTS

Ribotyping. Digestion with *Eco*RI generated 16 different RTs from the 486 *L. monocytogenes* isolates, and when 121 isolates representing all 16 RTs were further typed with *Pvu*II, 19 RTs were generated (Table 1). Different enzymes resulted in different sets of typical fragments (Figs. 1 and 2). Digestion with *Pvu*II generated one strong band with a fragment size of ca. 8 kbp in all the RTs, except for RT 1020-S-6 (Fig. 2). The similarity between the *Eco*RI RTs was higher (from 0.61 to 0.94) than that between the

FIGURE 1. *RiboPrint patterns generated with the* EcoRI *restriction enzyme. The patterns are composites of several individual patterns, the number of which varied from 3 to 287 as shown in Table 1.*
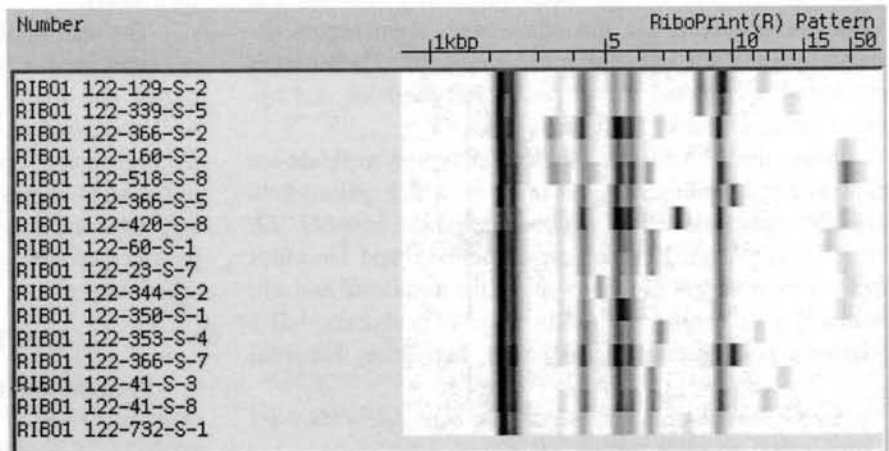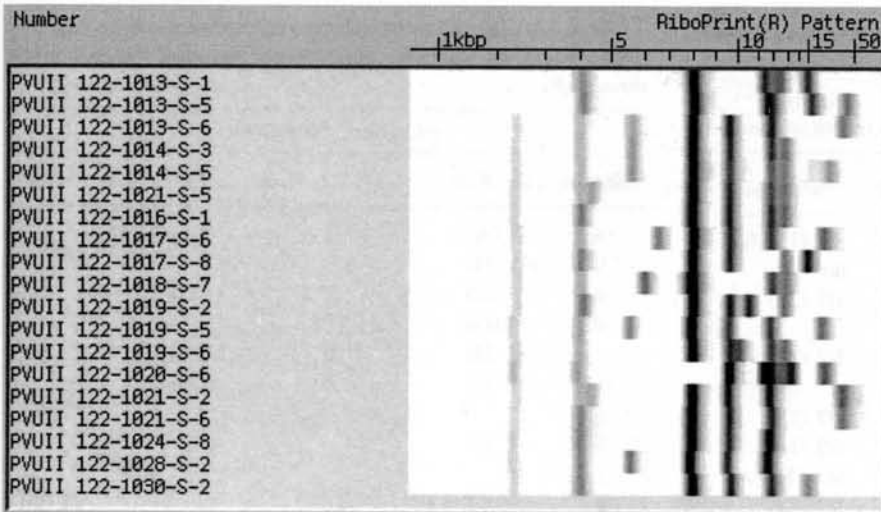
FIGURE 2. *RiboPrint patterns generated with the PvuII restriction enzyme. The patterns are composites of several individual patterns, the number of which varied from 2 to 34 as shown in Table 1.*

*Pvu*II RTs (from 0.22 to 0.96), indicating a higher discrimination for *Pvu*II than for *Eco*RI. The similarity of individual patterns to the composite pattern of their ribogroups was from 0.97 to 1.00, indicating very good reproducibility of the patterns. The combination of *Eco*RI and *Pvu*II RTs resulted in 23 final RTs (Table 1). Four of the RTs obtained with *Eco*RI (160-S-2, 518-S-8, 23-S-7, and 41-S-8) could be further divided with *Pvu*II into a total of nine RTs. By contrast, *Eco*RI could divide three *Pvu*II-based RTs (1014-S-3, 1014-S-5, and 1016-S-1) into five RTs.

