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TRACING THE SOURCES OF *LISTERIA MONOCYTOGENES* CONTAMINATION AND LISTERIOSIS USING MOLECULAR TOOLS

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ACADEMIC DISSERTATION

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ABBREVIATIONS

AFLP, amplified fragment length polymorphism
ALOA, Agar *Listeria* according to Ottaviani and Agosti
CAMP, Christine-Atkins-Munch-Petersen test
cfu, colony-forming units
CNS, central nervous system
DI, discrimination index
EB, *Listeria* enrichment broth
EHA, enhanced haemolysis agar
ESP, EDTA-sodium lauroyl sarcosine buffer with proteinase K
FDA, Food and Drug Administration
IDF, International Dairy Federation
ISO, International Organization for Standardization
LEB, *Listeria* enrichment broth
LMBA, *Listeria monocytogenes* blood agar
MLST, multilocus sequence typing
MRP, macrorestriction pattern
NCFA, Nordic Committee on Food Analysis
PALCAM, selective solid media containing polymyxin B, acriflavin, lithium chloride, ceftazidime, aesculin and mannitol
PCR, polymerase chain reaction
PCR-REA, polymerase chain reaction followed by restriction enzyme analysis
PFGE, pulsed-field gel electrophoresis
PMSF, phenylmethylsulfonyl fluoride
RAPD, randomly amplified polymorphic DNA
REA, restriction enzyme analysis
RTE, ready-to-eat products
TSBYE, tryptic soya broth with yeast extract
UPGMA, unweighted pair group method using arithmetic averages

ABSTRACT

Listeria monocytogenes is a causative agent of listeriosis, a severe foodborne disease associated with a high case fatality rate. To prevent product contamination with *L. monocytogenes,* it is essential to understand listerial contamination routes in the food processing industry. This will enable efficient prevention measures to be developed, thus reducing the number of cases of listeriosis. The aim of this study was to investigate *L. monocytogenes* contamination routes and sources of listeriosis using molecular typing techniques.

In low-capacity slaughterhouses, a high prevalence of *L. monocytogenes* was found in tonsils and tongues, and in two slaughterhouses, carcasses were contaminated. *L. monocytogenes* of tonsil and tongue origin was shown to be a possible source of contamination of slaughtering equipment, which may in turn spread the pathogen to carcasses. In a cold-smoked rainbow trout processing plant, the contamination was demonstrated to occur during processing. The dominating *L. monocytogenes* pulsotypes of the final product were associated with brining and slicing, and contaminants of raw fish were not detected in the final product. The most important contamination sites of cold-smoked rainbow trout were brining and slicing machines.

A typical feature of *L. monocytogenes* contamination was shown to be the existence of dominating strains in food processing plants. Persistent and sporadic strains from 11 food processing plants were compared with PFGE and AFLP typing. Thirteen of the 15 genotypes presented by persistent strains were specific to persistent strains, and 94% of the genotypes showed by sporadic strains were recovered only among sporadic strains. Even though *L. monocytogenes* strains causing persistent contamination differ from sporadic ones; they do not seem to be descended from a common ancestor and no specific evolutionary lineage of persistent strains has been found.

An outbreak of listeriosis was identified by continuous typing of human strains. The repeated isolation of specific PFGE pattern strains of rare serotype 3a in human listeriosis cases was the basis for recognition of the outbreak. In addition, typing of human *L. monocytogenes* strains enabled identification of patients included in the outbreak and confirmation of cessation of the outbreak. Moreover, the common vehicle of infections, butter, was determined by comparing strains from patients with those isolated from suspect food.

Typing results of strains isolated from food products may be used to aid in tracing vehicles of infections, by comparing patient strains with food strains. However, comparison of strains originating from foods of various producers showed that *L. monocytogenes* strains possessing identical pulsotypes can be found from products of several food manufacturers. As similar strains can be found in different product types of different producers and countries, the recovery of identical patterns from food and patient strains, does not necessarily prove that the food is the vehicle of infection. Thus in addition to molecular typing data, epidemiological investigations and establishment of the consumption of the suspected food item are of major importance.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to in the text by Roman numerals I to V:

I. Autio T., Säteri T., Fredriksson-Ahomaa M., Rahkio M., Lundén J., and Korkeala H. 2000. *Listeria monocytogenes* contamination pattern in pig slaughterhouses. J. Food Prot. 63: 1438–1442.

II. Autio T., Hielm S., Miettinen M., Sjöberg A.-M., Aarnisalo K., Björkroth J., Mattila-Sandholm T., and Korkeala H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. Appl. Environ. Microbiol. 65: 150–155.

III. Autio T., Keto-Timonen R., Lundén J., Björkroth J., and Korkeala H. 2003. Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Syst. Appl. Microbiol. In Press.

IV. Lyytikäinen O., Autio T., Maijala R., Ruutu P., Honkanen-Buzalski T., Miettinen M., Hatakka M., Mikkola J., Anttila V.-J., Johansson T., Rantala L., Aalto T., Korkeala H., and Siitonen A. 2000. An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in Finland. J. Infect. Dis. 181: 1838–1841.

V. Autio T., Lundén J., Fredriksson-Ahomaa M., Björkroth J., Sjöberg A.-M., and Korkeala H. 2002. Similar *Listeria monocytogenes* pulsotypes detected in several foods originating from different sources. Int. J. Food. Microbiol. 77: 83–90.

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1 INTRODUCTION

Listeria monocytogenes is a common bacterium in nature. It is a causative agent of listeriosis, which occurs both in humans and in animals. Invasive human listeriosis is a rare, but a severe disease associated with a high case fatality rate. *L. monocytogenes* was only discovered to be a foodborne pathogen in the 1980's, despite being recognized as a human pathogen as early as 1926. Several food products have been linked with epidemics and sporadic cases of listeriosis, including meat, dairy, vegetable and fish products. Annually, 17–41 listeriosis cases have been reported in Finland since 1995, but the source of infections has only been determined three times. Sporadic occurrence of the majority of cases makes tracing of vehicles of listeriosis difficult. Moreover, because the incubation period is long, there is often no food left for examination. In addition *L. monocytogenes* is found in a variety of food products, thus hindering the linking of a specific product with cases of listeriosis.

Modern processing and packaging techniques have enabled production of foods with extended shelf-lives. With regard to listeriosis, particularly processed foods, which support the growth of *L. monocytogenes* during a long shelf-life and are consumed without further heating, are of greatest concern. *L. monocytogenes'* psychrotrophic and facultatively anaerobic nature and its ability to grow in wide pH and a_w ranges makes it difficult to control in products and in food processing facilities. Thus, to prevent product contamination with *L. monocytogenes,* it is essential to understand listerial contamination routes in food processing industries. By evolving efficient prevention measures, cases of listeriosis can ultimately be prevented.

2 REVIEW OF LITERATURE

2.1 *Listeria monocytogenes* and listeriosis

2.1.1 *Listeria monocytogenes*

The history of *Listeria monocytogenes* begins in 1924, when Murray, Webb and Swann (1926) isolated a 1–2 µm long and 0.5 µm wide round-ended Gram-positive rod in dead laboratory rabbits and guinea-pigs in Cambridge and named it *Bacterium monocytogenes.* In 1927, Pirie described unusual deaths of gerbils in South Africa and named the discovered bacterium *Listerella hepatolytica* in honour of Lord Joseph Lister. Because the strains isolated by Murray et al. (1926) and Pirie (1927) showed great similarity, the bacterium was renamed *Listerella monocytogenes.* However, the generic name *Listerella* had previously been used for protozoan and Pirie (1940) proposed changing the name to *Listeria monocytogenes.* This name was accepted, even though it already existed in botanical taxonomy, an orchid named *Listeria,* and in zoology, a diptera called *Listeria* (Seeliger 1961). Genera of *Listeria* and *Brochothrix* are members of the family *Listeriaceae*, the order *Bacillales,* the class *Bacilli* and the phylum *Firmicutes.* Currently, the genus *Listeria* contains in addition to *L. monocytogenes,* five other species: *L. grayi, L. innocua, L. ivanovii* subspecies *londoniensis* and subspecies *ivanovii, L. seeligeri* and *L. welshimeri* (Boerlin et al. 1991, 1992, Rocourt et al. 1992). The main human and animal pathogen is *L. monocytogenes,* but a few human cases (Cummins et al. 1994, Lessing et al. 1994) and to a somewhat greater extent animal listeriosis (Low and Linklater 1985, Sergeant et al. 1991, Alexander et al. 1992, Gill et al. 1997), particularly ovine listeriosis, have been caused by *L. ivanovii.*

L. monocytogenes is widely spread in nature including soil, both cultivated and uncultivated fields, forests, sewage and aquatic environment (Weis and Seeliger 1975, Low and Donachie 1997). Healthy carriage of *L. monocytogenes* has also been well described in a variety of animal species as well as in humans (Low and Donachie 1997). The carriage rate in healthy people varies between <1% and 6%.

2.1.2 *L. monocytogenes* infections

In animals

Nearly all domestic animals are susceptible to Listeria infections, but animal listeriosis occurs most commonly in ruminants (Seeliger 1961). In monogastric animals, listeriosis is rare and primarily manifests itself as septicemia. The main clinical manifestations of ruminant listeriosis are encephalitis, septicemia and abortion (Low and Linklater 1985, Low and Donachie 1997). *L. monocytogenes* may also be shed in milk by both diseased and healthy animals, and prolonged excretion of bacteria has been described (Fthenakis et al. 1998, Wagner et al. 2000). Seasonal variation both in prevalence of listeriosis and in prevalence of *L. monocytogenes* in raw milk has been reported. The higher incidence in winter months has been suggested to be associated with indoor housing and silage feeding (Farber et al. 1988, Husu 1990).

In humans

L. monocytogenes can cause both invasive and non-invasive infections. Invasive listeriosis is a severe disease mainly associated with a specific risk group of people and the case fatality rate is high, whereas fairly mild non-invasive infections can also occur in healthy people (Crum 2002).

Invasive listeriosis is rare, with an annual incidence rate of 2–9 cases per million, but it is one of the most severe foodborne infections and has a high, approximately 30%, case fatality rate (Rocourt et al. 2001, Anonymous 2003a). Listeriosis characteristically occurs in persons with a predisposing condition or disease, such as pregnancy, neonatality, malignancy, transplantation, alcoholism, immunosuppressive therapy, diabetes, old age and HIV, among others (Schuchat et al. 1992, Skogberg et al. 1992, Mylonakis et al. 1998, Smerdon et al. 2001, Aouaj et al. 2002). The outcome of listeriosis includes central nervous system (CNS) infections, septicemia, abortions, neonatal infections and stillbirth. The incubation time of invasive listeriosis varies from one day to several weeks. The infectious dose is unknown and is suggested to vary among individuals (Crum 2002).

L. monocytogenes bacteremia in pregnant women expresses itself as self-limited flu-like symptoms, but it may result in abortion, stillbirth or premature birth of the infected baby (McLauchlin 1990). In neonates, listeriosis manifests as early-onset sepsis due to in-utero infection or as late-onset meningitis due to infection during passage through the birth canal or nosocomial transmission. Unlike the almost negligible fatality in mothers, the fatality in

early-onset sepsis in neonates is fairly high, 8–38% (McLauchlin 1990, Nolla-Salas et al. 1998).

In adults, bacteremia and CNS infections are the most frequent presentations of invasive listeriosis. The symptoms associated with bacteremia are fever and other non-specific symptoms such as malaise and fatigue. Patients with CNS infection present with fever, malaise, ataxia, seizures and altered mental status (Nieman and Lorber 1980, Skogberg et al. 1992, Chan et al. 2001b)

In addition to severe listeriosis, *L. monocytogenes* has been shown to cause non-invasive infections in healthy people; gastroenteritis and dermatitis are the most frequently reported (McLauchlin and Low 1994, Hof 2001). Mild gastrointestinal illness associated with *L. monocytogenes* was first demonstrated in 1994 by Riedo et al., followed by several other reports (Salamina et al. 1996, Dalton et al. 1997, Heitmann et al. 1997, Miettinen et al. 1999b, Aureli et al. 2000, Farber et al. 2000, Frye et al. 2002, Sim et al. 2002). *L. monocytogenes* is not routinely screened in cases of febrile gastroenteritis; thus, *L. monocytogenes* as a cause of febrile gastroenteritis may be underdiagnosed. The symptoms, which include fever, diarrhea, headache, body aches and vomiting, occur after a short, 18–28 h, incubation time. Foods linked with the illness have shown to contain high levels, $1.9 \times 10^5 - 1.6 \times 10^9$ cfu/g of *L. monocytogenes,* indicating that a large number of bacteria are needed to cause febrile gastroenteritis (Dalton et al. 1997, Miettinen et al. 1999b, Aureli et al. 2000, Frye et al. 2002, Sim et al. 2002).

