THE USE OF SEROTYPING AND PFGE-TYPING OF *LISTERIA MONOCYTOGENES* IN FOOD PROCESSING CONTAMINATION STUDIES AND HUMAN FOODBORNE INFECTIONS

MARIA SJÖMAN

Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Finland

ACADEMIC DISSERTATION

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Supervising Professor

Hannu Korkeala, DVM, M.Sc., Ph.D., Professor
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki, Finland

Supervised by

Hannu Korkeala, DVM, M.Sc., Ph.D., Professor
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki, Finland

Johanna Björkroth, DVM, Ph.D., Professor
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki, Finland

Reviewed by

Eija Trees, DVM, Ph.D., Docent
Centers for Disease Control and Prevention (CDC)
Atlanta, Georgia, USA

Patrick C Y Woo, MD, Professor
Department of Microbiology
The University of Hong Kong

Opponent

Pentti Huovinen, MD, Ph.D., Research Professor
National Institute for Health and Welfare (THL)
Turku, Finland

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For my daughter, Mathilda

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ALOA	Agar *Listeria* according to Ottaviani & Agosti
a_w	water activity
BHI	brain heart infusion
cfu	colony forming units
CDC	U.S. Centers for Disease Control and Prevention
CHEF	clamped homologous electric field
CSF	cerebrospinal fluid
ET	electrophoretic type
FDA	U.S. Food and Drug Administration
FIGE	field inversion gel electrophoresis
GAD	glutamate decarboxylase
GMP	good manufacturing practices
HACCP	hazard analysis and critical control point
ISO	International Organization for Standardization
KTL	Finnish National Public Health Institute
LEB	*Listeria* enrichment broth
LMBA	*Listeria monocytogenes* blood agar medium
LPM	lithium chloride phenyl ethanol moxalactam medium
MEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
NCFA	Nordic Committee on Food Analysis
PALCAM	polymyxin B, acriflavin, lithium chloride, ceftazidime, aesculin and mannitol medium
PCR	polymerase chain reaction
PCR-REA	polymerase chain reaction followed by restriction enzyme analysis
PFGE	pulsed-field gel electrophoresis
PIPLC	phosphatidylinositol phospholipase
RAPD	random amplification of polymorphic DNA
REA	restriction enzyme analysis
REDP	restriction enzyme digestion profile
RTE	ready-to-eat
TE	tris-EDTA

THL	Finnish National Institute for Health and Welfare
TSA	trypticase soy agar
TBE	tris-borate EDTA
USDA	U.S. Department of Agriculture
UPGMA	unweighted-pair group matching algorithm

ABSTRACT

Two different typing methods, serotyping and pulsed-field gel electrophoresis (PFGE) typing, were used to study *Listeria monocytogenes* contamination in food processing plants and human foodborne *L. monocytogenes* infections.

L. monocytogenes contamination was studied in two different types of food processing plants: one ice cream plant and two broiler abattoirs. A dominant *L. monocytogenes* PFGE type of serotype 1/2b was found to have persisted in this ice cream plant for at least seven years. This strain was found in the production environment, ice cream, and equipment, especially in the packaging machine. Two broiler abattoirs were evaluated for *L. monocytogenes* contamination of the processing environment, broiler meat, and broiler meat products. Contaminated sites in the broiler processing environment included the air chiller, the conveyor belt leading to the meat packaging area, and the skin removing machine, possibly suggesting important contamination points of broiler meat. The two broiler abattoirs harboured different *L. monocytogenes* PFGE types. Retail broiler meat samples were also analyzed in order to compare the results to those from the broiler abattoirs. Of the raw broiler meat pieces bought from retail stores, 62% tested positive for *L. monocytogenes*. PFGE types found in the retail raw broiler meat pieces were identical to the PFGE types found at the broiler abattoirs where they had been processed. PFGE characterized these industrial strains and enabled targeted cleaning and disinfection practices at these food processing plants.

