ELSEVIER

International Journal of
Food Microbiology 31 (1996) 161–171

International Journal of Food Microbiology

Pathways of *Listeria monocytogenes* contamination in the meat processing industry

Truls Nesbakken[a,*], Georg Kapperud[b], Dominique A. Caugant[b]

[a]*Department of Research and Development, Norwegian Meat Cooperative, P.O. Box 360, Økern, 0513 Oslo, Norway*

[b]*Department of Bacteriology, National Institute of Public Health, P.O. Box 4404, Torshov, 0403 Oslo, Norway*

Received 20 March 1995; revised 30 October 1995; accepted 27 December 1995

Abstract

One hundred and thirty-three isolates of *Listeria monocytogenes* from deboned fresh meat, production environment, cold cuts from five meat processing plants and from one plant producing cured dried sausages, were characterized using multilocus enzyme electrophoresis. On the basis of electrophoretically demonstrable allelic variation at 21 enzyme loci, 21 electrophoretic types (ETs) were distinguished. Analysis of the genetic relationships among the 21 ETs revealed two distinct clusters: Cluster A and Cluster B.

With the exception of two isolates from one plant, all isolates from deboned fresh meat belonged to Cluster B. During processing of cold cuts, however, isolates belonging to Cluster A became more frequent, and only one of the 37 isolates from cold cuts belonged to Cluster B. In contrast, six of the nine isolates from cured dried sausages had ETs in Cluster B.

One clone of Cluster A, ET-6 was isolated from cold cuts in four of six plants. This is one of the ETs most frequently recovered from patients in Norway. Isolates of ET-6 were further characterized using restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA. Six distinct restriction patterns were distinguished among the 44 ET-6 strains. In one plant, four different RFLP patterns could be identified. Two clone variants seemed to have colonized different areas in this plant for at least four years. However, in each of the other plants, all ET-6 isolates had the same RFLP patterns.

* Corresponding author. Tel. +47 22 092317; fax +47 22 220016; e-mail: truls.nesbakken@gilde.telemax.no

PII S0168-1605(96)00978-6

In only one of the five plants producing cold cuts was the same ET found both in the fresh meat and along the processing chain including the end-product. This indicates that the potential risk for contamination of the final end-product (cold cuts) posed by *L. monocytogenes* in the fresh meat might have been overestimated. It is possible that personal and general hygiene in the packing room are more significant with regard to *L. monocytogenes* contamination of cold cuts. Good manufacturing practices and attention to critical control points during processing in the meat plants are probably the most important prophylactic measures to avoid *L. monocytogenes* in cold cuts.

Keywords: Meat processing plant; Fresh meat; Production environment; Cold cuts; Cured dried sausages; Multilocus enzyme electrophoresis; Restriction fragment length polymorphism; *L. monocytogenes*

1. Introduction

Multilocus enzyme electrophoresis (MEE) is a powerful tool for studies on the epidemiology of listeriosis. This method allows the distribution of *Listeria monocytogenes* clones in the environment to be assessed, and the possible contamination pathways of foods to be defined. Differences observed in the distribution of *L. monocytogenes* clones could also reflect important differences between strains in their adaptation to diverse ecological niches, as well as differences in virulence among *L. monocytogenes* clones (Boerlin and Piffaretti, 1991).

MEE analysis of *L. monocytogenes* isolated from fish processing plants has shown that the electrophoretic types (ETs) dominating in processed fish and in the processing environment are different from those found on live fish and in sea water (Rørvik et al., 1995). The investigation of Boerlin and Piffaretti (1991) indicated that this may also be the case with slaughter animals, fresh meat and meat products.

We report here an investigation using MEE to characterize *L. monocytogenes* isolates from deboned fresh meat, production environment, wastes from slicers, cold cuts and cured dried sausages, in order to trace the dissemination of *L. monocytogenes* in meat processing plants. Isolates of the most frequently recovered ET (ET-6) were further differentiated by analysis of restriction fragment length polymorphism (RFLP) of the chromosomal DNA, using the enzyme *Hae*III.

2. Materials and methods

2.1. Sample collection and bacterial isolates

The samples were taken in connection with investigations of deboned fresh meat and end-products (wastes from the slicers, cold cuts and cured dried sausages) in each of six meat processing plants carried out five times a year from 1989 to 1993. When *L. monocytogenes* was isolated from cold cuts, the meat processing plant in

question was visited, and swab samples were collected from the critical control points in the production environment.