PFGE. Digestion with *Asc*I resulted in 42 macrorestriction patterns, and digestion with *Sma*I resulted in 24 patterns from the 121 isolates analyzed (Table 2). When the patterns were combined, 46 final PFGE types were generated.

Serotyping. Most of the isolates (109 of 121) belonged to the serotype 1/2 (Table 2). Only four of the isolates were of serotype 4b (3.3% of the isolates). Some isolates (6 of 121) were not typeable with the commercial kit used.

DI. The DI was 0.878 for ribotyping with *Eco*RI and 0.867 for ribotyping with *Pvu*II. The overall DI for ribotyping with both enzymes was 0.906. For PFGE, typing with *Asc*I DI was 0.960, and typing with *Sma*I was 0.920, the overall DI being 0.966. When these two subtyping methods were combined, a total of 50 final subtypes were generated, and the DI was 0.967.

Occurrence of RTs and PFGE types in different food sectors. Three of the 16 *Eco*RI RTs (60-S-1, 41-S-3, and 23-S-7) were present in all the food sectors, four only in the fish industry (366-S-2, 366-S-5, 366-S-7, and 339-S-5) and one only in the poultry (420-S-8) or dairy (732-S-1) samples (data not shown). When the occurrence of different RTs was investigated among the 121 isolates also typed with *Pvu*II, only two RTs (j and k) occurred in all the food sectors (Table 3). Of the RTs, 13 of 23 (56.5%) were present only in single plants (11 of 23 represented only by one isolate), and 10 of 23 (43.5%) occurred in from two to six plants. Of the PFGE types, none occurred in all the food sectors. However, PFGE type 43 (belonging to RT

j) occurred in the fish, meat, and poultry sectors (Table 4). Of the PFGE types, 36 of 46 (78.3%) were present only in single plants (24 of 46 represented only by one isolate), and 10 of 46 (21.7%) occurred in from two to four plants. None of the RTs or PFGE types found from only one food sector were found from more than one plant of this food sector, except for PFGE type 12, which was found from two meat plants but only one isolate from each plant.

Comparison of automated ribotyping and PFGE typing. Among the isolates of 12 plants (75.0% of the plants), the discrimination was the same with both methods (Table 5). However, from 6 of these 12 plants, there was only one isolate, and from the other 6 plants, the number of isolates was low (from 2 to 9). From the isolates of four plants (E [29 isolates], I [7 isolates], J [25 isolates], and P [25 isolates]), PFGE generated more PFGE types (8, 5, 11, and 14, respectively) than ribotyping RTs (5, 4, 7, and 9, respectively). PFGE could further divide 10 RTs into a total of 33 PFGE types (Table 2), and it could better distinguish isolates from different plants. By contrast, ribotyping could also divide three PFGE types into a total of six RTs (Table 2), indicating higher discrimination between these isolates than that obtained by PFGE.

The discrimination ability of ribotyping of isolates representing serotype 4b has been reported to be poor (*17, 39*). In this study, only four isolates, originating from two plants (three isolates and one isolate), belonged to serotype 4b. In ribotyping, all of these isolates matched to the same *Eco*RI and *Pvu*II RTs, but PFGE differentiated the isolates originating from different plants. However, this distinction was not restricted only to serotype 4b isolates but also applied to the other isolates. In the present study, ribotyping did not distinguish an isolate belonging to serotype 3 from some other isolates belonging to serotype 1/2. PFGE could distinguish these isolates.

DISCUSSION

The discrimination power of molecular typing methods depends on the restriction enzymes used, on the isolates analyzed, and on the interpretation of the patterns. The en-

TABLE 2. *The final ribotypes, PFGE types, and serotypes generated*

Ribotype (no. of isolates)	PFGE type (no. of isolates)	Serotype (no. of isolates)
a (4)	1 (3)	4b (4)
	2 (1)	
b (1)	3 (1)	3 (1)
c (5)	4 (5)	no[a] (5)
d (11)	5 (8)	1/2 (11)
	6 (3)	
e (3)	6 (1)	1/2 (3)
	7 (2)	
f (1)	6 (1)	1/2 (1)
g (1)	41 (1)	1/2 (1)
h (1)	28 (1)	no[a] (1)
i (1)	40 (1)	1/2 (1)
j (12)	42 (1)	3 (1)
	43 (5)	1/2 (11)
	35 (1)	
	44 (2)	
	45 (3)	
k (20)	8 (1)	1/2 (20)
	11 (1)	
	13 (13)	
	14 (1)	
	16 (2)	
	18 (1)	
	19 (1)	
l (4)	9 (1)	1/2 (4)
	10 (1)	
	12 (2)	
m (1)	15 (1)	1/2 (1)
n (1)	20 (1)	1/2 (1)
o (1)	17 (1)	1/2 (1)
p (1)	21 (1)	1/2 (1)
q (8)	22 (5)	1/2 (8)
	23 (2)	
	24 (1)	
r (4)	25 (3)	1/2 (4)
	26 (1)	
s (3)	27 (3)	1/2 (3)
t (18)	29 (1)	1/2 (18)
	30 (6)	
	31 (9)	
	32 (2)	
u (18)	33 (7)	1/2 (18)
	34 (1)	
	35 (4)	
	36 (3)	
	37 (1)	
	38 (1)	
	39 (1)	
v (1)	37 (1)	1/2 (1)
w (1)	46 (1)	1/2 (1)
Total		
23 (486/121[b])	46 (121)	3 (121)