Contact dermatitis caused by *L. monocytogenes* has been described, especially on the hands or arms of veterinarians and farmers (McLauchlin and Low 1994, Visser 1998). These cases have been associated with direct contact with infectious material during manual delivery of stillborn or aborting animal fetuses. Skin abrasions coinciding with a high dose of bacteria have been reported in this occupational disease (McLauchlin and Low 1994). Similarly to febrile gastroenteritis, *L. monocytogenes* dermatitis is mild and resolves successfully.

Foodborne *L. monocytogenes* infections

Even though *L. monocytogenes* was first time recognized as a human pathogen in 1929 (Nyfeldt 1929), the route of infections was unknown until the 1980s, when outbreaks of listeriosis were associated with consumption of contaminated food (Schlech et al. 1983, Fleming et al. 1985, Linnan et al. 1988). Food as a possible vehicle of listeriosis had been considered earlier,

and Dr. Seeliger summarized transmission routes in his monograph *Listeriosis* published in 1961 as follows:

"Like other zoonoses listeriosis is transmissible from animals to man. The chains of infection usually end "blind", i.e. in most cases the organisms are not known to be transmitted from a human host to others, with the exception of neonatal listeriosis which is transmitted from the pregnant to the foetus by the transplacental route. In most cases the transmission takes place by direct contact with diseased animals or their excretions, furthermore *by consumption of food containing the organisms* and by inhalation of infected dust." (Seeliger 1961)

There are several reasons for the late establishment of the foodborne nature of listeriosis. The symptoms of invasive listeriosis are severe and differ from classical foodborne diseases. Moreover, listeriosis is a rare disease, occuring mainly among a high-risk group of people, and majority of cases are believed to be sporadic. In addition, the long incubation time hinders tracing of vehicles of infections and often no food is left for microbiological analysis.

The first convincing evidence of foodborne transmission of *L. monocytogenes* was presented by Schlech et al. (1983), who demonstrated coleslaw as a vehicle of infection in an outbreak of listeriosis in Nova Scotia, Canada, in 1981 *L. monocytogenes* was assumed to be of animal origin since the cabbages used in coleslaw were fertilized with sheep manure originating from a herd with cases of ovine listeriosis. After the coleslaw outbreak, four epidemics, two in USA, one in Switzerland and one in Denmark, were linked to dairy products, thus generating concern about presence of *L. monocytogenes* in these products (Fleming et al. 1985, Linnan et al. 1988, Bille 1990, Jensen et al. 1994). Several epidemics have subsequently been described and various kinds of food products linked with the outbreaks (Table 1).

In Finland, Junttila and Brander (1989) described a listeria septicemia in an 80 year old man to be associated with consumption of home-made salted mushrooms. In 1997, five persons suffered from a febrile gastroenteritis caused by *L. monocytogenes* originating from cold-smoked rainbow trout (Miettinen et al. 1999b).

TABLE 1. Apparent and confirmed vehicles of epidemics of *Listeria monocytogenes* infections.

Implicated vehicle	Year	No. of cases (deaths)	Country	Reference
Coleslaw	1981	41 (18)	Canada	Schlech et al. 1983
Pasteurized milk	1983	49 (14)	USA	Fleming et al. 1985
Mexican-style cheese	1985	142 (48)	USA	Linnan et al. 1988
Soft cheese	1983-87	122 (34)	Switzerland	Bille 1990
Pâté	1987-89	366 (NK[b])	UK	McLauchlin et al. 1991
Blue mould or hard cheese	1989-90	27 (NK)	Denmark	Jensen et al. 1994
Shrimp[a]	1989	10 (0)	USA	Riedo et al. 1994
Processed meat or pâté	1990	11 (NK)	Australia	Watson and Ott 1990
Jellied pork tongue	1992-93	279 (85)	France	Goulet et al. 1993
Rillettes	1993	38 (1)	France	Goulet et al. 1998
Rice salad[a]	1993	18 (0)	Italy	Salamina et al. 1996
Chocolate milk[a]	1994	48 (0)	USA	Dalton et al. 1997
Rainbow trout	1994-95	8 (2)	Sweden	Ericsson et al. 1997
Raw milk cheese	1995	20 (0)	France	Goulet et al. 1995
Rainbow trout[a]	1997	5 (0)	Finland	Miettinen et al. 1999b
Corn and tuna salad[a]	1997	1566 (0)	Italy	Aureli et al. 2000
Meat products	1998	40 (4)	USA	Anonymous 1998b
Rillettes	1999-2000	10 (3)	France	de Valk et al. 2001
Jellied pork tongue	1999-2000	32 (5)	France	de Valk et al. 2001
Deli turkey meat	2000	29 (4)	USA	Anonymous 2000b
Ready-to-eat meat[a]	2000	7 (0)	NZ[c]	Sim et al. 2002
Ready-to-eat meat[a]	2000	21 (0)	NZ	Sim et al. 2002
Mexican-style cheese	2000-01	12 (0)	USA	Anonymous 2001
Turkey[a]	2001	16 (0)	USA	Frye et al. 2002

[a]Gastroenteritis; [b]NK, not known; [c]NZ, New Zealand

2.1.3 Isolation and identification of *L. monocytogenes*

Maintenance of pure *L. monocytogenes* cultures and isolation in cases where *L. monocytogenes* is the only bacteria present, such as in samples of blood or cerebrospinal fluid from listeriosis patients, are relatively easy, since the pathogen grows well on most common bacteriological media. However, isolation in complex samples, such as food, environmental and stool samples, containing abundant background flora and a low number of *Listeria*, requires increasing of *L. monocytogenes* before it can be detected. The first enrichment method, cold enrichment, was based on the psychrotrophic nature of *L. monocytogenes* and employed incubation in non-selective broth at a cold (2–5°C) temperature for several weeks to months (Seeliger 1961). This time consuming method has subsequently been replaced by methods involving selective enrichment and selective plating based on inhibition of the growth of background flora by adding such inhibitory agents as lithium chloride, nalidixic acid, acriflavine, cefotetan, ceftazidime, colistin, cycloheximide, fosfomycin and polymyxin B. Currently, several procedures including standard methods from FDA, IDF, ISO and NCFA, are available for detection of *L. monocytogenes* (Anonymous 1990, 1995, 1996, 1998a, Hitchins 1998, Anonymous 1999a). In several studies, various enrichment and plating procedures for detection of *L. monocytogenes* from different kind of samples have been compared, and none of the methods has proven to be suitable for all purposes (Warburton et al. 1991, 1992, Westöö and Peterz 1992, Waak et al. 1999, Scotter et al. 2001a, 2001b).

Selective enrichment, either one-step or two-step, is performed at 30–37°C. The most commonly used enrichment broths are EB (*Listeria* enrichment broth, Lovett et al. 1987) in one-step enrichment, and *Listeria* enrichment broths (LEBI and LEBII) by McClain and Lee (1988) and Fraser broths (half-Fraser and Fraser) by Fraser and Sperber (1988) in two-step enrichment procedures. EB consists of TSBYE supplemented nalidixic acid, acriflavine and cycloheximide as selective agents. The disadvantage of EB is its low buffering capacity compared with Fraser broths and LEBI/II. The secondary enrichment LEB and Fraser broths contain elevated concentrations of selective agents compared with the primary enrichment broths. In addition to the inhibitory agents (nalidixic acid and acriflavine) of LEB, Fraser broths contain lithium chloride for selectivity and ammonium citrate to detect the aesculin hydrolysis resulting in blackening of the broth.

Selective enrichment is followed by plating on selective solid media. Besides selective agents, most solid media contain indicator substrates, chromogens or blood to distinguish

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Listeria from other bacteria and/or *L. monocytogenes* from other *Listeria* species. The solid *Listeria* selective media commonly used are Oxford (Curtis et al. 1989) and PALCAM (van Netten et al. 1989), both of which contain aesculin with ferric ammonium citrate as indicatory substrates to differentiate *Listeria* from other bacteria. Combined used of these media provides high recovery rates, as they differ in their selectivity. Selective solid media specific for *L. monocytogenes* have been developed. To distinguish *L. monocytogenes* from other *Listeria* species, they contain chromogens (ALOA agar, Vlaemynck et al. 2000) or blood (enhanced haemolysis agar, EHA, Cox et al. 1991; *Listeria monocytogenes* blood agar, LMBA, Johansson 1998).

Typical colonies grown on selective media are confirmed as *Listeria* spp. by Gram staining, catalase reaction and motility at 25°C. Gram-positive, catalase positive rods showing tumbling motility at 25°C are further verified as *L. monocytogenes* by detection of ß-haemolysis, by testing the fermentation of rhamnose and xylose, and by CAMP-test. Several commercial rapid tests for identification of *Listeria* spp. and *L. monocytogenes* have been developed and are considered suitable alternatives to conventional tests.

2.2 Typing of *L. monocytogenes*

Various methods have been applied for subtyping of *L. monocytogenes* strains and several multi-centre studies conducted to evaluate different methods used for subtyping *L. monocytogenes* (Boerlin et al. 1991, Bille and Rocourt 1996, Brosch et al. 1996, Caugant et al. 1996, Gerner-Smidt et al. 1996, Schönberg et al. 1996, Swaminathan et al. 1996, Wernars et al. 1996). The choice of method depends most on the performance criteria of a method, such as typeability, discriminatory power, reproducibility, rapidity and ease of use, and the purpose of subtyping, e.g. phylogenetic analysis, epidemiological surveillance, outbreak investigations or food processing contamination analysis (Struelens et al. 1996).

2.2.1 Phenotyping

Serotyping

Serotyping has been a classical tool in subtyping of *L. monocytogenes.* Based on somatic (O) and flagellar (H) antigens, *L. monocytogenes* strains are divided into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). However, over 95% of strains isolated in human cases and in foods belong to serotypes 1/2a, 1/2b and 4b, thus

limiting the usefulness of serotyping in both epidemiological and contamination investigations (Farber and Peterkin 1991, Schönberg et al. 1996).

Phage typing

Phage typing has been shown to be an efficient method for large scale subtyping of *L. monocytogenes* (Audurier and Martin 1989), and a standard phage set with a standardized method has been described (Rocourt et al. 1985, McLauchlin et al. 1996). Even though phage typing shows high discrimination power, the high number of non-typeable strains, particularly serotype 1/2 strains is a major disadvantage of the method (Rocourt et al. 1985). Moreover, the method is only available at a limited number of reference laboratories because of the need to maintain stocks of biologically active phages and control strains.

2.2.2 Molecular typing

Ribotyping

Ribotyping is based on the use of nucleic acid probes targeting ribosomal genes after restriction enzyme analysis of chromosomal DNA (Grimont and Grimont 1986). Since ribosomal RNA is present and highly conserved in all bacteria, the method is commonly used for subtyping of different bacterial species, and a fully automated ribotyping system (RiboPrinter®) is commercially available (Farber 1996). The chromosomal DNA is first cut using frequent cutting restriction enzyme and fragments are separated in agarose gel electrophoresis. The separated DNA fragments are transferred onto membrane and hybridized with labelled probe containing 23S and 16S sequences. The visualization of fragments containing ribosomal gene/s usually results in 1 to 15 fragments per fingerprint.

Ribotyping has been used extensively in characterization of *L. monocytogenes* in taxonomical, epidemiological and contamination route investigations (Baloga and Harlander 1991, Graves et al. 1991, Arimi et al. 1997, Dalton et al. 1997, Wiedmann et al. 1997, Allerberger and Fritschel 1999, Gendel and Ulaszek 2000, de Cesare et al. 2001, Nadon et al. 2001, Suihko et al. 2002). The advantages of ribotyping are typeability and reproducibility, and thus, fingerprint databases based on automated ribotyping have been constructed. The main drawback with ribotyping is in its discriminating ability, particularly for serotype 4b it may be inadequate for epidemiological investigations (Swaminathan et al. 1996).