2.1.1. Deboned fresh meat

Altogether, 47 isolates were obtained from deboned fresh meat samples from the six meat processing plants (range 2–13) as shown in Tables 1 and 2.

2.1.2. Production environment

In all, 28 strains of *L. monocytogenes* were isolated from three of the six plants (range 0–12) as shown in Table 1.

2.1.3. Waste from the slicers

Fourteen *L. monocytogenes* strains were isolated from slicer waste collected throughout one working-day, from two of the five meat processing plants (Table 1).

2.1.4. Cold cuts and cured dried sausages

The cold cuts are produced from sausages, heat-treated at a temperature of at least 72°C. The sausages are sliced and vacuum packed (pH 6.2). Cured dried sausages are fermented, after which they are sliced and vacuum packed (pH below 5.0, water activity below 0.90). Thirty-five *L. monocytogenes* isolates were obtained from heat treated, sliced cold cuts, packed in vacuum or modified atmosphere, from five meat processing plants (range 1–15), while 9 isolates were recovered from the plant producing cured dried sausages (Tables 1 and 2).

2.1.5. Isolation and storage of L. monocytogenes

L. monocytogenes was enriched in two steps in selective broth (*Listeria* enrichment broth base (UVM Formulation, Oxoid CM 863) (Oxoid Ltd., Basingstoke, Hampshire, England). Ten μl of culture was plated onto Oxford agar (Oxoid CM 856 and SR 140) and blood agar. The plates were incubated at 37°C for 48 h (Oxford agar) and 24 h (blood agar). Four typical colonies were verified by haemolysis, Gram-staining, catalase reaction, fermentation of rhamnose and α-methyl-$\mathscr{D}$-mannoside, no fermentation of xylose, CAMP-test (Welshimer, 1981) against *Staphylococcus aureus* and *Rhodococcus equi*, and motility at 22°C. Only one isolate of *L. monocytogenes* was retained from each sample, strains exhibiting identical characteristics being recorded as one isolate. The bacterial cultures were stored at -70°C.

2.2. Multilocus enzyme electrophoresis

Procedures for protein extract preparation, starch-gel electrophoresis and selective enzyme staining were as described earlier (Kolstad et al., 1992; Selander et al., 1986). Twenty-one enzymes were assayed: IPO, indophenol oxidase; 6PG, 6-phosphogluconate dehydrogenase; G6P, glucose 6-phosphate dehydrogenase; ADK, adenylate kinase; PGI, phosphoglucose isomerase; GD1 and GD2, two glutamate dehydrogenases; PE2, PE3 and PE4, three peptidases; NSP, nucleoside phosphory-

Table 1
Electrophoretic types (ETs) (no. of isolates in parenthesis) and RFLPs of ET-6 as superscripts and distribution in meat processing plants 1, 2, 3, 4 and 5

Year	Deboned fresh meat		Production environment		Waste from the slicer		Cold cuts	
	Cluster A	Cluster B	Cluster A	Cluster B	Cluster A	Cluster B	Cluster A	Cluster B
Plant 1								
1989		18 (1)	6 (3)^{B,B,E}	18 (1)	6 (1)^{D}	12 (1)		
		11 (1)		12 (3)				
1990		11 (2)	6 (1)^{A}	7 (1)		12 (1)		
				8 (2)				
1991		23 (1)			6 (1)^{D}	12 (1)	6 (1)^{D}	
		11 (2)						
1992					6 (2)^{D,D}			
1993			6 (1)^{B}		6 (5)^{D,D,D,D,F}			
Plant 2								
1989		22 (3)						
1991	6 (2)^{A,A}	29 (4)	6 (5)^{A,A,A,A,A}		6 (2)^{A,A}		6 (4)^{A,A,A,A}	
Plant 3								
1989		31 (1)						
1991		24 (1)						
		27 (1)						
		8 (3)						
		12 (1)						
1992							6 (15)$^{A\ (all)}$	

Table 1 (continued)

Year	Deboned fresh meat		Production environment		Waste from the slicer		Cold cuts	
	Cluster A	Cluster B	Cluster A	Cluster B	Cluster A	Cluster B	Cluster A	Cluster B
Plant 4								
1989		23 (1)					6 (1)[C]	
1990		23 (2)						
		26 (1)						
		7 (2)						
		12 (2)						
1991		23 (1)						
		28 (3)						
		12 (1)						
Plant 5								
1989			3 (10)	23 (1)			21 (1)	7 (1)
							3 (12)	
1991		29 (1)						
		7 (6)						
		11 (1)						
		12 (1)						

lase; ACP, acid phosphatase; PGM, phosphoglucomutase; G3P, glyceraldehyde 3-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; EST, esterase; MPI, mannose phosphate isomerase; FUM, fumarase; ALD, alanine dehydrogenase; LAD, lactate dehydrogenase, and CAT, catalase.

Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches), and clustering was performed from a matrix of genetic distances by the average-linkage method (Sneath and Sokal, 1973).

2.3. Analysis of restriction fragment length polymorphism

Bacteria were cultured overnight at 37°C in 5% CO_2 atmosphere on blood agar plates, and one loopful of bacterial growth was transferred to 400 μl Tris-ethylene-diaminetetraacetic acid (EDTA) buffer (Sambrook et al., 1989). The bacteria were lysed by addition of 50 μl lysozyme solution (10 mg/ml). After incubation at 37°C for 2 h, 75 μl of 10% sodium dodecyl sulfate (SDS) solution with 0.7 mg/ml proteinase K was added. The mixture was incubated at 65°C for 10 min and subsequently blended with 100 μl of a solution containing 10.0 mg CTAB (*N*-acetyl-*N*,*N*,*N*,-trimethyl ammonium bromide) and 4.1 mg NaCl, which had been prewarmed to 65°C. The mixture was vortexed vigorously until the liquid content became white ('milky'), incubated at 65°C for 10 min, and mixed by vortexing with 750 μl chloroform/isoamyl alcohol (24:1), followed by centrifugation for 5 min at full speed (approx. 2500 × *g*) in a microcentrifuge. The aqueous supernatant was collected, extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and DNA was precipitated overnight at -20°C by addition of a double volume of 96% ethanol (Sambrook et al., 1989). The precipitate was washed once with 1 ml 70% ethanol and dried. The resulting DNA preparations were digested with the restriction enzyme *Hae*III under the conditions recommended by the supplier (Gibco BRL GmbH, Eggenstein, Germany). Electrophoresis was conducted for 18 h at 40 V on 0.7% horizontal agarose gels. A 1-kilobase (kb) DNA ladder (BRL, Gaithersburg, MD) was included as a size marker on each gel.

Table 2
ETs (no. of isolates in parenthesis) and distribution in meat processing plant 6

Year	Deboned fresh meat		Cured dried sausages	
	Cluster A	Cluster B	Cluster A	Cluster B
1989		7 (1) 11 (1)		
1991			3 (2) 1 (1)	25 (1) 28 (1) 30 (2) 32 (1) 33 (1)

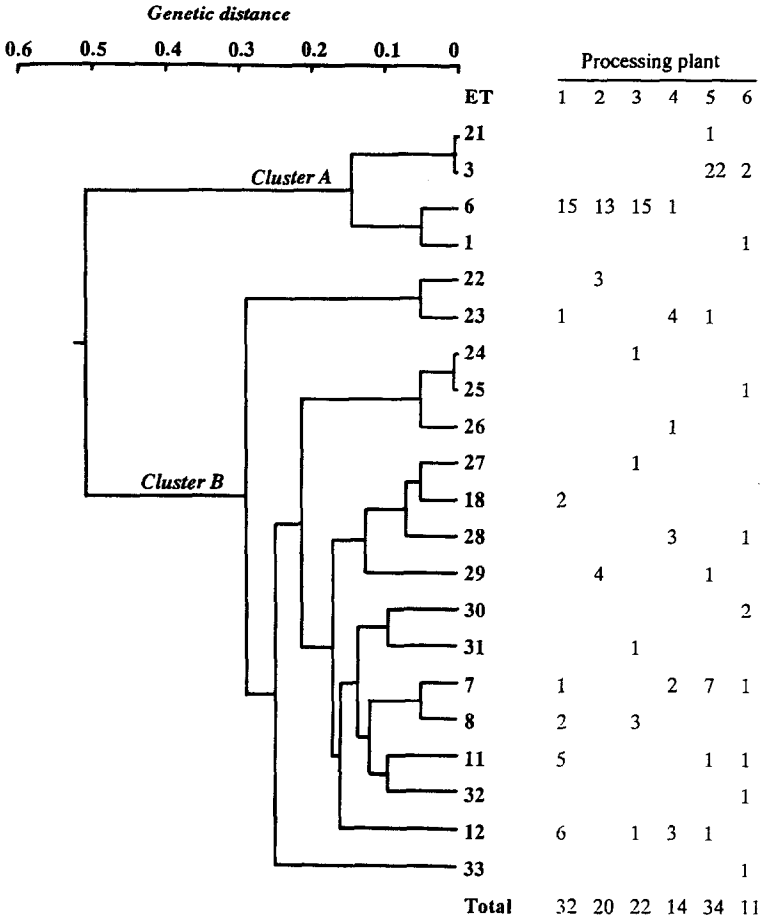

Fig. 1. Dendrogram demonstrating the genetic relationships of electrophoretic types (ETs) of 133 *L. monocytogenes* isolates and their distribution in six meat-processing plants.