[a] No result with the used antisera kit.

[b] For *Eco*RI patterns, $n = 486$, and for *Pvu*II patterns, $n = 121$.

TABLE 3. *Occurrence of ribotypes (a through w) in different food sectors indicated as numbers of isolates and plants (A through P)*

Ribotype	Food sector			
	Fish	Meat	Poultry	Dairy
a	3 (A)		1 (P)	
b	1 (D)			
c	5 (B)			
d	4 (D)	3 (I), 3 (J)		
e	2 (D)	1 (I)		
f	1 (D)			
g				1 (F)
h	1 (B)			
i			1 (P)	
j	1 (C), 1 (E)	2 (J)	7 (P)	1 (G)
k	12 (E)	3 (J), 1 (L)	3 (P)	1 (F)
l		2 (I), 1 (M)		1 (F)
m		1 (I)		
n		1 (O)		
o			1 (P)	
p		1 (E)		
q		5 (J)	3 (P)	
r		3 (J)	1 (P)	
s	3 (B)			
t	1 (D), 11 (E)	3 (J), 1 (L), 1 (N)		1 (F)
u	1 (C), 4 (E)	6 (J)	7 (P)	
v			1 (P)	
w				1 (H)

zymes used in this study, *Eco*RI and *Pvu*II in ribotyping and *Asc*I and *Sma*I in PFGE, are commonly used for the typing of *L. monocytogenes*. If the isolates originate from the same plant, the discrimination may be relatively low *(5)*. On the other hand, different plants, even in different countries, may have identical types *(6, 38)*. In the present study, the set of 121 isolates was a very representative group of isolates from one country for this kind of study, and high DIs were therefore obtained, 0.906 for ribotyping and 0.966 for PFGE, as well as some clear differences between the methods. The interpretation of patterns was different for PFGE patterns than for RiboPrint patterns, which may be reflected in the numbers of types.

Of the methods tested, PFGE had the best discrimination power between these isolates, as also reported by other authors *(13, 24, 26)*. It was also the best indicator of different plants, which is very important in cases of epidemics, when this method has commonly been used *(12, 22, 25, 29)*. Although it is generally considered laborious and time-consuming and thus also too expensive for preliminary screening of hundreds of isolates as required in extensive hygiene surveys for tracing contamination sources *(38)*, the recently standardized protocol that enables typing in 30 h *(16)* makes PFGE more competitive in speed when compared with automated ribotyping.

The advantages of automated ribotyping are based on its automation to perform the analyses, the standardization of the procedure, and on the availability of identification databases for the genetic fingerprints generated. Four batches (32 samples) can be loaded during a working day, and

TABLE 4. *Occurrence of PFGE types (1 through 46) in different food sectors indicated as numbers of isolates and plants (A through P)*

PFGE type	Food sector			
	Fish	Meat	Poultry	Dairy
1	3 (A)			
2			1 (P)	
3	1 (D)			
4	5 (B)			
5	4 (D), 1 (E)	3 (J)		
6	2 (D)	3 (I)		
7	1 (D)	1 (I)		
8				1 (F)
9				1 (F)
10		1 (I)		
11		1 (J)		
12		1 (I), 1 (M)		
13	12 (E)	1 (J)		
14		1 (J)		
15		1 (I)		
16			2 (P)	
17			1 (P)	
18			1 (P)	
19		1 (L)		
20		1 (O)		
21		1 (K)		
22		5 (J)		
23			2 (P)	
24			1 (P)	
25		3 (J)		
26			1 (P)	
27	3 (B)			
28	1 (B)			
29	1 (D)			
30		3 (J), 1 (L), 1 (N)		1 (F)
31	9 (E)			
32	2 (E)			
33	1 (C), 2 (E)	4 (J)		
34		1 (J)		
35	1 (E)		4 (P)	
36			3 (P)	
37			2 (P)	
38		1 (J)		
39	1 (E)			
40			1 (P)	
41				1 (F)
42				1 (G)
43	1 (C), 1 (E)	2 (J)	1 (P)	
44			2 (P)	
45			3 (P)	
46				1 (H)