Methods involving PCR amplification

Several typing methods involving polymerase chain reaction (PCR) have been developed. Some of the methods employ only PCR amplification (randomly amplified polymorphic DNA, RAPD), whereas in others PCR amplification is performed either before (PCR-REA) or after (amplified fragment length polymorphism, AFLP) restriction enzyme analysis. Because of PCR amplification step these methods require only a small amount of DNA.

RAPD employs short (9–10 bp) primers with sequences chosen at random, thus prior sequence knowledge of template DNA is not needed. The annealing temperatures used in RAPD are relatively low (25–40°C) compared with conventional PCR reactions. Short primers combined with low-stringency amplification conditions allows primer annealing at several locations of the genome, resulting in amplification of multiple fragments (Power 1996). RAPD is rapid and relatively simple, but its reproducibility is low and development of standardized protocols has proven to be difficult (Wernars et al. 1996). Several studies have demonstrated RAPD to be a highly discriminative method for rapid subtyping of *L. monocytogenes;* however, the poor reproducibility has limited general use of the method and construction of RAPD fingerprint databases (Lawrence et al. 1993, Niederhauser et al. 1994, Lawrence and Gilmour 1995, Wernars et al. 1996, Allerberger et al. 1997, Wagner et al. 1998, 1999, Malak et al. 2001).

In PCR-REA, known sequences are amplified under stringent PCR conditions and the resulting amplicon is digested with a restriction enzyme. PCR-REA methods are relatively rapid, simple and reproducible. However, disadvantages include needing prior knowledge of the region studied and variable discriminatory power (Farber 1996). PCR-ribotyping in which the spacer regions existing between rRNA coding genes are targeted is probably the most common form of PCR-REA. Other common targets are virulence genes, but any region of interest can be amplified and digested. In addition to PCR-ribotyping (Sontakke and Farber 1995, Franciosa et al. 2001), PCR-REA has been applied to determine polymorphism of virulence-associated genes (*hly*A, *iap, prf*A, *mpl, act*A, *inl*A and *inl*B) of *L. monocytogenes* (Vines et al. 1992, Ericsson et al. 1995, Wiedmann et al. 1997, Saito et al. 1998, Giovannacci et al. 1999, Unnerstad et al. 1999, Jeffers et al. 2001).

Similar to PCR-REA, amplified fragment length polymorphism (AFLP) utilizes both digestion with restriction enzyme and PCR amplification, but here amplification is performed after digestion. The method involves digestion of DNA with two restriction enzymes,

ligation of adaptors, selective amplification of sets of restriction fragments and analysis of the amplified fragments (Vos et al. 1995). The DNA is digested with two restriction enzymes, one with an average cutting frequency and the other with a higher cutting frequency that results in hundreds of fragments. The fragments are ligated to double-stranded oligonucleotide adapters, which are complementary to the sequence of restriction sites and are designed such that the original restriction site is not restored after ligation. Ligation is followed by two PCR amplifications under stringent conditions with primers specific to adapters. To reduce the number of amplified fragments, in the second PCR, primers have a sequence corresponding to that of the adapter, restriction site and one or more nucleotides of the original target DNA, referred to as selective nucleotides. On average if one selective nucleotide is added one of four of the ligated fragments are amplified. The resulting 30–200 fragments are separated by polyacrylamide gel electrophoresis, and thus, automatic sequencing apparatuses are widely used. Only a few studies have been conducted to characterize *L. monocytogenes* with AFLP, but they demonstrated AFLP to be a discriminative and reproducible method (Aarts et al. 1999, Keto-Timonen et al. 2003). A simplified variation of AFLP using one restriction enzyme and resolving of fragments in agarose gel has also been described (Ripabelli et al. 2000, Guerra et al. 2002).

Pulsed-field gel electrophoresis (PFGE) typing

Pulsed-field gel electrophoresis (PFGE) typing is based on cutting an entire bacterial genome into relatively large (10 kb to 800 kb) and few (5 to 20) visualized DNA fragments. The large fragments are not separated or are poorly separated by conventional gel electrophoresis, and thus, pulsed-field gel electrophoresis, where the orientation of the electric field is changed periodically, is used. In PFGE typing, the bacterial DNA is isolated *in situ* to obtain unshared DNA. The cells are embedded in agarose prior to lysis with detergent and enzymes. The entire genome is digested with an infrequently cutting restriction enzyme and the resulting fragments are separated by PFGE (Römling et al. 1994). PFGE typing is a highly discriminative and reproducible method and is the typing method of choice for many bacteria and PFGE fingerprint databases have been constructed (Swaminathan et al. 2001).

PFGE typing has been widely used in characterization of *L. monocytogenes* isolates and standardized protocols have been proposed (Brosch et al. 1991, Buchrieser et al. 1991, 1993, Jacquet et al. 1995, Brosch et al. 1996, Destro et al. 1996, Unnerstad et al. 1996, Giovannacci

et al. 1999, Miettinen et al. 1999a, 1999b, 2001b, Dauphin et al. 2001, Graves and Swaminathan 2001, Vela et al. 2001, Lundén et al. 2002). PFGE typing is considered to be the current gold standard for typing of *L. monocytogenes* due to its high discriminatory ability and reproducibility (Wiedmann 2002). Therefore, national networks for comparing PFGE fingerprints are in use at least in Finland and in USA (Graves and Swaminathan 2001, Rantala et al. 2001).

DNA sequencing based methods

The whole genome sequences of one *L. monocytogenes* and one *L. innocua* strain have recently been published, and sequencing of another *L. monocytogenes* strain and one *L. ivanovii* strain are in progress (Glaser et al. 2001, Anonymous 2003c). This knowledge will probably lead to development of sequencing based methods also for typing purposes. Until now, pyrosequencing and multilocus sequence typing have been applied for typing *L. monocytogenes.*

Pyrosequencing, a real-time DNA sequencing technique employing detection of released inorganic pyrophosphate by enzymatic reactions, has been used for determination of single nucleotide polymorphism in the human genome (Ronaghi and Elahi 2002). The method is rapid and a large number of samples can be screened inexpensively. However, it is suitable only for determination of short, up to 50 nucleotides, sequences, and difficulty in completing incorporation of nucleotides in homopolymeric regions may limit the use of this method. To the author's knowledge, pyrosequencing has been used for typing *L. monocytogenes* only once. Unnerstad et al. (2001) applied pyrosequencing to detect single-nucleotide polymorphisms in *inlB* gene and demonstrated grouping 106 *L. monocytogenes* strains into four categories.

Multilocus sequence typing (MLST) is a newly developed typing method that determines variation of multiple genes or gene fragments by using DNA sequencing. MLST targets slowly diversifying genes (e.g. housekeeping genes), virulence genes and/or intergenic regions (Maiden et al. 1998, Enright and Spratt 1999). The most common MLST scheme involves sequencing 450–500 bp long fragments of seven housekeeping genes. For each gene, the different sequences are designed as alleles, and sequences that differ at even a single nucleotide are assigned as different alleles. Combination of different alleles results in different sequence types. MLST is expensive and technically demanding, but the advantage is that sequence data is truly portable, enabling exchange of typing data and placing of one

expanding global MLST database in World-Wide Web sites (Chan et al. 2001a, Anonymous 2003b). To the author's knowledge, only one study has examined application of MLST in subtyping *L. monocytogenes* (Cai et al. 2002). In that study, the MLST approach targeting two housekeeping genes, one stress response gene, two virulence genes and two intergenic regions was developed. This method is promising and should be fully evaluated with large sets of epidemiologically well-defined isolates.

2.3 *L. monocytogenes* in foods and food processing

2.3.1 Properties of *L. monocytogenes* related to food and food processing

L. monocytogenes, a facultative anaerobic bacterium, is able to grow in foods packaged under vacuum or modified atmosphere (Buchanan et al. 1989). The optimal growth temperature is between 30 and 37°C, but it is able to grow at refrigeration temperatures as low as 0.5°C and can even survive freezing (Seeliger and Jones 1986, Junttila et al. 1988). Growth is limited by temperatures over 45°C, and *L. monocytogenes* is destroyed by pasteurization at 71.6°C in 15 s (Petran and Zottola 1989, Lovett et al. 1990). *L. monocytogenes* can grow within a wide pH range (4.3–9.6), with optimal growth occurring in a neutral or slightly alkaline environment. Minimum a_w value for growth of *L. monocytogenes* is 0.90 and it survives for extended periods even at lower a_w values (Petran and Zottola 1989, Lou and Yousef 1999). *L. monocytogenes* can grow even in 12% NaCl (w/v) and survive in 26% NaCl (w/v), thus limitation of its growth in foods by salt is unrealistic (Lou and Yousef 1999). In general, *L. monocytogenes* is sensitive *in vitro* to disinfectants commonly used by the food processing industry (van de Weyer et al. 1993). However, it has been shown that organic material reduces the activity of disinfectants (Best et al. 1990, Aarnisalo et al. 2000), and bacteria growing on biofilms are more resistant than planktonic cells (Ronner and Wong 1993). Moreover, differences between *L. monocytogenes* strains in sensitivity to disinfectants have been demonstrated, and adaptation and cross-adaptation to disinfectants can occur (Jacquet and Reynaud 1994, Aarnisalo et al. 2000, Aase et al. 2000, Romanova et al. 2002, Lundén et al. 2003a).

2.3.2 Prevalence of *L. monocytogenes* in raw materials

L. monocytogenes is widespread in the environment. It is commonly found in soil and water and on plant material and is also excreted by a variety of animals, and thus, may be introduced to the initial processing, such as slaughtering process and milking (Seeliger 1961, Fenlon et al.

1996, Fenlon 1999). The prevalence of *L. monocytogenes* in raw materials according to European studies is shown in Table 2.

In general, the prevalence of *L. monocytogenes* in raw milk is low and the contamination level is also often very low (Meyer-Broseta et al. 2003). *L. monocytogenes* is known to be secreted in milk by both diseased and healthy animals (Fthenakis et al. 1998, Wagner et al. 2000) and contamination of milk may also be due to environmental contamination from farm environment and faecal material due to poor hygiene practice (Sanaa et al. 1993, Hassan et al. 2001). Seasonal variation in the prevalence of *L. monocytogenes* in raw milk has been reported, with a higher prevalence in cold weather months; an association has been observed with silage feeding and in-door housing (Husu 1990, Husu et al. 1990b, Meyer-Broseta et al. 2003).

L. monocytogenes has been found both in pork and beef slaughterhouses (van den Elzen and Snijders 1993, Gill and Jones 1995, Saide-Albornoz et al. 1995, Jericho et al. 1996, Sammarco et al. 1997, Korsak et al 1998). Its prevalence varies between slaughterhouses, although the plants may be of similar overall design and line speed (van den Elzen and Snijders 1993, Gill and Jones 1995, Saide-Albornoz et al. 1995, Sammarco et al. 1997, Korsak et al 1998). The source of contamination has been suggested to be live animals, as they are known to harbour *L. monocytogenes* in faeces, tonsils and hide (Skovgaard and Morgen 1988, Buncic 1991), and similar strains have been recovered from live animals and the slaughterhouse environment (Giovannacci et al. 1999). However, the contamination of carcasses has been suggested to originate from the slaughterhouse environment and the different recoveries at plants may be attributable to varying hygienic conditions (Gobat and Jemmi 1991, Nesbakken et al. 1994, Saide-Albornoz et al. 1995, Borch et al. 1996). All of these surveys have been conducted in high-capacity slaughterhouses, and thus, the prevalence and contamination in low-capacity facilities is unknown. These two slaughterhouse types differ significantly not only in capacity but also in lay-out and working routines, and therefore, the results obtained in high-capacity slaughterhouses may not be generalized to low-capacity slaughterhouses.