L. monocytogenes isolates from human invasive infections from 1990-2001 in Finland were studied in order to detect a possible clustering of cases. The most common *L. monocytogenes* serotype was 1/2a. Altogether 81 different PFGE types were found using one restriction enzyme (*Asc*I). A strain of an identical PFGE type of serotype 1/2a had appeared in 1994, accounting for 12% of the invasive infections.

In addition to the human invasive infections, we studied two foodborne *L. monocytogenes* outbreaks in Finland. In the first outbreak in 1997, five healthy persons fell ill with febrile gastroenteritis. The outbreak was associated with the consumption of vacuum-packed cold-smoked rainbow trout containing *L. monocytogenes*. Indistinguishable *L. monocytogenes* PFGE types of serotype 1/2a were isolated from the incriminated fish product lot and human stool samples. This same *L. monocytogenes* PFGE type was identical

to the most common PFGE type in human invasive *L. monocytogenes* infections in Finland during 1994-2001.

The second outbreak studied occured in 1999, when 25 patients acquired listeriosis from butter, 6 of whom died. This outbreak was initially identified with the help of timely serotyping and PFGE typing, and resulted from a globally uncommon strain of *L. monocytogenes* of serotype 3a and unique PFGE type.

For regular epidemiological surveillance of human *L. monocytogenes* infections with PFGE typing, the use of one restriction enzyme is sufficient, but in the case of contamination studies or suspected outbreaks, the use of at least two restriction enzymes is recommended in order to increase the discrimination power of PFGE typing.

LIST OF ORIGINAL PUBLICATIONS

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

I Miettinen[1], M. K., Björkroth, K. J. and Korkeala, H. 1999. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 46: 187-192.

II Miettinen[1], M. K., Palmu, L., Björkroth, K. J. and Korkeala, H. 2001. Prevalence of *Listeria monocytogenes* in broilers at the abattoir, processing plant, and retail level. J. Food Prot. 64: 994-999.

III Lukinmaa, S., Miettinen[1], M., Nakari, U.-M., Korkeala, H. and Siitonen, A. 2003. *Listeria monocytogenes* isolates from invasive infections: Variation of sero- and genotypes during an 11-year period in Finland. J. Clin. Microbiol. 41: 1694-1700.

IV Miettinen[1], M. K., Siitonen, A., Heiskanen, P., Haajanen, H., Björkroth, K. J. and Korkeala, H. 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J. Clin. Microbiol. 37: 2358-2360.

V Lyytikäinen, O., Autio, T., Maijala, R., Ruutu, P., Honkanen-Buzalski, T., Miettinen[1], 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. Inf. Dis. 181: 1838-1841.

[1] The previous name of the author of this thesis was Maria Miettinen.

1 INTRODUCTION

"About 44 years ago, shortly after the causative agent of listeriosis had been encountered in wild rodents, there was an epizootic among veldt rodents in the De Aar area along the Tiger River in South Africa. Sand rats were dying in numbers, and an organism was isolated, not virulent for most rodents but fatal to gerbils by ingestion. On the principle prevailing at that time – of casting out devils by Beelzebub – the so-called Tiger River virus, or ***Listeria monocytogenes*** *of today, was used in an effort to destroy gerbils over a belt of South African country some 20 miles long, with some success. Among those entrusted with the control of plague there were advocates of introducing Listeria into other continents for rodent control. This is understandable, because the first proved human infection was not reported until 1929, and the livestock infections, much later still. But there can be hardly any doubt that infection with this organism was associated with illness and death in man and other vertebrates long before the cause was isolated and identified".*

- From the foreword by Dr. Dr. h. c. K.F. Meyer in the second edition of Dr. H.P.R. Seeliger's (1961) monograph "Listeriosis"

Today our knowledge of *Listeria monocytogenes* has expanded tremendously, and research in many areas continues on this fascinating bacterium. Attitudes towards *L. monocytogenes* have changed from using it for possible rodent control to controlling the bacterium itself in the modern food processing environment. *L. monocytogenes* is an uninvited guest in food processing establishments, and much effort is required to keep it from contaminating processed food. Even if food processing practices are carefully planned and based on a hazard analysis and critical control point (HACCP) systems and good manufacturing practices (GMP) are followed, including proper cleaning and disinfection, problems with *L. monocytogenes* contamination are possible (Autio *et al.* 2004). A *L. monocytogenes* contamination problem in a food processing plant may cause a national listeriosis outbreak, since the food product lots are often very large, and the distribution area may include an entire country or even several continents.