TABLE 5. *Discrimination between the* Listeria monocytogenes *isolates of different plants by automated ribotyping and PFGE (n = 121)*

Food sector	Plant	No. of isolates	No. of ribotypes	No. of PFGE types
Fish	A	3	1	1
	B	9	3	3
	C	2	2	2
	D	9	5	5
	E	29	5	8
Dairy	F	4	4	4
	G	1	1	1
	H	1	1	1
Meat	I	7	4	5
	J	25	7	11
	K	1	1	1
	L	2	2	2
	M	1	1	1
	N	1	1	1
	O	1	1	1
Poultry	P	25	9	14

a slightly higher value for typing with *Eco*RI (DI 0.878) than with *Pvu*II (DI 0.867), although they had generated 16 and 19 RTs, respectively. This is because the formula takes into account not only the number of types generated but also their frequencies *(21)*. A similar result was reported by Kerouanton et al. *(24)*, who obtained 13 and 12 types with DIs of 0.857 and 0.886, respectively. It appears that the formula does not work logically in all cases.

The availability of several identification databases, one provided by the manufacturer DuPont Qualicon and others created by the individual users of each instrument, e.g., the own database of the Technical Research Centre of Finland, makes the isolation procedure of *L. monocytogenes* much more rapid. It is possible to ribotype the first pure culture colonies without any conventional time-consuming cultivations on different media and different preliminary tests. The system will identify whether the isolate is *L. monocytogenes,* some other *Listeria* spp., or some other bacterial species within 8 h *(9)*. This is very important if a *Listeria* contamination is suspected and if the product must possibly be withdrawn from the market. Currently, the DuPont Qualicon database includes 101 *Eco*RI identification patterns for *Listeria* spp., of which 57 are for *L. monocytogenes*. The Technical Research Centre of Finland database includes 69 patterns for *Listeria* and 54 patterns for *L. monocytogenes* (34 with *Eco*RI, 19 with *Pvu*II, and 1 with *Pst*I). For identification purposes, the *Eco*RI is recommended, because it is the main enzyme in the system. Further discrimination, if necessary, may be carried out with the other enzymes. De Cesare et al. *(10)* recently studied the suitability of 15 different enzymes for discrimination of *L. monocytogenes* isolates in automated ribotyping, and *Pvu*II had the highest DI (0.992). The next most potential enzymes after *Pvu*II and *Eco*RI (DI 0.950) could be *Bst*EII (DI 0.925), *Ban*I (DI 0.917), and *Xho*I (DI 0.900).

In particular, the discrimination ability of ribotyping

the instrument then concurrently analyzes the batches, each batch lasting 8 h. On the basis of the results of the present work and also of some earlier studies *(10, 15)*, the discrimination power of automated ribotyping can be improved by the optimization of the set of enzymes, and probably, different enzymes could be used at the same time. All the investigators quoted have obtained more RTs with *Pvu*II than with *Eco*RI. However, in the present study, the commonly used formula of Hunter and Gaston *(21)* resulted in

among isolates of serotype 4b has been claimed to be poor *(17, 39)*. In the present study, only 4 of 121 isolates originating from two plants belonged to serotype 4b. In ribotyping, all of these isolates matched to the same *Eco*RI and *Pvu*II RTs, but PFGE differentiated the isolates originating from different plants. However, the number of isolates was too low to make any definitive conclusions. De Cesare et al. *(10)* obtained relatively good discrimination between isolates of serotype 4b.

In the results of the present study, specific RTs or PFGE types could not be connected, especially to specific food sectors (fish, meat, poultry, and dairy), which is in agreement with the results of our earlier studies in Nordic countries *(38)*. However, many food plants appeared to have their own plant-specific *L. monocytogenes* types, although the number of isolates in this study was too low for any statistical analysis. The "house types" may be very troublesome because of their strong attachment to surfaces *(27, 28)*. *L. monocytogenes* can form biofilms on different surfaces *(8)* and may be difficult to remove by cleaning procedures from all parts of the equipment *(40)*. Therefore, these biofilms may contaminate the processing procedures either continuously or else occasionally, when a piece of the biofilm is dislodged to contaminate the processing procedures.

On the basis of the results and experience obtained during this study, automated ribotyping can be considered a good method for hygiene control purposes in food processing plants for *L. monocytogenes*. However, in epidemiological studies, identical results obtained with the automated ribotyping system must be confirmed by PFGE.

ACKNOWLEDGMENTS

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