The prevalence of *L. monocytogenes* in poultry carcasses is higher than in pork or beef carcasses (Table 2). However, live poultry is unlikely to be a common reservoir of *L. monocytogenes*, as poultry flocks before slaughter have shown to be negative or contain low levels of *L. monocytogenes* (Husu et al. 1990a, Ojeniyi et al. 1996, 2000). Moreover, the presence

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of *L. monocytogenes* in carcasses has been associated with a high rate of *L. monocytogenes* on processing equipment and in the environment, indicating that these are the source of carcass contamination (Ojeniyi et al. 1996, Uyttendaele et al. 1997, Ojeniyi et al. 2000, Miettinen et al. 2001b).

Although the natural niche of *L. monocytogenes* is probably soil and vegetation, it is also isolated in both fresh and marine water, with the highest prevalence in coastal areas and in polluted waters (Ben Embarek 1994). In fish, *L. monocytogenes* is found on such external surfaces as skin, gills, heads and slime, and the contamination of fish most likely depends on the presence of the bacteria in the surrounding waters (Eklund et al. 1995). The contamination rate of slaughtered salmon differs among slaughterhouses, and it has been suggested that some slaughterhouses may be colonized by *L. monocytogenes* (Rørvik 2000).

TABLE 2. Prevalence of *Listeria monocytogenes* in raw materials in Europe.

Sample	No. of positive samples/total (%)	Country	Reference
Raw milk			
Cow	1/59 (2)	Finland	Husu et al. 1990b
	25/1459 (2)	France	Meyer-Broseta 2003
	28/774 (4)	Spain	Gaya et al. 1998
	3/294 (1)	Sweden	Waak et al. 2002
Carcasses			
Poultry	132/552 (24)	Belgium, France	Uyttendaele et al. 1997
	103/320 (32)	Denmark	Ojeniyi et al. 1996
	15/100 (15)	Spain	Capita et al. 2001
Beef	11/50 (22)	Belgium	Korsak et al. 1998
	NK/NK (2)	Finland	Husu 1991
	0/13 (0)	Norway	Rørvik and Yndestad 1991
	0/144 (0)	Switzerland	Gobat and Jemmi 1991
	0/20 (0)	The Netherlands	van den Elzen and Snijders 1993
Pork	1/49 (2)	Belgium	Korsak et al. 1998
	0/25 (0)	Norway	Rørvik and Yndestad 1991
	0/480 (0)	Norway	Nesbakken et al. 1994
	0/480 (0)	Sweden	Nesbakken et al. 1994
	0/36 (0)	Switzerland	Gobat and Jemmi 1991
	4/90 (4)	The Netherlands	van den Elzen and Snijders 1993
Raw meat			
Poultry	112/410 (27)	Belgium, France	Uyttendaele et al. 1997
	8/17 (47)	Denmark	Skovgaard and Morgen 1988
	38/61 (62)	Finland	Miettinen et al. 2001b
	13/17 (76)	Greece	Samelis and Metaxopoulos 1999
	55/90 (61)	Norway	Rørvik and Yndestad 1991
	19/30 (63)	UK	MacGowan et al. 1994
Beef	12/94 (13)	The Netherlands	van den Elzen and Snijders 1993
	1/4 (25)	UK	MacGowan et al. 1994
Pork	13/34 (38)	Greece	Samelis and Metaxopoulos 1999
	107/296 (36)	The Netherlands	van den Elzen and Snijders 1993
	4/15 (27)	UK	MacGowan et al. 1994
Lamb	7/17 (41)	UK	MacGowan et al. 1994
NK	106/343 (31)	Denmark	Nørrung et al. 1999
Raw fish			
Salmon	0/16 (0)	Italy	Pourshaban et al. 2000
	0/50 (0)	Norway	Rørvik et al. 1995
	6/7 (86)	Norway	Dauphin et al. 2001
	0/40 (0)	Norway	Vaz-Velho et al. 1998
	16/215 (7)	Norway, Faroe Islands	Fonnesbech Vogel et al. 2001a
	7/8 (88)	UK	Dauphin et al. 2001
	2/18 (11)	NK	Dauphin et al. 2001
Rainbow trout	1/48 (2)	Portugal	Vaz-Velho et al. 1998
NK	3/24 (13)	UK	MacGowan et al. 1994
	33/232 (14)	Denmark	Nørrung et al. 1999
	4/27 (15)	Switzerland	Jemmi and Keusch 1994

NK, not known

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2.3.3 Occurrence of *L. monocytogenes* in food processing facilities

L. monocytogenes has been found in various food processing facilities, including dairy plants, fish processing plants, meat and poultry processing plants and ready-to-eat product processing plants. In processing plants, it is more commonly found in processing environment and other non-food contact sites than in food contact sites. Therefore processing environment contamination does not necessarily translate into contamination of equipment within the same plant (Pritchard et al. 1995).

Sites positive for *L. monocytogenes* on non-food contact surfaces have been floors, walls, trucks, drains, shoes, doors and door handles, and it has also been detected on sanitizing floor mats and foot baths (Klausner and Donnelly 1991, Jacquet et al. 1993, Franco et al. 1995, Pritchard et al. 1995, Nesbakken et al. 1996, Samelis and Metaxopoulos 1999, Fonnesbech Vogel et al. 2001a, Miettinen et al. 2001a, Norton et al. 2001, Suihko et al. 2002). The food contact site most commonly contaminated is processing equipment, but gloves and aprons of personnel, and brine solutions have also been found to harbour *Listeria.* Contaminated processing equipment is numerous and varied, including conveyors, tanks, cooling and freezing machines, and skinning, dicing, slicing, cutting, filling and packaging machines (Klausner and Donnelly 1991, Cotton and White 1992, Jacquet et al. 1993, Eklund et al. 1995, Franco et al. 1995, Pritchard et al. 1995, Salvat et al. 1995, Johansson et al. 1999, Miettinen et al. 1999a, Samelis and Metaxopoulos 1999, Aguado et al. 2001, Dauphin et al. 2001, Fonnesbech Vogel et al. 2001a, Miettinen et al. 2001a, Norton et al. 2001, Chasseignaux et al. 2002, Lundén et al. 2002, Suihko et al. 2002). All of above-mentioned equipment is complex, with narrow openings and hard-to-reach sites, hindering efficient cleaning and disinfection. It is therefore not surprising that *L. monocytogenes* has also been found on cleaned and disinfected surfaces.

Temperature and state of surface have been shown to be associated with *L. monocytogenes* contamination. Low surface temperatures are related to the presence of *L. monocytogenes*, while at relatively high surface temperatures (>10°C) *L. monocytogenes* is often absent (Chasseignaux et al. 2002). Other microflora presumably outcompete and outnumber *L. monocytogenes* at rather high temperatures, whereas at low temperatures growth of psychrotrophic *L. monocytogenes* is favoured. The state of surface is also important, with smooth stainless steel surfaces being more often associated with absence of *L. monocytogenes* than granular, stripped or damaged surfaces (Chasseignaux et al. 2002).

2.3.4 Prevalence of *L. monocytogenes* in foods

Several studies have been conducted to establish the prevalence of *L. monocytogenes* in food products (Tables 3–5). Both non-heat treated and heat treated products have been shown to harbour *Listeria,* and it has been found in numerous products with a variable prevalence. Comparison of results of different surveys is often problematic since food classification, sampling strategies and examination methods differ.

TABLE 3. Prevalence of *Listeria monocytogenes* in dairy products in Europe.

Sample	No. of positive samples/total (%)	Country	Reference
Soft cheese	1/251 (0.4)	UK	MacGowan et al. 1994
	18/60 (30)	France	Jacquet et al. 1993
	10/69 (14)	France	Beckers et al. 1987
	10/90 (11)	Norway	Rørvik and Yndestad 1991
	90/1752 (5)	UK	Greenwood et al. 1991
Blue mould cheese	16/151 (11)	France	Jacquet et al. 1993
Hard cheese	1/66 (2)	UK	Greenwood et al. 1991
Cheese	1/10 (10)	Austria	Rudolf and Scherer 2001
	0/4 (0)	Denmark	Rudolf and Scherer 2001
	0/46 (0)	Denmark	Loncarevic et al. 1995
	14/73 (19)	Denmark	Nørrung et al. 1999
	1/40 (3)	Finland	Keto and Rahkio 1998
	5/150 (3)	France	Rudolf and Scherer 2001
	18/174 (10)	France	Loncarevic et al. 1995
	11/120 (9)	Germany	Rudolf and Scherer 2001
	1/31 (3)	Germany	Loncarevic et al. 1995
	4/23 (17)	Italy	Rudolf and Scherer 2001
	1/36 (3)	Italy	Loncarevic et al. 1995
	0/27 (0)	Sweden	Loncarevic et al. 1995
	0/22 (0)	Switzerland	Rudolf and Scherer 2001
Butter	0/17 (0)	Italy	Pourshaban et al. 2000
Yoghurt	4/180 (2)	UK	Greenwood et al. 1991
Ice cream	0/5 (0)	Finland	Fieandt and Mäkelä 1994
	6/1129 (0.5)	Finland	Miettinen et al. 1999b
	2/150 (2)	UK	Greenwood et al. 1991
NK[a]	166/3541 (5)	France	Goulet et al. 2001

[a]NK, not known

Of dairy products, cheeses are most commonly contaminated with *Listeria*, with prevalence varying from 0% to 30% and the highest prevalence occurring in soft cheeses (Table 3). Prevalence in meat, poultry and ready-to-eat (RTE) products has varied from 0%

to 24% (Table 4). Although fish products have less often been linked to cases of listeriosis than meat or dairy products, they are the most contaminated with prevalences ranging from 0% to 50 % (Table 5). Particularly contaminated are products that are not heat-treated, such as cold-smoked and gravad fish (6–50%), whereas in heat-treated fish products the prevalence is shown to be less than 12%.

TABLE 4. Prevalence of *Listeria monocytogenes* in meat products, poultry products and ready-to-eat (RTE) products in Europe.

Sample	No. of positive samples/total (%)	Country	Reference
Meat products			
Heat-treated	613/6809 (9)	Denmark	Nørrung et al. 1999
	45/772 (5)	Denmark	Nørrung et al. 1999
	1/88 (1)	Finland	Fieandt 1993
	3/60 (5)	Greece	Samelis and Metaxopoulos 1999
	4/35 (11)	Norway	Rørvik and Yndestad 1991
	15/255 (6)	Switzerland	Jemmi et al. 2002
	6/68 (9)	UK	MacGowan et al. 1994
	3/29 (10)	Yugoslavia	Buncic 1991
Pâté	0/31 (0)	UK	MacGowan et al. 1994
	37/3065 (3)	UK	Nichols et al. 1998
Preserved, not heat-treated	57/357 (16)	Denmark	Nørrung et al. 1999
	4/132 (3)	Switzerland	Jemmi et al. 2002
	77/328 (24)	Denmark	Nørrung et al. 1999
Fermented	0/4 (0)	Greece	Samelis and Metaxopoulos 1999
	10/335 (3)	Finland	Pelttari 1990
	0/70 (0)	Norway	Rørvik and Yndestad 1991
	21/142 (15)	Switzerland	Jemmi et al. 2002
	4/21 (19)	Yugoslavia	Buncic 1991
RTE	355/3283 (11)	France	Goulet et al. 2001
Poultry products			
Heat-treated	18/139 (13)	Belgium, France	Uyttendaele et al. 1997
	7/80 (9)	Denmark	Ojeniyi et al. 1996
	4/55 (7)	Denmark	Ojeniyi et al. 2000
	17/528 (3)	UK	Nichols et al. 1998
RTE meals			
	425/3861 (11)	Denmark	Nørrung et al. 1999
	2/315 (0.6)	Finland	Fieandt and Mäkelä 1994
	143/3166 (5)	France	Goulet et al. 2001
	11/151 (7)	Switzerland	Jemmi et al. 2002
	9/72 (13)	Switzerland	Jemmi et al. 2002
	9/75 (12)	UK	MacGowan et al. 1994

In addition to the overall prevalence of *L. monocytogenes* in food products, the level of contamination is important; 100 cfu/g at the time of consumption has been estimated to be of low risk to the consumer (Anonymous 1999b). Large studies, including over 10 000 food samples, conducted in Denmark and France have revealed *L. monocytogenes* levels in various foods (Nørrung et al. 1999, Goulet et al. 2001). In Denmark, Nørrung et al. (1999) found 1.3% of heat-treated meat products and preserved meat and fish products to be contaminated at levels >100 cfu/g in 1994–1995 and in 1997 and 1998, 0.3% and 0.6% of samples, respectively. In a French study, 1.1%, 1.8%, 0.3% and 0.5% of RTE meat products, dairy products, prepared salads and seafood-fish products, respectively, contained >100 cfu/g of *L. monocytogenes* during 1993–1996. In smaller studies, the percentage of samples containing >100 cfu/g has varied from 0% to 1% (Rørvik and Yndestad 1991, Harvey and Gilmour 1993, Wilson 1996, Nichols et al. 1998, Jemmi et al. 2002).