L. monocytogenes food processing contamination studies must be conducted when *L. monocytogenes* isolates have been isolated in the food processing environment and the need arises to identify the exact problem spots in the process. Careful planning and performance is needed for the sampling, isolation, and typing of *L. monocytogenes* for contamination study purposes (Lundén *et al.* 2005). The typing method to be selected requires an understanding of both the strengths and limitations of the chosen typing technique (Foxman *et al.* 2005). Molecular typing methods are valuable tools in contamination studies of different *L. monocytogenes* strains found in the food processing environment. They enable comparison at the genotype level visualized by the "fingerprints" obtained. An understanding of molecular typing methods and food processing environments is needed to interpret typing results. The contamination site may turn out to be a machine in food processing, which might then require constant special cleaning attention as part of corrective actions.

Almost 35% of all deaths caused by foodborne bacterial pathogens in the USA in 1999 was attributable to *L. monocytogenes* (Mead *et al.* 1999). Every year foodborne diseases cause 76 million illnesses and 5000 deaths in the USA. The incidence of listeriosis is relatively low, but it was associated with the highest hospitalization risk and was the second-leading cause of death for known foodborne pathogens (CDC [Centers for Disease Control and Prevention], 2000). These are important reasons for investigating *L. monocytogenes* infections carefully in order to prevent people from eating contaminated food and acquiring infection. The identification of *L. monocytogenes* is not always sufficient when one is trying to investigate a possible foodborne outbreak. Establishing an epidemiological link between incriminated food and patients is a difficult task to perform and requires systematic cooperation between the food industry, authorities at all levels, and researchers (Lukinmaa *et al.* 2004b). Different molecular typing methods enable comparison of *L. monocytogenes* genotypes from food and patients and aid in identifying possible epidemiological links in order to pinpoint the causative food.

2 REVIEW OF THE LITERATURE

2.1 *Listeria monocytogenes*

2.1.1 History

In 1911, a Swedish scientist, later a professor in food hygiene, named Hülpers isolated bacteria from a liver necrosis in a rabbit. He found that the bacteria were pathogenic for mice. He performed different coloring tests, observed growth at different temperatures, and performed motility tests. Because Hülpers was unable to identify this bacterium, he called it *Bacillus hepatica* according to the isolation site (Hülpers 1911). Unfortunately, Hülpers's strain was not preserved for later confirmation. The species was later described in 1926 when Murray, Webb and Swann isolated bacteria from dead laboratory rabbits and guinea pigs. They called it *Bacterium monocytogenes* because of the monocytosis it caused in the animals (Murray *et al.* 1926). One year later, Pirie isolated the bacterium from wild gerbils with "Tiger River Disease" in South Africa, who named it *Listerella hepatolytica* to honor Lord Joseph Lister, and because of the typical liver infections it induced in experimentally infected animals (Pirie 1927). These two identical species were named *Listerella hepatolyticus.* In Denmark in 1929, Nyfeldt reported the first confirmed isolation from humans (Nyfeldt 1929). In the 1940s, the present name *Listeria monocytoges* was established (Pirie 1940).

2.1.2 Genus *Listeria*

The genus *Listeria,* together with the genus *Brochotrix*, belongs to the *Listeriaceae* family, the order *Bacillales,* the class *Bacilli* and the phylum *Firmicute* (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi Benson *et al.* 2000, Wheeler *et al.* 2000, Garritty and Holt 2001). Today the genus comprises the following seven species: *L. monocytogenes, L. innocua, L. ivanovii* subsp. *ivanovii* and, *L. ivanovii* subsp. *londoniensis, L. seeligeri, L. welshimeri, L. grayi* and only recently described *L. marthii* (Seeliger and Jones 1986, Rocourt *et al.* 1992, Boerlin *et al.* 1997, Graves *et al.* 2009). The first genome of *L. monocytogenes* was sequenced in 2001 (Glaser *et al.* 2001).

