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Characterization of *Listeria monocytogenes* isolates from the meat, poultry and seafood industries by automated ribotyping

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Abstract

A total of 564 *Listeria monocytogenes* isolates were characterized by automated ribotyping. The samples were taken from equipment, personnel and the environment after cleaning procedures and during food processing, as well as from raw materials and products from six meat, two poultry and five seafood processing plants located in the Faroe Islands, Finland, Iceland, Norway and Sweden. Altogether, 25 different ribotypes (RTs) were generated. Two RTs occurred in the samples from all three food sectors—meat, poultry and seafood. Four RTs occurred in meat and poultry plant samples and other four RTs occurred in meat and seafood plant samples. Five RTs occurred only in meat plant samples, five only in poultry plant samples and five only in seafood plant samples. Eight of the thirteen plants had their own in-house *L. monocytogenes* ribotype. There was geographical differences between the RTs, but no correlation between RTs and food sectors was detected. The discrimination power of automated ribotyping was satisfactory to trace the contamination sources in the food processing plants clearly indicating the sites at which improved cleaning procedures were necessary. In addition, it was possible to screen a large number of isolates with two instruments located at different institutes and to make a reliable combination of the results.

Keywords: *Listeria monocytogenes*; Ribotyping; Meat; Poultry; Seafood

1. Introduction

The natural habitats of the bacterium *Listeria monocytogenes* are soil, water and plant material, particularly plant material undergoing decay (Fenlon, 1999). *L. monocytogenes* has been recognized as an animal pathogen for over 70 years (Wesley, 1999), but only for 20 years as a food-borne human pathogen (Slutsker and Schuchat, 1999). In the 1990s, it was

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frequently isolated from all the main food sectors, such as unfermented dairy products (Ryser, 1999a), cheese and other dairy products (Ryser, 1999b), meat products (Farber and Peterkin, 1999), poultry and egg products (Cox et al., 1999), products of plant origin (Brackett, 1999) and fish and seafood products (Jinneman et al., 1999). As a widely distributed environmental contaminant, *L. monocytogenes* may contaminate food at any point in the food chain. In addition, it is very tolerant to different environmental extremes. It can grow at 0–45 °C, survive for long periods in frozen or dried foods and tolerate acidic conditions and high salt concentrations (Lou and Yousef, 1999). It may grow on production lines and in the production environment, and may survive in difficult-to-clean equipment and production areas (Gravani, 1999). The occurrence of *L. monocytogenes* in even the most hygienic food processing conditions is difficult to prevent totally.

Subtyping of *L. monocytogenes* isolates is essential in epidemiological studies, and recently subtyping methods have also become extremely important in the control of process hygiene connected to the risk management system in food processing plants. Pulsed-Field Gel Electrophoresis (PFGE) has been considered to be the best method for molecular characterization of *L. monocytogenes* (Graves et al., 1999). However, the method is time-consuming and too laborious for routine hygiene control purposes. Hitherto, ribotyping has been the only fully automated standardized subtyping method commercially available, introduced by DuPont Qualicon™ (Wilmington, DE, USA) in 1995. This system characterizes and identifies bacterial isolates by generation of ribosomal RNA fingerprints (RiboPrint® patterns) from bacteria (Bruce, 1996). The standardization on the system makes it possible to combine the results carried out by different instruments. In developing the identification database, the manufacturer has analysed over 1300 *L. monocytogenes* isolates (Bruce et al., 1995; Hubner et al., 1995). The results generated from these studies were used in the development of the current DUP (DuPont Qualicon™) identification database which includes 48 different RiboPrint® identification patterns (genetic fingerprints) for *L. monocytogenes*.

This work was a part of the NordSafety P97070-project funded by the Nordic Industrial Fund (NI) and the participating industrial partners. The aim of this study was to examine the occurrence of different molecular subtypes of *L. monocytogenes* in 13 different food processing plants in the participating countries and to compare them with each other and with other available data. Two instruments of the relatively new subtyping method, the automated RiboPrinter® System, were used for that purpose. In addition, the usefulness of the system in hygiene control was evaluated.