Another interesting aspect of prevalence of *L. monocytogenes* is possible changes in occurrence over time, since due to large outbreaks of listeriosis, several countries have extended *L. monocytogenes* control measures. In France, prevention measures were progressively introduced by the food industry, and control measures were expanded from dairy products to all food products in 1993. Food monitoring data showed success of the measures, as the percentage of products containing >100 cfu/g decreased from 1.3% in 1993–1994 to 0.8% in 1995–1996. The percentage of samples with <100 cfu/g also decreased but to a lesser extent (Goulet et al. 2001). In Finland, the annual food control plan in 2000 focused on the fish processing industry, and recommendation for *Listeria* control at meat and fish plants was implemented (Anonymous 2000a). The contamination of fish products was reduced from 15–50% in 1996–1998 to 5% in 2000, and similarly, the proportion of samples containing >100 cfu/g decreased from 5.8% to 0% (Keto and Rahkio 1998, Lyhs et al. 1998, Johansson et al. 1999, Hatakka et al. 2001). However, in 2001, the contamination rate of fish products was again rather high (14%), and 0.6% of samples contained levels >100 cfu/g, emphasizing the need for constant attention to *Listeria* control (Hatakka et al. 2002).

TABLE 5. Prevalence of *Listeria monocytogenes* in fish products in Europe.

Sample	No. of positive samples/total (%)	Country	Reference
Not heat-treated			
Cold-smoked fish	59/1000 (6)	Denmark	Fonnesbech Vogel et al. 2001a
	153/420 (36)	Denmark	Jørgesen and Huss 1998
	3/82 (4)	Finland	Pelttari 1990
	9/62 (14)	Finland	Lyhs et al. 1998
	5/30 (17)	Finland	Johansson et al. 1999
	7/50 (14)	Finland	Keto and Rahkio 1998
	10/232 (4)	Finland	Hatakka et al. 2001
	46/356 (13)	Finland	Hatakka et al. 2002
	13/32 (41)	France	Dauphin et al. 2001
	7/65 (11)	Norway	Rørvik et al. 1995
	3/26 (12)	Sweden	Loncarevic et al. 1996
	1/16 (6)	Switzerland	Jemmi and Keusch 1994
	114/814 (14)	Switzerland	Jemmi et al. 2002
Gravad fish	51/176 (29)	Denmark	Jørgesen and Huss 1998
	2/30 (7)	Finland	Pelttari 1990
	16/32 (50)	Finland	Johansson et al. 1999
	14/43 (32)	Finland	Lyhs et al. 1998
	9/29 (31)	Finland	Keto and Rahkio 1998
	5/82 (6)	Finland	Hatakka et al. 2001
	12/58 (21)	Sweden	Loncarevic et al. 1996
Preserved, not heat-treated	62/282 (22)	Denmark	Nørrung et al. 1999
	35/335 (11)	Denmark	Nørrung et al. 1999
Marinated fish	48/125 (38)	Switzerland	Jemmi et al. 2002
Heat-treated			
Hot-smoked fish	1/14 (7)	Finland	Pelttari 1990
	1/95 (1)	Finland	Lyhs et al. 1998
	0/12 (0)	Finland	Keto and Rahkio 1998
	1/48 (2)	Finland	Johansson et al. 1999
	1/66 (2)	Sweden	Loncarevic et al. 1996
	0/33 (0)	Switzerland	Jemmi and Keusch 1994
	57/471 (12)	Switzerland	Jemmi et al. 2002
Other	11/124 (8)	Denmark	Jørgesen and Huss 1998
	9/122 (7)	UK	Nichols et al. 1998
NK			
	204/1966 (10)	France	Goulet et al. 2001
	4/38 (11)	Iceland	Heinitz and Johnson 1998
	4/18 (22)	Ireland	Heinitz and Johnson 1998
	31/131 (24)	Norway	Heinitz and Johnson 1998
	20/124 (16)	UK	Heinitz and Johnson 1998

NK, not known

2.4 Tracing bacterial contamination in the food processing industry

The understanding of bacterial contamination routes in the food processing industry is crucial for preventing unwanted bacteria in products. Detailed contamination analyses enable tracing the sources and routes of bacteria. Contamination analyses involve intensive sampling of various sites, including raw material, products, processing equipment and environment, food handlers and air, among others. Product sampling at different stages of processing with concurrent environmental sampling has been found to be an effective approach for tracing bacterial contamination in the food processing industry (Mäkelä and Korkeala 1987, Björkroth and Korkeala 1997). In sample examination, conventional methods, such as determination of total bacterial counts, genus and species identification and phenotypic tests, have been commonly used to evaluate bacterial contamination (Rahkio et al. 1992, Mäkelä et al. 1992, Nerbrink and Borch 1993, Rahkio and Korkeala 1997, Shumaker and Feirtag 1997). However, identification of the same genus and species in different samples, e.g. environmental and final product samples, does not necessarily establish a causal relationship. In addition, quantitative methods have been demonstrated to be of limited use in, for instance, lactic acid bacterial contamination analyses (Nerbrink and Borch 1993, Björkroth and Korkeala 1996).

Contamination analysis combined with characterization of isolates of interest by molecular subtyping techniques has proven to be an effective approach to trace both pathogen and spoilage bacterial contamination (Destro et al. 1996, Nesbakken et al. 1996, Björkroth and Korkeala 1997). By comparison of isolates recovered at different stages of the process, in processing environment and on equipment, in air and in the final product, it is possible to define the specific sources and sites of product contamination. The molecular approach has been used in contamination studies of spoilage organisms, such as lactic acid bacteria and *Pseudomonas fluorescens*, and various pathogens, including *Bacillus cereus, Escherichia coli, L. monocytogenes* and *Yersinia enterocolitica;* and a number of methods, e.g. multilocus enzyme electrophoresis, plasmid profiling, analysis of fatty acid profiles, ribotyping, RAPD, AFLP and PFGE typing, have been successfully applied (Dykes et al. 1993, Destro et al. 1996, Nesbakken et al. 1996, Björkroth and Korkeala 1997, Lin et al. 1998, Raleya et al. 1998, Fredriksson-Ahomaa et al. 2000, Geornaras et al. 2001).

Sources and routes of bacterial contamination are numerous. Animal raw material has proved to be a contamination source of several spoilage organisms and pathogens. For

example, *Y. enterocolitica* contamination of pig carcasses originates from tonsils, is spread to carcasses and subsequently to meat and has been shown to be associated with slaughtering technique (Fredriksson-Ahomaa et al. 2001). Similarly, raw material was identified as a source of major spoilage strains in vacuum-packaged sliced cooked meat products, and the contamination of the product surfaces after cooking was confirmed to be airborne (Björkroth and Korkeala 1997). However, the sources of contamination may be various as shown that *E. coli* in poultry carcasses may originate from farm and processing environments as well as from processing equipment (Geornaras et al. 2001).

Unlike many other organisms, *L. monocytogenes* contamination is associated with strains existing in processing facilities, and it has been suggested that animal strains do not easily contaminate meat and are replaced by other better adapted strains (Boerlin and Piffaretti 1991, Nesbakken et al. 1996). A specific feature of *Listeria* contamination is prolonged processing plant contamination since some *L. monocytogenes* strains have been established to cause persistent plant contamination over several months or years (Unnerstad et al. 1996, Miettinen 1999a, Lundén et al. 2002). While sites of contamination are numerous, processing equipment is a particularly important source, as the contamination often occurs after heat treatment (Rørvik et al. 1995, Ojeniyi et al. 1996, Miettinen 1999a, Rørvik 2000, Aguado et al. 2001, Norton et al. 2001, Lundén et al. 2003a, 2003b).

3 AIMS OF THE STUDY

L. monocytogenes contamination routes and sources of listeriosis were investigated using molecular typing techniques. Specific aims of this thesis were as follows:

1. to determine the prevalence of *L. monocytogenes* in pig tonsils, on carcasses and offals and in the slaughterhouse environment and the sources of carcass *L. monocytogenes* contamination in pig slaughterhouses (I).

2. to determine *L. monocytogenes* contamination routes in the food processing industry (I, II).

3. to study similarity of persistent and sporadic *L. monocytogenes* using PFGE and AFLP typing (III).

4. to use continuous typing of human strains in epidemiological investigation and to trace the source of listeriosis (IV).

5. to study the distribution of different pulsotypes of *L. monocytogenes* isolated in foods of various origins to evaluate interpretation of results of *L. monocytogenes* contamination analysis and to evaluate the usefulness of *L. monocytogenes* typing data obtained from food products in tracing vehicles of infections (V).

4 MATERIALS AND METHODS

4.1 Sampling in contamination analyses (I, II)

4.1.1 Pig slaughterhouses

A total of 373 samples were collected in ten low-capacity slaughterhouses in study I. The samples were taken from carcasses, pluck sets and the slaughterhouse environment. The carcasses and pluck sets were sampled by swabbing with 7.5 cm × 7.5 cm sterile gauze-pads premoistened in sterile 0.1% peptone water. Sponge sampling technique (Technical Service Consultants Ltd, Heywood, Lancashire, UK) was used in sampling the environment, equipment and aprons of employees, and drains were examined by collecting 25 ml of drain water. Environmental samples were taken in slaughterhouses 2, 4, 5, 7 and 8 prior to slaughtering, and in slaughterhouses 1, 3, 6, 9 and 10 after slaughtering.

4.1.2 Cold-smoked fish processing plant

In study II, a total of 587 samples were collected, of which 493 were for contamination analyses and 94 for evaluating the success of the eradication program. Contamination analyses consisted of monitoring two production lots by sampling both fish and the processing environment. Raw fish in a rainbow trout slaughterhouse and the fish during processing were sampled after each processing stage. Samples from the processing environment (surfaces, drains), machines and employees (aprons, gloves) were taken both before starting daily production and during production, when the lots were handled in the area. The processing environment, machines and equipment were examined by swabbing with 7.5 cm × 7.5 cm sterile gauze-pads or with sterile swabs premoistened in sterile 0.1% peptone water. Floor drains were examined by taking 25 ml of water from drains. Samples from the gloves of the fish handlers were taken by washing the gloves with 100 ml of 0.1% peptone water. Liquid samples (minimum 500 ml) were taken from the brine solution, rinsing water and tap water. Air samples were taken during processing and during the washing of the plant after production by four sampling methods: sedimentation, liquid impingement (AGI-30, Ace Glass Inc, Vineland, NJ, USA), a portable high-volume Surface Air System impactor sampler (Air Sampler SAS-SUPER90, International, Milano, Italy) and a Reuter centrifugal air sampler (RCS) (Biotest AG, Dreieich, Germany).

4.2 *L. monocytogenes* strains and samples in a listeriosis outbreak investigation (IV)

L. monocytogenes strains isolated from human cases are sent from microbiological laboratories to the Laboratory of Enteric Pathogens at the National Public Health Institute and belong to the culture collection of the Laboratory of Enteric Pathogens (IV, Fig. 1). The strains were continuously serotyped for O antigens at the Laboratory of Enteric Pathogens, and complete serotyping and PFGE typing was performed at the Department of Food and Environmental Hygiene. National and local food authorities inspected the dairy, and the production line, dairy environment and butter were sampled. From the hospital kitchen, 7 g butter packages and other foods served without further heating in the units in which the majority of patients were treated were collected.

4.3 *L. monocytogenes* strains (III, V)

Strains used in studies III and V belong to the culture collection of the Department of Food and Environmental Hygiene and originate from various food processing facilities and processed foods. In study III, 17 strains representing persistent strains and 38 sporadic strains originating from 11 food processing plants were examined (III, Table 1). In each plant the selection of strains was based on previous PFGE typing results and occurrence of pulsotypes in a plant. Persistent strains, which were recurrently recovered in the processing plant over a minimum of one year, and isolated in both the processing equipment and the final product, were included. Processing equipment associated with persistent strains were conveyors, freezers, and slicing, brining, dicing and packaging machines. Sporadic strains were pulsotypes sporadically recovered in the raw material, in the processing environment or on processing equipment but not found in the final product.