3. Results

Among the 133 isolates of *L. monocytogenes* investigated by MEE 21 ETs were distinguished. The genetic relationships between the 21 ETs are shown in a dendrogram (Fig. 1). ETs previously identified (Kolstad et al., 1992; Rørvik et al., 1995) were given the same numbers, while new ones were numbered consecutively according to their position in the dendrogram, starting with number 21. ETs were grouped into two clusters diverging from one another at a genetic distance greater than 0.5: Cluster A consisting of ETs 1, 3, 6 and 21 and Cluster B comprising ETs 7, 8, 11, 12, 18 and 22–33. The distribution of isolates in each of the six meat processing plants is also presented in Fig. 1, and in Tables 1 and 2.

The 47 strains isolated from fresh meat belonged to 14 distinct ETs. The predominant clone, ET-11, was represented by seven isolates recovered from fresh meat in three different processing plants. In plant 1, ET-11 isolates were identified in samples collected in three consecutive years. Of the 47 isolates from fresh meat, all except two (95.6%) belonged to clones of Cluster B.

The 28 strains from the processing environment belonged to seven ETs, among which two, represented by 10 isolates each, belonged to Cluster A. ET-6 was represented by five isolates in each of plants 1 and 2.

L. monocytogenes was isolated from slicer waste in two plants. In both plants, strains of ET-6 were identified. In plant 1, although ET-6 strains were found in waste from the slicer from 1989 to 1993, strains of ET-12 in Cluster B were also detected in 1989–1991.

Strains of ET-6, and ET-6 only, were detected in the processed product in four of the five plants producing cold cuts. In plant 5, three distinct clones were identified, although 12 of the 14 isolates were ET-3, the predominant clone in the production environment. While 34 of 35 isolates (97.1%) from cold cuts belonged to Cluster A, six of nine isolates (66.7%) from cured dried sausages belonged to clones of Cluster B. It is also noteworthy that the nine isolates from cured dried sausages represented seven different ETs.

Of the 133 isolates, 44 (33.1%) could not be differentiated using MEE and were assigned to ET-6. These 44 ET-6 strains were examined using RFLP analysis, and six distinct patterns were distinguished (Fig. 2). All 13 isolates of ET-6 from plant 2, and all 15 from plant 3, had the same RFLP pattern (A). The single ET-6 isolate from plant 4 had a unique RFLP pattern (C). In plant 1, however, the 15 ET-6 strains showed five different patterns. Patterns A, B and E were found for strains from the production environment, and patterns D and F for the strains isolated from the waste from the slicer. The single isolate from cold cuts was also pattern D.

4. Discussion

The use of multilocus enzyme electrophoresis has shown that the natural population of *L. monocytogenes* is very diverse. In spite of this genetic diversity, most human infections are caused by only a few ETs of *L. monocytogenes* (Piffaretti et al., 1989). Most isolates causing human disease, both outbreaks and sporadic cases, belong to clones of Cluster A. Our ET-3 strains were undistinguishable by MEE from those associated with the Swiss epidemic (Piffaretti et al., 1989). ET-6 was responsible for a small outbreak (six cases) in the county of Trøndelag, Norway, in 1992, and has also caused a number of sporadic cases in recent years in Norway (D.A. Caugant, unpublished data). Although ET-6 was identified from cold cuts in four of six plants, only in plant 2 were ET-6 isolates with the same RFLP pattern (A), found not only in cold cuts, but also in samples from fresh meat, the production environment, and slicer waste. In two plants, the same ETs within cluster A were isolated both from the production environment and cold cuts, but not from fresh meat. In one of these plants (plant 1), the ET-6 isolates were

differentiated by RFLP. The patterns of the isolates from the environment were different from those in waste from the slicer and cold cuts, and both as regards the production environment and slicer waste, isolates with the same pattern (B and D, respectively) were found in samples taken several years apart. This suggests that two clone-variants of ET-6 have colonized different areas of this meat processing plant, and have persisted for at least four years.