2. Materials and methods

2.1. Sampling and preliminary screening

The *L. monocytogenes* isolates analyzed in this study originated from 36 hygiene surveys (two or three surveys in each plant) carried out in six meat, two poultry and five seafood processing plants located in the Faroe Islands, Finland, Iceland, Norway and Sweden during 1998 and 1999. The samples were taken from equipment, personnel and the environment, after cleaning procedures and when the process had been run for 2 h, as well as from raw materials and products. The enrichment and cultivation methods NCFA-Listeria no. 136 (Anon, 1990) (plants I, IV, VII, VIII, IX, X, XI) or USDA (FSIS) (McClain and Lee, 1989) (plants II, III, V, VI, XII, XIII) were used. From each sample, depending on the plant, one or three isolates identified by conventional methods as *L. monocytogenes* (dark colonies on Oxford agar, β-haemolysis-positive on Blood agar, Gram-positive, catalase-positive and motile rods identified by API Listeria strips according to the manufacturer's instructions (bioMérieux, Marcy-l'Etoile, France) were subjected to ribotyping.

2.2. Ribotyping

Ribotyping was performed using the standard method of the automated ribotyping device RiboPrinter® System (DuPont Qualicon™) according to the manufacturer's instructions (Bruce, 1996). Bacterial cells were taken from Brain Heart Infusion agar (Difco, Detroit, MI, USA) plates incubated aerobically at 37 °C over night. To ensure the reproducibility of the ribotypes (RTs), all new ribogroups (ribotypes)

generated at VTT (Technical Research Centre of Finland) were analyzed three times.

A total of 534 isolates were ribotyped at VTT and 109 at SIK (Swedish Institute for Food and Biotechnology). The system generates its own ribogroups in the database of each instrument. Thus, altogether, 77 isolates from 564 were ribotyped by both instruments. Finally, the RiboPrint® patterns of VTT and SIK were