Strains used in study V were isolated from the products (meat, poultry, dairy, fish, vegetable and other food products) of 41 producers during 1988−1999. The strains were recovered from products of Belgium, Denmark, Estonia, Finland, France, Hungary, Norway, Poland and Spain (V, Table 1).

4.4 Isolation of *L. monocytogenes* (I, II, IV)

One-step enrichment in EB (*Listeria* Enrichment broth, Oxoid, Basingstoke, UK) according to NCFA (Anonymous 1990) was used in study I. *L. monocytogenes* isolation by two-step enrichment methods was performed in studies II and V. In study II, enrichment broths

LEB1 (primary *Listeria* Enrichment Broth, Oxoid) and LEB2 (secondary *Listeria* Enrichment Broth, Oxoid) were used according to the NCFA method (Anonymous 1990), and in study IV, isolation was performed according to the ISO method (Anonymous 1996) using half-Fraser (Half Fraser broth, Oxoid) and Fraser (Fraser broth, Oxoid) enrichment broths. In study II, sedimentation and impingement air samples were preincubated at 30°C for 6–8 h before primary enrichment.

Direct plating of final product samples in study II was performed on Oxford plates as described by Loncarevic et al. (1996). In study IV, enumeration of *L. monocytogenes* was done according to the ISO method (Anonymous 1998a), with modifications as described by Johansson (1998).

Selective plates used were Oxford (Oxoid) (I and II) and LMBA (I and IV) [trypticase soy agar base (Difco, Detroit, MI, USA), 10 g/l lithium chloride, 10 mg/l polymyxine B sulphate (Sigma Chemicals, St. Louis, MO, USA), 20 mg/l ceftazidime (Abtek Biologicals Ltd, Liverpool, England), 5% sterile defibrinated sheep blood] (I), and PALCAM (Oxoid) (IV).

For identification, ten suspected colonies (in study I, five from both selective agar plates) from selective agar plates were streaked on blood agar, and β-haemolytic colonies were further identified by Gram staining, catalase reaction, motility at 25°C and using API Listeria (BioMériux SA, Marcy l'Etoile, France). In study I, when no ß-haemolytic colonies were observed, non-haemolytic colonies were further identified as described above.

4.5 Serotyping of *L. monocytogenes* (I, II, IV, V)

Serotyping of *L. monocytogenes* strains was performed with commercial antisera by agglutination methods according to manufacturers' instructions (I, II, IV, V: Denka Seiken, Tokio, Japan; IV: Bacto Listeria O antisera, Difco).

4.6 Genotyping of *L. monocytogenes* (I, II, III, IV, V)

4.6.1 DNA isolation

In situ DNA isolation was performed as described by Björkroth et al. (1996), with the following modifications. The plugs were lysed for only 3 h. A single 2–h (I, II) or 1–h (III, IV, V) wash with ESP at 50°C was used. Proteinase K was inactivated by Pefabloc SC or PMSF (Roche Diagnostics Gmbh, Mannheim, Germany) at 37°C over night. In study III,

DNA used for AFLP analysis was isolated according to method of the Pitcher et al. (1989), with the modifications described by Keto-Timonen et al. (2003).

4.6.2 PFGE typing (I, II, III, IV, V)

Restriction enzymes *Asc*I (I, II, III, IV, V), *Apa*I (I, III, IV, V) and *Sma*I (II, III) were used for cleavage of *L. monocytogenes* DNA. The samples were electrophoresed through 1.0% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts, Rockland, ME, USA) in 0.5 × TBE (45 mM Tris, 4.5 mM boric acid, pH 8.3, 1 mM sodium EDTA) at 200 V at 14°C in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden). The pulse times ramped from 1 s to 15 s for 18 h for *Sma*I and from 1 s to 35 s for 18 h for *Asc*I and *Apa*I. The gels were stained with ethidium bromide and photographed under UV translumination (II) or digitally photographed with an Alpha Imager 2000 documentation system (I, III, IV, V) (Alpha Innotech, San Leandro, CA, USA). Lambda Ladder PFG marker (II) or Low Range PFG marker (New England Biolabs, Beverly, MA, USA) (I, III, IV, V) were used for fragment size determination.

4.6.3 AFLP analysis (III)

AFLP analysis was performed as previously described by Keto-Timonen et al. (2003). Briefly, genomic DNA (400 ng) was digested with restriction enzymes *Hin*dIII and *Hpy*CH4IV (New England Biolabs), followed by ligation of adapters. The digested and ligated DNA was amplified by PCR with *Hin*dIII and *Hpy*CH4IV +0/+0 primers (MWG-Biotech AG, Edersberg, Germany), followed by selective PCR amplification with +A/+A primer combination. AFLP fragments were separated in 7% polyacrylamide gel on an LI-COR automated DNA sequencer (LI-COR Global IR2 4200LI-1 Sequencing System, LI-COR, Lincoln, NE, USA). IRDye800 50–700 bp sizing standard (LI-COR) was used for fragment size determination, and a DNA control sample was included in each AFLP analysis set and loaded onto every AFLP gel.

4.6.4 Numerical analysis of patterns (II, III, V)

Numerical analysis of MRP was performed using computer software (GelCompar, GelComparII or BioNumerics2.5; Applied Maths, Sint-Martens-Platen, Belgium). The similarity between complete MRP patterns was expressed as a Dice coefficient (position tolerance 1.0% and 1.2% for *Asc*I and *Apa*I, respectively). The clustering and construction of dendrograms were performed by the unweighted pair group method using arithmetic

averages (UPGMA). The similarity between AFLP patterns was calculated with the Pearson coefficient correlation using optimization value 1.0%, and the clustering and dendrogram construction was performed by UPGMA.

4.6.5 Discrimination index (III)

The discriminatory power of PFGE and AFLP was compared with Simpson's index of diversity, as presented by Hunter and Gaston (1988). The index estimates the probability of two strains from a test population being placed into different typing groups. The discrimination index (DI) is given by the following equation:

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

where N is the total number of strains, s is the total number of types described and n_j is the number of strains belonging to jth type.

5 RESULTS

5.1 Prevalence and characterization of *L. monocytogenes* in pig slaughterhouses (I)

L. monocytogenes was detected in six slaughterhouses, with a prevalence varying from 0% to 40% (Table 6). Nine percent of pluck set samples were positive for *L. monocytogenes,* the highest prevalence being in tongue and tonsil samples, 14% and 12%, respectively. Environmental samples positive for *L. monocytogenes* originated from splitting saws, door, table and drain. Characterization of 58 *L. monocytogenes* isolates by PFGE typing with restriction enzymes *Asc*I and *Apa*I yielded 18 pulsotypes (Table 6).

TABLE 6. Prevalence of *Listeria monocytogenes* and distribution of *L. monocytogenes* pulsotypes in ten low-capacity slaughterhouses.

Slaughter-house	No. of samples positive for *L. monocytogenes*/no. of samples (pulsotypes)			
	Carcasses	Pluck sets	Environment	Total [%]
1	2/5 (I)	5/25 (I, II, IIIa)	2/6 [a] (I)	9/36 [25]
2	0/5	0/25	0/6 [b]	0/36 [0]
3	0/5	0/25	1/7 [a] (IIIb, IVa)	1/37 [3]
4	0/5	1/25 (IX)	0/8 [b]	1/38 [3]
5	0/5	0/25	0/8 [b]	0/38 [0]
6	4/5 (IIIc)	1/25 (IIIc)	1/9 [a] (IIIc)	6/39 [15]
7	0/5	15/25 (IVb, IVc, VI, VII, VIII, X, XI, XII, XIIIa, XIIIb)	0/7 [b]	15/37 [40]
8	0/5	0/25	1/8 [b] (V)	1/38 [3]
9	0/5	0/25	0/8 [a]	0/38 [0]
10	0/5	0/25	0/6 [a]	0/36 [0]
Total [%]	6/50 [12]	22/250 [9]	5/73 [7]	33/373 [9]

[a]Samples taken after slaughtering.
[b]Samples taken prior slaughtering.

5.2 Prevalence and characterization of *L. monocytogenes* in a cold-smoked fish processing plant and evaluation of an eradication program (II)

Only one sample (2%) from a total of 60 fish sampled from the fish slaughterhouse contained *L. monocytogenes,* and none of the 49 samples of filleted fish, skinned fish and pooled skin samples were positive for *L. monocytogenes.* The number of positive *L. monocytogenes*

fish samples clearly increased after the brining stage. Thirteen percent (16/122) of the environmental samples taken before production and 30% (19/65) of samples taken during production were positive for *L. monocytogenes.* The most contaminated sites were the areas of brining, slicing and packaging (Table 7). Fresh brine solution and its ingredients were not contaminated with *L. monocytogenes*, but recirculated brine solution was found to contain *L. monocytogenes* both during and at the end of both production lots. None of the glove samples from workers in the pre-brining area contained *L. monocytogenes*, but two of five gloves from workers in post-brining area were contaminated. Characterization with *Sma*I and *Asc*I resulted in the isolates being divided into nine different pulsotypes. Pulsotype I, the dominating pulsotype for the whole plant, was associated with brining, pulsotype II was also associated with brining and pulsotype III was associated with slicing and packaging area. The pulsotype recovered in raw fish was not detected in the processing plant. At most, four pulsotypes were detected in a single sample, and twenty-three samples were contaminated with two or more pulsotypes. In the numerical analysis of *Asc*I MRPs, isolates formed four clusters at a similarity level of 75% (II, Fig. 1). The isolates from final products belonged to clusters 1, 2 and 3. Non-product isolates in cluster 1 were mostly associated with brining, and in cluster 2 with slicing. The isolates from raw fish and those of possible raw fish origin were in cluster 4. All strains belonged to serogroup 1/2.

Based on the results of contamination analyses, an eradication program was planned to decrease the level of *L. monocytogenes* contamination in the cold-smoked fish processing plant. Skinning, slicing and brining machines were disassembled and thoroughly cleaned and disinfected, and the components were placed in hot water (80°C), heated in an oven (80°C) or treated with gas flame. The production line, the floors and the walls were treated thoroughly with hot steam. A total of 94 samples were taken in the five months after eradication from critical contamination points determined during sampling for contamination analysis. Processing equipment, recirculated brine solution and final products on the sell-by date were sampled, and none was positive for *L. monocytogenes.*

TABLE 7. Prevalence of *Listeria monocytogenes* and distribution of *L. monocytogenes* pulsotypes in a cold-smoked rainbow trout processing plant.

Sampling site	No. of samples positive /total	Pulsotypes (no. of isolates)
Slaughterhouse		
Raw fish	1/60	VII (4)
Processing plant		
Arrival area		
Ice-packs	1/7	III (2)
Fish	0/35	
Environment	0/6	
Air	0/9	
Skinning, brining and smoking areas		
Environment	4/48	I (4), II (2), IX (2)
Machines	15/62	I (16), II (10), III (4)
Brine solution	4/6	I (8)
Employees[a]	4/9	I (4), III (2), VII (2)
Fish after skinning	0/10	
Skins	0/4	
Fish after brining	7/10	I (15), II (13)
Fish after smoking	8/10	I (19), II (3), IV (6), V (1)
Air	0/66	
Slicing and packaging area		
Environment	5/19	I (6), III (4)
Machines	5/22	I (4), III (6)
Employees	2/10	I (3), III (1)
Fish after slicing	7/10	I (17), III (7), VI (1)
Air	0/42	
Cleaning area[b]	2/12	II (2), III (2)
Departure area		
Environment	0/6	
Air	0/8	
Final product		
Enrichment	22/22	I (64), II (4), III (2), IV (3), V (1)
Direct plating	9/22	I (41), II (3), III (1), IV (10), VI (4)

[a]Samples were taken from aprons and gloves of employees.
[b]The cleaning area was situated between the brining and slicing areas and was used for cleaning smoking tracks.