L. monocytogenes is generally considered the only species pathogenic to humans. However, some reports note the possibility that *L. ivanovii* (Lessing *et al.* 1994, Cummins *et al.* 1994, Snapir *et al.* 2006, Guillet *et al.* 2010) and *L. grayi* cause disease in humans (Todeschini *et al.* 1998). According to Todeschini *et al.* (1998), a severly immunocompromised patient with advanced Hodgkin's disease suffered from bacteremia caused by *L. grayi*. Ovine and bovine infections with *L. ivanovii* have also been reported (Sergeant *et al.* 1991, Alexander *et al.* 1992, Gill *et al.* 1997, Chand and Sadana 1999). *L. monocytogenes* and *L. ivanovii* are both ß-hemolytic on blood agar, but *L. ivanovii* is characterised by a wider ß-hemolytic zone than that of *L. monocytogenes*.

2.1.3 Isolation

L. monocytogenes is a ubiquitous bacterium that can be isolated from a variety of environmental sources, such as soil, water, sewage sludge, silage, decaying plant material, and the feces of humans and animals (Fig. 1) (Husu *et al.* 1990b, Rocourt 1994, Sahlström *et al.* 2004, Paillard *et al.* 2005, Ivanek *et al.* 2006, Lyautey *et al.* 2007, Kerouanton *et al.* 2009; Mohammed *et al.* 2010). Isolation methods have improved from direct culturing to cold enrichment and further to the selective enrichment of samples. Samples usually contain other bacteria as well, which makes isolation of *L. monocytogenes* challenging. Cold enrichment used to take several months to perform, which was clearly too long (Gray *et al.* 1948).

Today selective two-step enrichment broths, such as LEB (*Listeria* enrichment broth) (FDA, NCFA), half-Fraser, and Fraser (ISO, USDA), are in use for isolation from food (Lovett *et al.* 1987, Cook 1998, Fraser and Sperber 1988, McClain and Lee 1988, Anonymous 1990, Hitchins 1995). Agar media have developed from colorless tryptose or McBride agars, which were examined after incubation with oblique transillumination for the presence of characteristic blue colonies, to selective agars such as Oxford, PALCAM (polymyxin B, acriflavin, lithium chloride, ceftazidime, aesculin and mannitol), LPM (lithium chloride phenyl ethanol moxalactam medium), ALOA® (Agar *Listeria* according to Ottaviani & Agosti), and LMBA (*Listeria monocytogenes* blood agar medium) and Rapid'L.mono (Henry 1933, Lee and McClain 1986, VanNetten *et al.* 1988, Curtis *et al.* 1989, Foret and Dorey 1997, Ottaviani *et al.* 1997, Johansson 1998, Jaradat *et al.* 2002). Selective agars contain selective agents, such as various antibiotics, or indicative agents, such as esculin and