From an epidemiological and food hygienic point of view, it is of great concern that *L. monocytogenes* grows at refrigeration temperatures in cold cuts (Glass and Doyle, 1989). For this reason, when end-product contamination with *L. monocytogenes* was detected in meat processing plants, production lots of cold cuts containing *L. monocytogenes* were destroyed, and processing lines investigated. In addition, critical control points were checked and special cleaning and disinfection measures were introduced.

Generally, isolates from deboned fresh meat belonged to Cluster B. During processing, however, isolates belonging to Cluster A became more and more frequent. This is reflected by the distribution of the isolates in Clusters A and B in

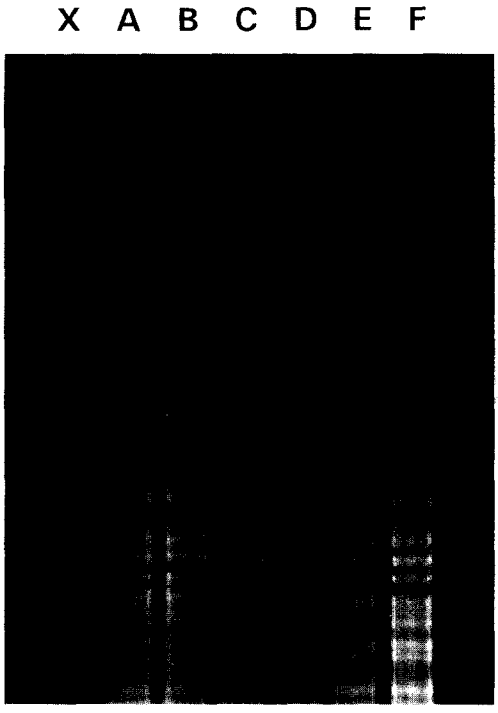

Fig. 2. Restriction endonuclease (*Hae*III) cleavage patterns (RFLPs) of total DNA from *L. monocytogenes* strains belonging to ET-6. Lanes A through F show the six different patterns detected (Table 1). The following strains are presented: Plant 2, production environment, 1991 (lane A); Plant 1, production environment, 1989 (lane B); Plant 4, cold cuts 1989 (lane C); Plant 1, waste from the slicer, 1993 (lane D); Plant 1, production environment, 1989, (lane E); Plant 1, waste from the slicer, 1992 (lane F). The size marker in lane X is a 1-kilobase DNA ladder.

the production environment, wastes from slicers and in cold cuts. However, in the cured dried sausages, most ETs fell into Cluster B. Only three of nine isolates in the end-product were represented by Cluster A. These results suggest that for cured dried meat products, *L. monocytogenes* isolates may originate from the fresh meat.

The results of this investigation suggest that the potential risk for contamination of the final end-products (cold cuts) posed by the presence of *L. monocytogenes* in fresh meat may have been overestimated. Nevertheless, in one plant the same ET was found in the fresh meat and along the processing line, including the end-product. It is possible that personal and general hygiene in the packing room could be more significant than fresh meat with regard to *L. monocytogenes* contamination of cold cuts. Good manufacturing practices and critical control points during the processing are probably the most important prophylactic measures to avoid *L. monocytogenes* contamination in cold cuts. A very small percentage of humans (often $< 1\%$) appear to be healthy carriers of *L. monocytogenes* (Jensen, 1993), although slaughterhouse workers have a higher carrier rate (4.8%) (Bojsen-Møller, 1972). Moreover, higher faecal carrier rates of 11.9% among office personnel and 13.3% among slaughterhouse workers have also been reported (Schuchat et al., 1991). Workers in food plants seem to carry *Listeria* spp. on their hands more frequently than office personnel in the same plant ($P < 0.015$) (Kerr et al., 1993). Thus, it is likely that the ETs of significance in the end-products are brought into the plant by humans.