5.3 Characterization of persistent and sporadic *L. monocytogenes* strains (III)

The 17 persistent and 38 sporadic strains showed 14 and 35 pulsotypes, respectively. AFLP analysis revealed 36 AFLP types, of which 14 and 28 were found among persistent and sporadic strains, respectively. The combination of PFGE and AFLP typing results yielded a

total of 48 genotypes. Thirteen of the 15 genotypes presented by persistent strains were specific to persistent strains, and similarly 94% (33/35) of genotypes of sporadic strains were recovered only among sporadic strains. AFLP further separated two pulsotypes (8 and 12), whereas PFGE distinguished strains of nine AFLP types. The discriminatory powers of AFLP, PFGE and their combination determined using Simpson's index of diversity (DI) were 0.982, 0.993, and 0.994, respectively. Numerical analysis of PFGE and AFLP patterns yielded two distinct clusters, both consisting of persistent and sporadic strains. Neither clusters nor fragments specific to persistent or sporadic strains were found in PFGE or AFLP patterns.

5.4 Use of PFGE typing in a listeriosis outbreak investigation (IV)

In early 1999, strains indistinguishable by PFGE typing and belonging to rare serotype 3a were repeatedly isolated in human cases. A total of 25 outbreak-associated patients were identified between 1 June 1998 and 30 April 1999. Most of the patients were treated at the same tertiary care hospital.

A *L. monocytogenes* strain of the same serotype and sharing an identical macrorestriction pattern had been isolated from an in-house control sample of a Finnish dairy plant producing butter in 1997. Delivery of butter from this dairy to the hospital had begun on 1 June 1998. The outbreak strain was also detected in butter samples from the hospital kitchen and several lots of 7, 10 and 500 g butter packages from the dairy and wholesale store. The levels of *L. monocytogenes* in butter were 5–60 cfu/g and one sample had 1.1×10^4 cfu/g. In the dairy environmental samples, the outbreak associated *L. monocytogenes* strain was recovered from packaging machines, a screw conveyor on the butter wagon and two drains.

Outbreak control measures included stopping of butter production and a recall of products. The production facility was thoroughly cleaned and disinfected. The success of *L. monocytogenes* elimination was evaluated by control samples from the process line, the environment and several test butter batches, and no *L. monocytogenes* was detected.

5.5 Distribution of different pulsotypes of *L. monocytogenes* isolated in foods of various origins (V)

Characterization of 295 strains isolated in various food products with restriction enzymes *Asc*I and *Apa*I resulted in 66 pulsotypes. The strains isolated in meat (n=100), poultry (n=40), dairy (n=14), fish (n=76), vegetables (n=39) and other products (n=26) were divided

into 19, 10, 6, 20, 15 and 8 different pulsotypes, respectively. In all, 39% (116/295) of strains possessed pulsotypes (17, 24, 27, 28, 31, 43, 51, 58, 61 and 63) common to two or more product types. The number of different pulsotypes detected in products of a single producer varied from one to eight. In several cases, the same pulsotypes were detected in the products of a single producer over many years. However, 17 pulsotypes were detected in foods of more than one producer with no apparent association with each other. These pulsotypes were possessed by 132 out of 295 (45%) strains. Seven of these pulsotypes were recovered in products of several producers as well as in different product types and were presented by 29% (85/295) of strains. Strains possessing identical restriction patterns were recovered from foods of different countries and also over different years.

Six different serotypes (1/2a, 1/2b, 1/2c, 3a, 4b and 4c) were detected when at least one strain representing each pulsotype was serotyped. Some serotype 1/2a and 3a strains had an identical, either *Asc*I or *Apa*I restriction pattern, as had some serotype 4b and 4c strains. Numerical analysis of macrorestriction patterns yielded two clusters (I and II) by both enzymes and their combination (V, Figs 1–3). Serotypes 1/2b, 4b and 4c formed cluster I, and serotypes 1/2a, 1/2c and 3a cluster II.

6 DISCUSSION

6.1 Contamination routes in food processing (I, II, III, IV, V)

6.1.1 Contamination routes in pig slaughterhouses (I)

Eight out of ten low-capacity slaughterhouses were contaminated with *Listeria.* Similarly, Gill and Jones (1995), Sammarco et al. (1997), Saide-Albornoz et al. (1995) and Korsak et al. (1998) have described a variable occurrence of *Listeria* among high-capacity slaughterhouses.

A high prevalence of *L. monocytogenes* was found in tonsils (12%).The contamination rate of tonsils in other studies has varied from 7.1% to 45% (Buncic 1991, Kanuganti et al. 2002). The reason for the high rate in tongues (14%) may be the slaughtering process, where the tonsils are removed together with pluck set. The contamination may also spread from the tonsils when they hang together on a hook (Fredriksson-Ahomaa et al. 2001).

Characterization of strains yielded 18 pulsotypes, 15 of which were recovered from pluck sets, suggesting that with animals a wide range of *L. monocytogenes* strains enter the plant. In two slaughterhouses, carcasses were contaminated with *L. monocytogenes.* In these slaughterhouses, the saws used for splitting the carcasses were also contaminated with the pathogen. Moreover, the isolates from the saws and carcasses shared identical restriction patterns in PFGE typing, suggesting saws as a possible contamination site of carcasses in these slaughterhouses. These results concur with those of Nesbakken et al. (1994) and Giovannacci et al. (1999), who have proposed slaughterhouse equipment and the environment as possible sources of carcass contamination.

6.1.2 Contamination routes in a cold-smoked fish processing plant (II)

Only 2% of raw fish contained *L. monocytogenes*, but the frequency of fish containing *L. monocytogenes* clearly rose after brining. Moreover, the most contaminated sites of the processing plant were the brining and post-brining areas, and during production the brine solution became contaminated with *L. monocytogenes* originating from brining machine. In addition, the gloves of employees working in the production line after brining were positive for *L. monocytogenes*, whereas no *L. monocytogenes* was detected on the gloves of employees working pre-brining. These findings indicate that contamination of fish occurred during processing and that brining is the critical point of *L. monocytogenes* contamination.

In PFGE typing, nine pulsotypes were recovered, some of which were dominating in the plant, whereas other pulsotypes were sporadically recovered. The contaminant of raw fish was not detected in the processing plant or in the final products. Pulsotype I, the major contaminant of the processing plant, was associated with brining, being the only pulsotype detected in the recirculated brine solution. Pulsotype II was also associated with the brining stage; it was found in the injection brining machine, in fish during processing and in the final product. Moreover, fish after brining contained only pulsotypes I and II.

Characterization of isolates revealed slicing as another important contamination site of the product. In the slicing and packaging area strains of pulsotypes I and III were recovered and both pulsotypes were found on the slicing machine. The fish, which contained pulsotype I, had probably contaminated the slicing machine with this pulsotype, and thereafter the machine continued to spread the pulsotype. Pulsotype III, also found on the slicing machine, was present in fish only after the slicing stages. These findings indicate that the slicing machine had been a specific site of pulsotype III contamination.

Rørvik et al. (1995) have previously showed that contamination of smoked fish primarily occurs during processing, but no specific contamination sites were established. To the author's knowledge, this is the first time that specific sites of cold-smoked fish contamination have been identified. Other researchers have pointed out also the importance of injection brining and slicing in contamination of cold-smoked fish (Johansson et al. 1999, Dauphin et al. 2001, Fonnesbech Vogel et al. 2001a).

The role of raw fish in contamination of fish products has been examined in many studies. Eklund et al. (1995) suggested that raw fish is the primary contamination source; however lacking molecular typing, these conclusions were based on the incidence of *L. monocytogenes* in raw salmon. Other researchers using molecular typing techniques have suggested, similarly to our study, that raw fish is not the essential source of final product contamination (Rørvik et al. 1995, Dauphin et al. 2001, Fonnesbech Vogel et al. 2001a, 2001b, Norton et al. 2001).

6.1.3 Common features of *L. monocytogenes* contamination (I, II, III, IV, V)

In a cold-smoked fish processing plant (II), three *L. monocytogenes* strains were found to dominate, and in study III, one to three persistent strains were recovered from different processing plants. Some pulsotypes were recurrently recovered from the same product of the same producer in the biodiversity study (V), suggesting long-term occurrence of these strains

in the processing plant. Moreover, the strain associated with listeriosis outbreak was shown to have colonized the dairy processing plant (IV). This existence of dominating strains seems to be a feature typical of *L. monocytogenes* contamination in various types of food processing plants (Jacquet et al. 1993, Nesbakken et al. 1996, Ojeniyi et al. 1996, Unnerstad et al. 1996, Giovannacci et al. 1999, Miettinen et al. 1999a, Pourshaban et al. 1999, Ojeniyi et al. 2000, Pourshaban et al. 2000, Senczek et al. 2000, Fonnesbech Vogel et al. 2001a, 2001b, Harvey and Gilmour 2001, Lundén et al. 2002, Suihko et al. 2002), and it has been shown that strains may persist in processing facilities for years (Unnerstad et al. 1996, Miettinen et al. 1999a).

Contamination of products mostly occurs during processing (Fig. 1). Strains causing contamination are often recovered from processing equipment such as mechanical saws, brining, slicing, dicing, freezing, and packaging machines and conveyors (I, II, III). These machines are therefore considered essential niches of the bacteria and important sites of product contamination (Rørvik et al. 1995, Ojeniyi et al. 1996, Miettinen 1999a, Rørvik 2000, Aguado et al. 2001, Norton et al. 2001, Lundén et al. 2003a, 2003b). Transfer of a persistent strain from one plant to another has also been demonstrated to be associated with contaminated processing machine (Lundén et al. 2002). The aforementioned equipment is very complex, often possessing narrow openings and dead areas, and its routine dismantling for efficient cleaning and disinfection procedures is cumbersome.

The role of employees in the spreading of *L. monocytogenes* contamination is unclear. In study II, the gloves of fish handlers were found to be contaminated, but in that case the gloves most likely became contaminated when the workers handled the fish, rather than the gloves themselves being the source of contamination. However, contaminated gloves may subsequently spread *L. monocytogenes* to other sites. Destro et al. (1996) reported similar findings in a shrimp processing plant and suggested that food handlers did not appear to play a major role in the dissemination of *L. monocytogenes* throughout the plant. However, rotation of assigned duties between departments has been found to be the strongest expressed risk factor for *L. monocytogenes* contamination of smoked salmon (Rørvik et al. 1997).

While airborne contamination has been associated with bacterial contamination (Björkroth and Korkeala 1997, Rahkio and Korkeala 1997), in *L. monocytogenes* contamination, air-mediated contamination may not be of major importance. In intensive air sampling of a fish processing plant, none of the air samples was positive for *L. monocytogenes*. Similarly other researchers have not found any *Listeria* in air samples in food processing plants (Jacquet et al.

1993, Salvat et al. 1995). Nevertheless, Spurlock and Zottola (1991) experimentally showed that high populations of *L. monocytogenes* survive in aerosol suspensions for hours, thus practices encouraging extensive aerosol formation should be avoided in a food processing environment.