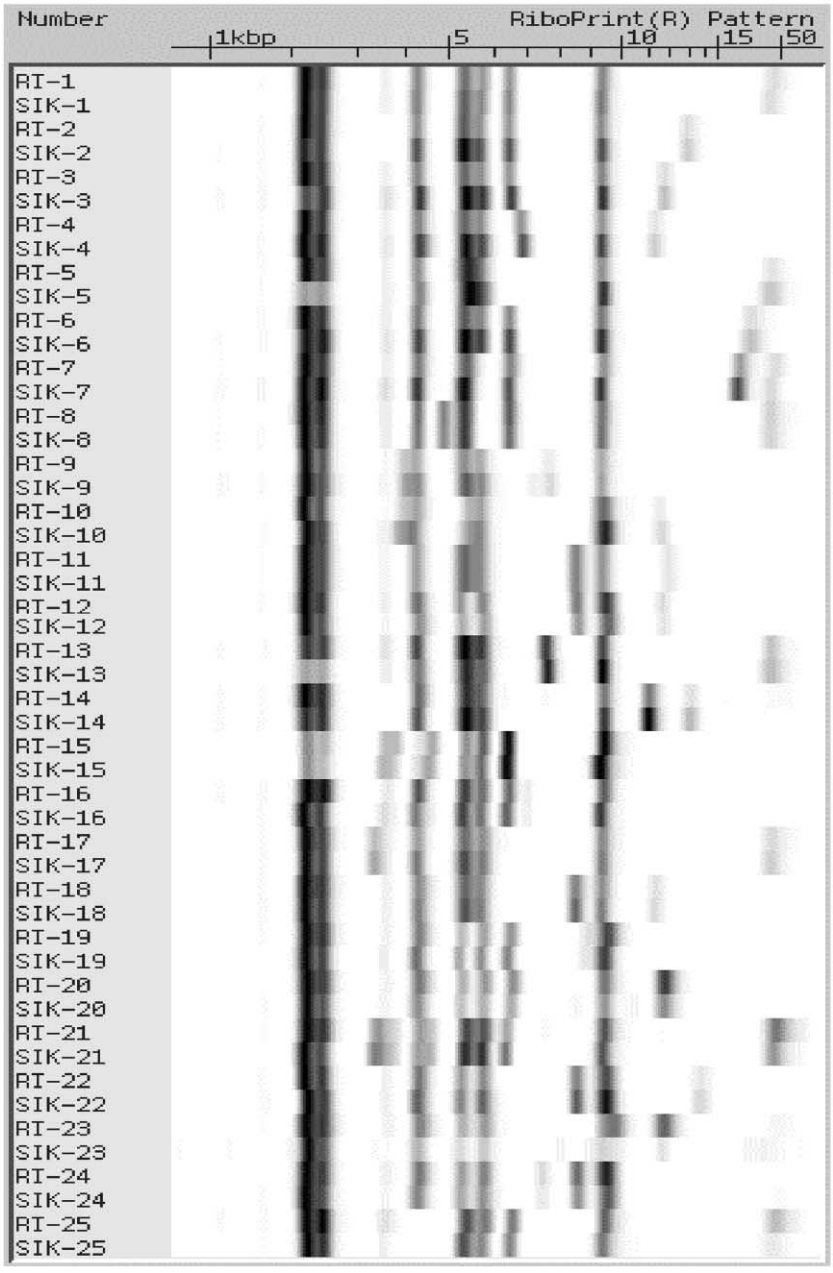

Fig. 1. RiboPrint® patterns of 25 ribotypes of *L. monocytogenes* generated by the VTT (RTs 1–25) and SIK (SIKs 1–25) instruments. The patterns are composites of individual patterns, the number of which varied from 1 to 160 as shown in Table 1.

downloaded electronically and were compared with each other and with the patterns in the DUP identification database (Version 11.2c 1999).

3. Results

3.1. Ribotypes generated from L. monocytogenes isolates

A total of 564 *L. monocytogenes* isolates were ribotyped with the RiboPrinter® System and characterized based on the system's software and visual assessment of the data into 25 ribogroups or ribotypes (RTs) (Fig. 1). Each ribotype pattern generated between seven and nine fragments. The sizes of the most typical fragments were two between 2.0 and 2.5 kbp (kilobase pair used in the system), one about 4.0 kbp, two or three between 5.0 and 6.0 kbp, one between 6.0 and 7.0 kbp and one between 9.0 and 9.5 kbp. Similarity between these 25 RTs ranged from 0.55 to 0.97 and the similarity of RTs to identification reference patterns in the DUP database varied from 0.80 to 0.99 (VTT patterns). Based on the preset identification similarity threshold of 0.85, strains within 23 of the 25 RTs were automatically identified as *L. monocytogenes* by the system's software. Although the patterns of the strains representing the remaining

Table 1
L. monocytogenes ribotypes (RTs 1–25 generated at VTT and SIKs 1–25 generated at SIK), their comparison between VTT and SIK instruments (databases), as well as with DUP database patterns and the numbers of ribotyped isolates originated from the meat, poultry and seafood industries in this study

RTs (number of patterns[a])		Similarity between patterns	Identical to DUP strain	Lineage[b]	Number of isolates		
VTT	SIK				Meat	Poultry	Seafood
RT-1 (160)	SIK-1 (60)	0.99	DUP 1039	II	39	28	102
RT-2 (12)	SIK-2 (6)	0.99	DUP 1030	II	8	5	3
RT-3 (78)	SIK-3 (2)	0.97	new	unknown	12	60	0
RT-4 (12)	SIK-4 (2)	0.98	new	unknown	7	3	0
RT-5 (23)	SIK-5 (2)	0.94	DUP 1046	II	17	4	0
RT-6 (26)	SIK-6 (2)	0.99	new	unknown	2	20	0
RT-7 (3)	SIK-7 (1)	0.98	new	unknown	1	0	0
RT-8 (4)	SIK-8 (5)	0.99	new	unknown	4	0	0
RT-9 (3)	SIK-9 (1)	0.97	DUP 1053	II	1	0	0
RT-10 (3)	SIK-10 (3)	0.96	new	unknown	3	0	0
RT-11 (8)	SIK-11 (7)	0.98	new	unknown	6	0	0
RT-12 (6)	SIK-12 (5)	0.99	new	unknown	0	3	0
RT-13 (3)	SIK-13 (1)	0.97	new	unknown	0	1	0
RT-14 (3)	SIK-14 (1)	0.97	new	unknown	0	1	0
RT-15 (3)	SIK-15 (1)	0.97	DUP 10144	III	0	1	0
RT-16 (4)	SIK-16 (5)	0.99	new	unknown	0	3	0
RT-17 (75)	SIK-17 (4)	0.99	DUP 1045	II	7	0	58
RT-18 (95)	SIK-18 (6)	1.00	new	unknown	2	0	92
RT-19 (46)	SIK-19 (2)	0.99	DUP 1044a	I	1	0	45
RT-20 (7)	SIK-20 (6)	0.97	new	unknown	4	0	5
RT-21 (10)	SIK-21 (5)	0.98	DUP 1047	III	0	0	6
RT-22 (5)	SIK-22 (3)	0.97	new	unknown	0	0	3
RT-23 (3)	SIK-23 (1)	0.84	new	unknown	0	0	1
RT-24 (9)	SIK-24 (4)	0.98	new	unknown	0	0	5
RT-25 (3)	SIK-25 (1)	0.95	new	unknown	0	0	1
Total (604)	(136)				114	129	321