Although faeces and skin of slaughter animals are considered to be sources of *L. monocytogenes* contamination, the slaughterhouse environment has recently been more often implicated as an important source. As regards pigs, recent studies have shown a much higher incidence of *L. monocytogenes* in the environment of the cutting room as well as on the primary cuts produced in this slaughterhouse area, compared to the incidence at earlier stages of slaughtering (Van den Elzen and Snijders, 1993; Wendtland and Bergann, 1994). Often, only a few or no isolates of *L. monocytogenes* are recovered from fresh pig carcasses (Nesbakken et al., 1994). Results of an investigation using randomly amplified polymorphic DNA (RAPD) (Van den Elzen et al., 1995) indicated that *L. monocytogenes* strains originating from pigs do not account for the contamination of the primary cuts. The significance of *L. monocytogenes* strains in the production environment has been emphasized by Boerlin and Piffaretti (1991): certain ETs among *L. monocytogenes* strains have often been isolated from meat, but not from animals. These findings indicate that contamination of meat with *L. monocytogenes* might originate mainly from the environment in which the meat is processed rather than from the animals themselves. This could explain the differences in the distribution of *L. monocytogenes* serovars and ETs isolated from slaughter animals, carcasses, fresh meat and processed meat. The results from two other investigations (Røtterud and Nesbakken, 1991; Røtterud and Nesbakken, 1994) indicate that the presence of *L. monocytogenes* in cold cuts seems to be connected with contamination after heat processing rather than insufficient heat processing. These findings also support the conclusions from the present investigation.

References

Bojsen-Møller, J. (1972) Human listeriosis: diagnostic, epidemiological and clinical studies. Acta Pathol. Microbiol. Scand. (B) S 229:1–157.

Boerlin, P. and Piffaretti, J.C. (1991) Typing of human, animal, food, and environmental isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis. Appl. Environ. Microbiol. 57:1624–1629.

Glass, A.G. and Doyle, M.P. (1989) Fate of *Listeria monocytogenes* in processed meat products during refrigerated storage. Appl. Environ. Microbiol. 55:1565–1569.

Jensen, A. (1993) *Listeria* in faecal and genital specimens. Med. Microbiol. Lett. 2:125–130.

Kerr, K.G., Birkenhead, D., Seale, K., Major, J. and Hawkey, P.M. (1993) Prevalence of *Listeria* spp. on the hands of food workers. J. Food Prot. 56:525–527.

Kolstad, J., Caugant, D.A. and Rørvik, L.M. (1992) Differentiation of *Listeria monocytogenes* isolates by using plasmid profiling and multilocus enzyme electrophoresis. Int. J. Food Microbiol. 16:247–260.

Nesbakken, T., Nerbrink, E., Røtterud, O.J. and Borch, E. (1994) Reduction of *Yersinia enterocolitica* and *Listeria* spp. on pig carcasses by enclosure of the rectum during slaughter. Int. J. Food Microbiol. 23:197–208.

Piffaretti, J.C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Musser, J.M., Selander, R.K. and Rocourt, J. (1989) Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA, 86:3818–3822.

Rørvik, L.M., Caugant, D.A. and Yndestad, M. (1995) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. Int. J. Food Microbiol. 25:19–27.

Røtterud, O.J. and Nesbakken, T. (1991) *Listeria monocytogenes* i kjøttindustrien-forekomst og forebyggende tiltak. Nor. Vet. Tidsskr. 103:697–705.

Røtterud, O.J. and Nesbakken, T. (1994) Varmeinaktivering av *Listeria monocytogenes* i kjøttpålegg. Nor. Vet. Tidsskr. 106:379–387.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schuchat, A., Swaminathan, B. and Broome, C. (1991) Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4:169–183.

Selander, R.K., Caugant, D.A., Ochman, H., Musser, J.M., Gilmour, M.N. and Whittam, T.S. (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51:873–884.

Sneath, P.H.A. and Sokal, R.R. (1973) Numerical Taxonomy. W.H. Freeman and Co., San Francisco, CA, USA.

Van den Elzen, A.M.G. and Snijders, J.M.A. (1993) Critical points in meat production lines regarding the introduction of *Listeria monocytogenes*. Vet. Quart. 15: 143–145.

Van den Elzen, A.M.G., Klaassen, C.C.M., Voskamp, P. and Snijders, J.M.A. (1995) Characterisation of *Listeria monocytogenes* from pigs and pork at a slaughterhouse using random amplification of polymorphic DNA; a pilot study. In: Proceedings II: 41 st Annual Int. Congress of Meat Science and Technology, San Antonio, TX, pp. 243–244.

Welshimer, H.J. (1981) The genus *Listeria* and related organisms. In: The Procaryotes. Starr, M.P., Stolp, H., Trüper, H.G., Barlows, A. and Schlegel, H.G. (Eds.), Springer-Verlag, Berlin, pp. 1680–1687.

Wendtland, A. and Bergann, T. (1994) *Listeria monocytogenes*: occurrence in a factory for slaughtering, carving and meat processing. Fleischwirtsch. 74:1329–1331.