Even though product contamination is associated with processing, the initial origin of processing plant contamination remains obscure. *L. monocytogenes* is known to be present in raw materials both of animal and plant origin and in nature (Seeliger 1961, Fenlon et al. 1996, Fenlon 1999), and thus, may be introduced into the processing environment by several ways such as via incoming raw materials, other materials, incoming traffic and people (Fig. 1). While the diversity of strains found in raw material is wide – for example in the study of pig slaughterhouses 15 different *L. monocytogenes* pulsotypes were recovered from tonsils of pigs (I) – all incoming strains do not survive and cause persistent contamination of the processing plant (II, III). There are probably several reasons for this. Bacteria in food processing facilities are exposed to variety of energy starvation and environmental stress; thus the ability of strains to tolerate stress may impact their survival in processing environment. It is possible that *L. monocytogenes* strains, which are better than other able to adapt to the ambient environmental conditions are selected and will survive and subsequently may cause persistent contamination in food processing facilities (Norwood and Gilmour 1999, Lundén et al. 2000, Harvey and Gilmour 2001, Lundén et al. 2003a). Eradication of *Listeria* contamination has proven difficult and presumes specific measures such as structural changes, dismantling of machines and targeted cleaning procedures including use of hot steam, hot air and hot water (II, IV). Hygienic aspects should therefore be emphasized in design of processing plants, processing lines and particularly processing equipment to prevent persistent contamination of the machines and in turn the spread of *Listeria* to products. In addition to specific niches, the lay-out and maintenance of facilities as well as working and cleaning routines may affect the persistence of contamination. Well-maintained facilities have been demonstrated to have a preventive effect on *Listeria* contamination in processing plants (Rørvik et al. 1997).
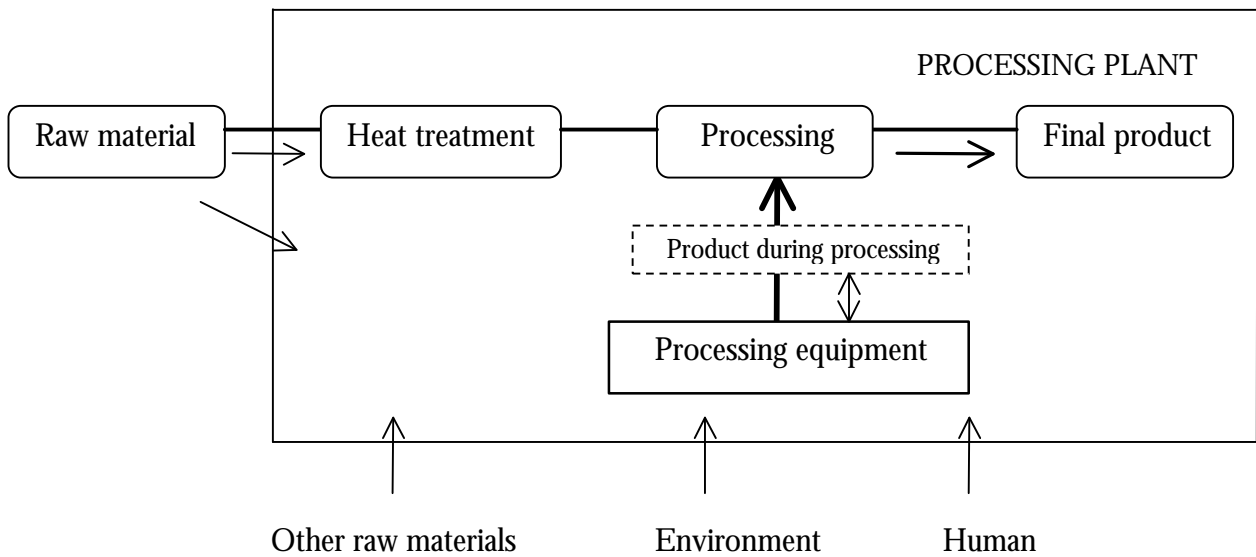
FIGURE 1. *Listeria monocytogenes* contamination routes in food processing plants. The arrows show the direction and significance of *L. monocytogenes* contamination.

6.1.4 Characterization of persistent and sporadic *L. monocytogenes* strains (III)

In study III, the genetic dissimilarity between strains causing persistent plant contamination and sporadic strains was established by PFGE typing and AFLP. Both persistent and sporadic strains showed wide diversity, by means of the number of different patterns obtained by PFGE and AFLP. However, 13 out of 15 genotypes presented by persistent strains were specific to persistent strains, and 94% (33/35) of the genotypes of sporadic strains were recovered only among sporadic strains. The results indicate that *L. monocytogenes* strains causing persistent contamination differ from sporadic ones. However, in the numerical analysis of AFLP patterns, neither persistent nor sporadic strains formed any specific clusters. Moreover, no difference was observed in the frequencies of sporadic or persistent strains between the two known genomic groups of *L. monocytogenes,* indicating that although persistent strains differ from sporadic strains they are not descended from a common ancestor and no specific evolutionary lineage exists for persistent strains.

6.1.5 Molecular tools in contamination tracing (I, II, III, V)

Several molecular typing techniques have been used for tracing *L. monocytogenes* contamination in the food processing industry. Methods most applied are ribotyping, RAPD and PFGE

typing (Destro et al. 1996, Miettinen et al. 1999a, Lundén et al. 2002, Fonnesbech Vogel 2001a, 2001b, Harvey and Gilmour 2001, Suihko et al. 2002, Aarnisalo et al. 2003). All these methods have excellent typeability, but they differ in discriminatory ability and reproducibility. The advantage of ribotyping is availability of a fully automated system and database composition, however, its discriminatory ability is lower than that of PFGE typing and RAPD. RAPD is a rapid, simple and discriminative method, but low reproducibility, and thus, difficulties in fingerprint database construction has limited its use. In our studies, PFGE typing and AFLP were used.

PFGE typing showed excellent typeability, reproducibility and discriminatory power (DI 0.993), and thus, proved to be an effective tool for tracing *L. monocytogenes* contamination in the food processing industry. Of the restriction enzymes (*Asc*I, *Sma*I, *Apa*I *and Not*I) most commonly used in PFGE typing of *L. monocytogenes, Asc*I, *Sma*I and *Apa*I were used in our studies. In study I, *Asc*I was more discriminative than *Sma*I. *Apa*I also resulted in less MRPs than *Asc*I, but unlike in the case of *Sma*I, the combination of *Apa*I with *Asc*I MRPs resulted in increased discrimination (study V). This is in accordance with other studies demonstrating that discrimination of PFGE typing can be increased by use of more than one restriction enzyme (Brosch et al. 1994, Fredriksson-Ahomaa et al. 1999, Miettinen et al. 1999a). The analysis of MRPs can be done visually or by using computer softwares. Computerized analysis of macrorestriction patterns is a widely used and effective way of evaluating similarity of patterns. However, two *Asc*I MRPs (AscI MRP 11 and 13) were found to be identical in numerical analysis, even though in visual examination a four-band difference was present (V, Fig. 1) due to minor weight differences between the fragments. Therefore, when using computer softwares visual examination of restriction patterns should also be included, especially if only one restriction enzyme is used.

AFLP was used to evaluate similarity between persistent and sporadic *L. monocytogenes* strains. AFLP analysis showed high discriminatory power (DI 0.98), and the results were in concordance with those of PFGE. Reproducibility is often a concern in PCR based typing methods, but AFLP showed high reproducibility by means of high similarity cut-off value (95%) used for AFLP type definition. AFLP proved to be a valuable tool for strain characterization of *L. monocytogenes* and can be successfully applied to analyse *L. monocytogenes* routes and ecology in food processing industries. Compared with PFGE typing, AFLP has high throughput, possibilities for automation and the applicability not only for strain

characterisation but also for determining molecular evolution of bacteria (Keim et al. 1997, Kühn et al. 1997, Mougel et al. 2002, Keto-Timonen et al. 2003). However, AFLP is more complicated than PFGE typing and requires an automated sequencer. In addition, AFLP patterns are complex and their analysis requires the use of computer softwares.

6.2 PFGE typing as a tool in epidemiological investigations (I, II, IV, V)

6.2.1 Outbreak investigation (IV)

An outbreak of listeriosis occurring mainly among persons with intense immunosuppression was discovered. The outbreak involving 25 cases was shown to be caused by *L. monocytogenes* serotype 3a and was transmitted by an unusual vehicle, pasteurized butter. To the author's knowledge, this was the first outbreak caused by this rare serotype.

Repeated isolation of specific PFGE pattern strains of rare serotype 3a in human listeriosis cases was the basis for identification of the outbreak. Outbreak cases were shown to be clustered in a patient population spending long periods at the same hospital. In addition, a strain of the same PFGE pattern and serotype was previously isolated in an in-house control sampled in a Finnish dairy, and this dairy was the only deliverer of butter to the hospital in which the majority of cases were treated since summer 1998. The outbreak strain was detected in butter samples from the hospital kitchen, several lots of 7, 10 and 500 g butter packages from the dairy and wholesale store, and the dairy environmental samples.

Co-operation between researchers and authorities was of major importance in identification of the outbreak, determination of the vehicle of infection and prevention of further cases. Continuous subtyping of human *L. monocytogenes* strains enabled recognition of the outbreak, identification of patients included in an outbreak and confirmation of the outbreak's cessation. Moreover, the common vehicle of infections was determined by comparing strains from patients with those isolated from suspect food. This study emphasizes the effectiveness of comprehensive, laboratory-based surveillance with continuous typing of bacteria in improving outbreak recognition.

The cost-effectiveness of subtyping surveillance of *L. monocytogenes* has not been evaluated to the author's knowledge. Elbasha et al. (2000) analysed the costs and benefits of a subtype-specific surveillance system for identifying outbreak-associated *E. coli* O157:H7 infections in USA and estimated that if five cases were averted annually, the system would recover all its costs. They also pointed out that the system becomes even more cost-effective

if the resources that would have been wasted in investigating apparent increases in sporadic cases of infections are taken into account.

6.2.2 Detection and biodiversity of *L. monocytogenes* strains isolated from foods (I, II, V)

A single sample may harbour more than one *L. monocytogenes* pulsotype. In study I, five samples contained two different pulsotypes, and in study II, 23 of the samples had more than one pulsotype. At most, four different *L. monocytogenes* were found in a single sample (II). In addition, some *L. monocytogenes* pulsotypes would not have been recovered if only one detection method had been used. These results are in agreement with previous findings (Danielsson-Tham et al. 1993, Loncarevic et al. 1996), and stress the importance of characterization of several isolates per sample in epidemiological investigations.

The products of several food manufacturers were found to harbour *L. monocytogenes* strains possessing identical pulsotypes. Strains sharing identical restriction patterns were recovered in foods from different countries and years, and in the numerical analysis of MRPs, no association was found between clustering of strains and producer, product type, country or year of isolation. These results indicate that *L. monocytogenes* strains are not geographically specific.

In epidemiological studies, fingerprint databases of food strains may be used to aid in tracing vehicles of infections by comparing patient strains with food strains. However, it is essential to understand the limitations of food strain typing databases because recovery of indistinguishable fingerprints may direct an investigation towards establishing the vehicle of infections, but it may also mislead the investigation. As similar strains can be found in different product types of different producers and countries, the recovery of identical patterns from food and patient strains does not prove that the food is the vehicle of infection. Therefore, in addition to molecular typing data, epidemiological investigations and establishment of the consumption of the suspected food item are of major importance.

7 CONCLUSIONS

1. Eight out of ten low-capacity slaughterhouses were contaminated with *Listeria.* A high prevalence of *L. monocytogenes* was found in tonsils and tongues, 12% and 14%, respectively. The reason for the high rate in tongues may be the slaughtering process, where the tonsils are removed together with pluck set. In two slaughterhouses, carcasses were contaminated with *L. monocytogenes,* and in both cases, the saws used for splitting the carcasses were also contaminated with the same. Splitting saws were shown to be a possible contamination site of carcasses in these slaughterhouses.

2. A typical feature of *L. monocytogenes* contamination was shown to be existence of dominating strains in food processing plants. The contamination of cold-smoked rainbow trout was shown to occur during processing, and processing equipment was demonstrated to be important site of contamination. Air-mediated contamination was deemed of minor relevance. The eradication of *Listeria* contamination required specific measures, such as structural changes, dismantling of machines and targeted cleaning procedures including the use of hot steam, hot air and hot water.

3. Thirteen of the 15 genotypes presented by persistent strains were specific to persistent strains, and 94% of the genotypes of sporadic strains were recovered only among sporadic strains. The results showed that *L. monocytogenes* strains causing persistent contamination differ from sporadic ones, but are not descended from a common ancestor and no specific evolutionary lineage of persistent strains exists.

4. The repeated isolation of specific PFGE pattern strains of rare serotype 3a in human listeriosis cases was the basis for identifying an outbreak of listeriosis. Continuous subtyping of human *L. monocytogenes* strains enabled recognition of the outbreak, identification of patients included in an outbreak and confirmation of the outbreak's cessation. Moreover, the common vehicle of infections, butter, was determined by comparing strains from patients with those isolated from suspect food.

5.	The products of several food manufacturers were found to harbour *L. monocytogenes* strains possessing identical pulsotypes. Strains sharing identical restriction patterns were recovered in foods from different countries and years, and in the numerical analysis of MRPs, no association was found between clustering of strains and producer, product type, country or year of isolation. As similar strains can be found in different product types of different producers and countries, the recovery of identical patterns from food and patient strains does not necessarily prove that the food is the vehicle of infection.

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