"New" indicates RTs which were considered to be different from those present in the DUP database.
[a] Total number of generated patterns matching a particular ribogroup in one instrument.
[b] From Wiedmann et al. (1997).

two ribotypes, RT-7 and RT-25, contained the conserved fragments as described by Bruce et al. (1995), the similarity values of the patterns to the set of DUP identification reference patterns were less than 0.85 and they could not be automatically identified by the system's software. The pattern of RT-15 was clearly different from the other *L. monocytogenes* patterns (Fig. 1). The first two fragments (2.0–2.5 kbp) of the fingerprint pattern of RT-15 were exceptionally weak compared to those of all the other patterns and the fragments 6.0–6.5 and 9.0–9.5 kbp were exceptionally strong.

The ribogroup patterns (RTs) generated were composites of individual patterns, at VTT from 3 to 160 and at SIK from 1 to 60 (Table 1). The similarity between the relevant ribogroups varied between 0.84 (RT-23) and 1.00 (RT-18). The low similarity of RT-23 was due to the low DNA mass in the SIK-23 pattern (Fig. 1), which was generated only once. The other similarities were on the same level as when only one instrument and one database was used. However, the first two fragments of the patterns SIK-5 and SIK-13 were weaker than those of RT-5 and RT-13, probably also due to the low DNA mass used.

The RTs were also compared with those in the current DUP identification database. The data revealed that eight RTs had similarity values greater than 0.97 and were in fact identical to DUP patterns previously described by Wiedmann et al. (1997) (Table 1). The strains of these DUP patterns were also studied for their lineages.

The generated RTs were also imported into a second analysis, program BioNumerics (Applied Maths, St. Martens-Latem, Belgium), and a dendrogram was generated based on UPGMA (Unweight Pair Group Method using Arithmetic averages) and Pearson correlation coefficients (Fig. 2). No correlation between the RTs and food sectors was observed. RT-15 was again clearly different from the other RTs.

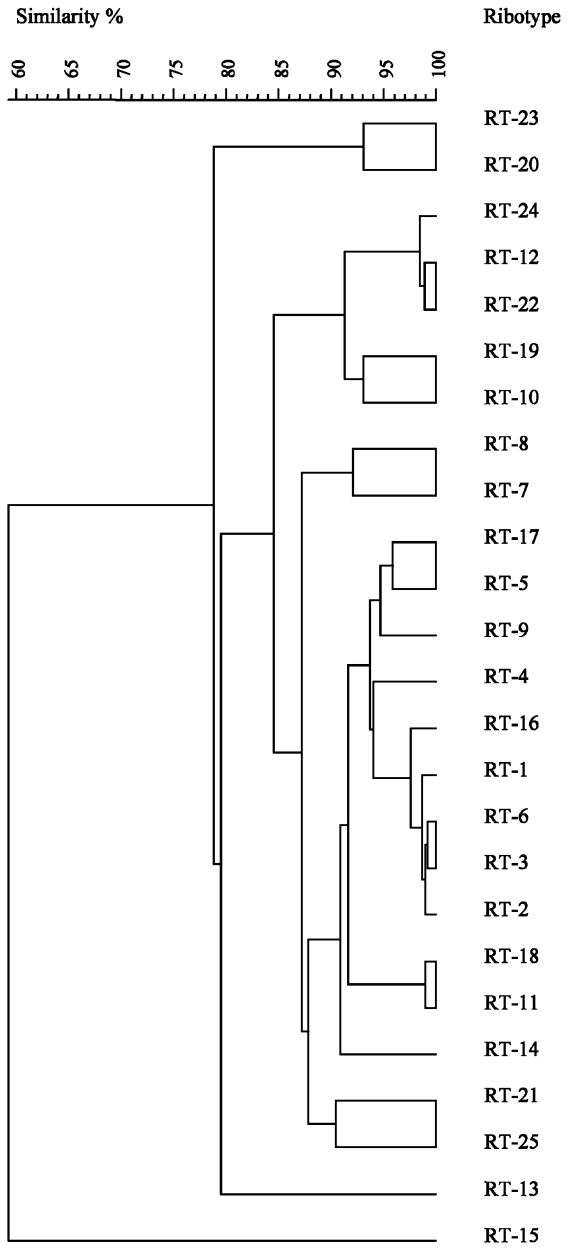

Fig. 2. Dendrogram of the generated 25 ribotypes (RTs) of *L. monocytogenes* produced by using the Pearson correlation coefficient and UPGMA algorithm.

3.2. Occurrence of different ribotypes

The occurrence of *L. monocytogenes* positive samples varied from 0% to 24.1% in the samples from different plants (Table 2). The number of *L. monocytogenes* isolates analyzed from each plant varied from 0 to 108. The ribotypes RT-1 and RT-2 occurred in all the food sectors studied—meat, poultry and seafood—and the RiboPrint® similarity between them was 0.91. RT-1 was the most common type, being present in 10 of 12 plants where *L. monocytogenes*

Table 2
Ribotypes (RTs) and numbers of *L. monocytogenes* isolates originating from meat (I–VI), poultry (VII–VIII) and seafood (IX–XIII) plants

Ribotypes	Plant/number of isolates													Total
	Meat						Poultry		Seafood					
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	
RT-1	12	13	3	5	6	–	9	19	–	–	2	21	79	169
RT-2	5	–	3	–	–	–	3	2	3	–	–	–	–	16
RT-3	12	–	–	–	–	–	60	–	–	–	–	–	–	72
RT-4	7	–	–	–	–	–	3	–	–	–	–	–	–	10
RT-5	17	–	–	–	–	–	4	–	–	–	–	–	–	21
RT-6	2	–	–	–	–	–	20	–	–	–	–	–	–	22
RT-7	–	–	–	1	–	–	–	–	–	–	–	–	–	1
RT-8	–	–	–	4	–	–	–	–	–	–	–	–	–	4
RT-9	–	–	–	1	–	–	–	–	–	–	–	–	–	1
RT-10	–	–	–	–	3	–	–	–	–	–	–	–	–	3
RT-11	–	–	6	–	–	–	–	–	–	–	–	–	–	6
RT-12	–	–	–	–	–	–	3	–	–	–	–	–	–	3
RT-13	–	–	–	–	–	–	1	–	–	–	–	–	–	1
RT-14	–	–	–	–	–	–	–	1	–	–	–	–	–	1
RT-15	–	–	–	–	–	–	–	1	–	–	–	–	–	1
RT-16	–	–	–	–	–	–	–	3	–	–	–	–	–	3
RT-17	7	–	–	–	–	–	–	–	42	11	5	–	–	65
RT-18	–	–	–	2	–	–	–	–	–	92	–	–	–	94
RT-19	–	–	–	–	1	–	–	–	–	–	45	–	–	46
RT-20	–	–	3	–	1	–	–	–	–	–	–	–	5	9
RT-21	–	–	–	–	–	–	–	–	1	5	–	–	–	6
RT-22	–	–	–	–	–	–	–	–	3	–	–	–	–	3
RT-23	–	–	–	–	–	–	–	–	–	–	1	–	–	1
RT-24	–	–	–	–	–	–	–	–	–	–	–	5	–	5
RT-25	–	–	–	–	–	–	–	–	–	–	–	1	–	1
Number of isolates	62	13	15	13	11	0	103	26	49	108	53	27	84	564
Total samples for listeria analysis	238	202	204	113	132	142	199	112	110	281	238	301	250	2522
Listeria positive (%)	18.1	11.4	7.4	28.3	6.1	11.3	42.2	50.9	11.8	23.1	5.9	9.0	20.0	17.7
L. monocytogenes positive (%)	8.8	2.5	2.5	12.4	3.0	0	20.6	24.1	8.2	22.1	5.9	6.3	20.0	10.7

occurred and clearly prevailing in plants II (100% of the isolates studied), VIII (73%), XII (78%) and XIII (93%). The ribotypes from RT-3 to RT-6 occurred in meat and poultry plant samples and were most typical to plants I and VII. RT-3 was especially established in plant VII (58%). The ribotypes from RT-7 to RT-11 occurred only in meat plant samples and those from RT-12 to RT-16 only in poultry plant samples. Due to the low numbers of isolates in these RTs they may be regarded as sporadic cases or they may be very specific for those food plants. The ribotypes from RT-17 to RT-20 occurred in meat and seafood plant samples. RT-17 was established in plant IX (86%), RT-18 in plant X (85%) and RT-19 in plant XI (85%). The ribotypes from RT-21 to RT-25 occurred only in seafood plant samples as sporadic cases.

From each sample of eight plants, three isolates were ribotyped. On average, in 15% of samples ($n = 118$) two and in 2% of samples even three different RTs were detected. The variation between the plants was from 0% (plant XIII) to 35% (plant I).

3.3. Tracing the contamination source of product samples

Of the 362 product samples analyzed, 17% were listeria positive and 9% *L. monocytogenes* positive (Table 3). The RTs present in the products could be

Table 3
Tracing of the contamination source of the products in different plants

Plant	Product	No. of *Listeria* samples			Ribotype	Source of contamination
		Analyzed	Listeria positive	*L. monocytogenes* positive		
I	Finished	2	1	1	RT-1	skinning machine, slicing machine, door
	In process	2	0	0	–	
II	Finished	16	4	1	RT-1	floor *, scraper *, apron
	In process	20	2	0	–	
III	Finished	27	0	0	–	
	In process	11	3	1	RT-18	door
IV	Finished	0	0	0	–	
	In process	15	10	5	RT-8	crates *, table *
					RT-1	conveyer belt *
					RT-18	not found
					RT-7	not found
					RT-9	not found
V	Finished	13	1	1	RT-1	floor
	In process	8	1	1	RT-20	outdoor
VI	Finished	22	7	0	–	
		8	0	0	–	
VII	Finished	8	4	0	–	
	In process	0	0	0	–	
VIII	Finished	10	7	4	RT-1	trimming board *, tables *, drain *, knife/gloves
	In process	2	0	0	–	
IX	Finished	6	0	0	–	
	In process	0	0	0	–	
X	Finished	38	16	16	RT-18	floor *, conveyor belt *, drain *, tray, line, jacket *, shoe *, ice
	In process	33	1	1	RT-18	brine
XI	Finished	37	2	2	RT-19	brine, drain, transporter *, line *, shoes, waste, deicing tank
					RT-23	not found
	In process	23	0	0	–	
XII	Finished	23	1	1	RT-25	not found
	In process	20	0	0	–	
XIII	Finished	3	0	0	–	
	In process	15	0	0	–	
Total		362	60	34		

* Also detected after cleaning.

traced into the process in eight plants. From the other plants, the samples taken were free of *L. monocytogenes* or the RTs detected were sporadic and were not found from the process. The samples were taken after cleaning procedures and after 2 h from the start of the process. The results indicated that in some plants (e.g., in IV, VIII and X) the cleaning procedures had not been satisfactory. After cleaning the same RTs were already present in some equipment in contact with the final product or in noncontact environments (Table 3, sites with an asterisk) and also later in samples taken during the process. The most problematic sites were conveyor belts (27% isolates of relevant RTs after cleaning) and floors including drains (40%), which were also the worst sites during the process (22% and 16%, respectively).

4. Discussion

Slightly different strategies were used to carry out the surveys in different countries. The number of isolates characterized from different plants varied from 0 to 108 depending on the occurrence of *L.*

monocytogenes and the number of isolates ribotyped from each sample. Thus, the results obtained better describe those plants from which the numbers of samples (over 100 per survey) and isolates analyzed were high. In this study, we traced only the RTs of product samples. However, it is also possible to trace, e.g., the contamination of the process or the effectiveness of a cleaning agent or procedure. Our results demonstrated that in some plants the same RTs were present not only during the process but already after cleaning before the start of the process. Furthermore, the same RTs appeared to be present continuously, because they were detected in different surveys carried out, e.g., in summer and winter. This clearly indicates that these plants are badly contaminated with a prevailing ribotype, probably due to biofilm formation, which has also been reported to be possible with *L. monocytogenes* (Blackman and Frank, 1996; Ronner and Wong, 1993; Spurlock and Zottola, 1991; Sinde and Carballo, 2000). The listeria-free raw material may be contaminated in the process, e.g., by brine, as also reported by Autio et al. (1999). In two plants, improved procedures were already carried out between the first and second survey. In one plant, the corrective actions were so effective that no listeria were detected during the second survey, which was focused on samples taken after cleaning. In the other plant, eight different ribotypes were detected during the first survey, but only the prevailing RT was detected during the second survey. Depending on the site at which this tolerant ribotype was established, the next step in controlling contamination in this plant would be to open a difficult-to-clean machine or change the cleaning strategy or cleaning agent.

When the generated RTs were compared with the *L. monocytogenes* patterns in the DUP database, eight were identical with those generated earlier. The lineages, based on ribotype and the alleles *act*A, *hly* and *inl*A, are proposed to give some evidence for differences in pathogenic potential (Wiedmann et al., 1997). It has been concluded that the strains from lineages I (strains from food-borne epidemic outbreaks) and III (strains only from animal related cases) were more pathogenic than the strains of lineage II (no food-borne epidemic strains). Most of our isolates identical with a DUP pattern belonged to lineage II. However, the isolates of RT-19 belonged to the lineage I and those of RT-15 and RT-21 to lineage III. Furthermore, the relationship of most of our ribotypes to these lineages remained unknown.

The discrimination power of PFGE has been considered to be the best for *L. monocytogenes* isolates and the other subtyping methods are usually compared with it. It has been reported that in PFGE the restriction enzyme *Asc*I generated from 8 to 14 fragments, whereas *Apa*I generated between 11 and 23 fragments (Brosch et al., 1991; Buchrieser et al., 1993; Carriere et al., 1991; Nakama et al., 1998; Johansson et al., 1999). In this study, the automated ribotyping with *Eco*RI generated from seven to nine fragments, as also reported by Bruce et al. (1995). It is clear that two enzymes, *Asc*I and *Apa*I, used in PFGE have more cutting sites in the DNA of *L. monocytogenes* than one enzyme, *Eco*RI, used in standard automated ribotyping. It has also been reported that the discrimination power of ribotyping has not been adequate in epidemiological cases especially among the *L. monocytogenes* isolates of serotype 4b (Louie et al., 1996; Kerouanton et al., 1998; Swaminathan et al., 1996). In order to improve these weaknesses of automated ribotyping, restriction enzymes other than *Eco*RI had to be applied, as Gendel and Ulaszek (2000) have already demonstrated. In their study, some of the *Eco*RI ribogroups (total 11 RTs) could be further divided by *Pvu*II (16 RTs).

It can be concluded that discrimination power of automated ribotyping is sufficient for hygiene control purposes, it is reproducible allowing work to be conducted with two or more instruments, and the data can be stored for later use. It is rapid, simple to carry out, standardized, objective, labour-saving and versatile. The disadvantages of the system are that it is very expensive to purchase, the running costs are high, it is applicable only for bacteria and effective use of the system requires good characterization and identification databases. Own databases must be constructed by the users to augment those provided by the manufacturer. However, the databases grow continuously with the new samples analysed, which makes the system more and more valuable for its users after each batch.

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