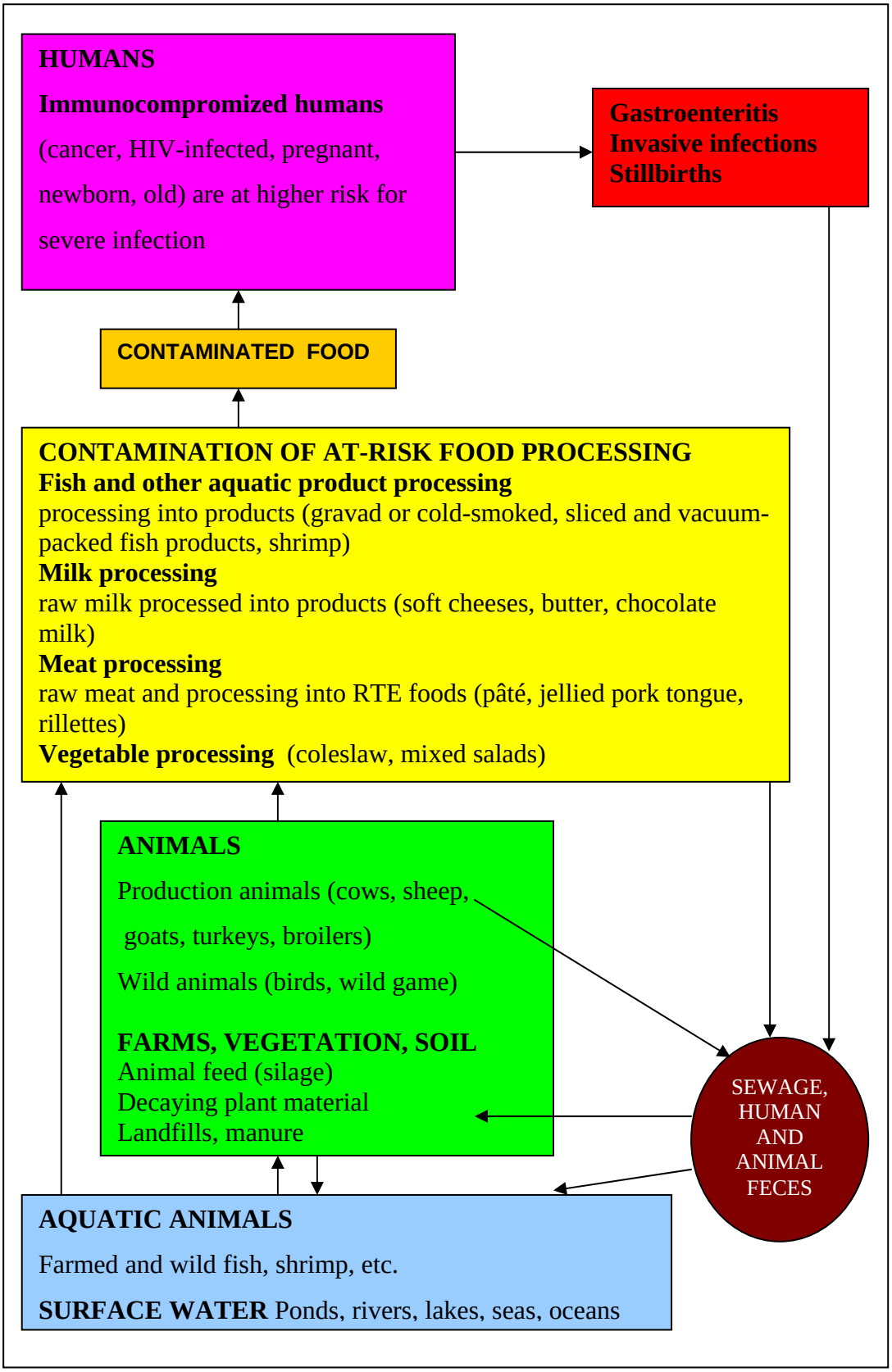

FIGURE 1. Relationship of *L. monocytogenes* in the environment and the human risk group

ferric substances. Of these, LMBA is very useful, because it contains sheep blood, and hemolytic colonies are easily identified and easy to select for confirmation of *L. monocytogenes* (Johansson 1998).

Chromogenic media may be used to enhance the detection of *L. monocytogenes* among many other *Listeria* sp. colonies on a plate. A few commercial chromogenic *L. monocytogenes* media are available on the market, such as RAPID'L.MONO® (Bio-Rad, Marnes la Coquette, France), CHROM agar® Listeria (Mast Diagnostic, Reinfeld, Germany) and BCM® *Listeria monocytogenes* plating medium (Biosynth International, Naperville, USA) (Restaino *et al.* 1999, Allerberger 2003). The detection of *L. monocytogenes* is based on the specific detection of phosphatidylinositol phospholipase C (PIPLC) of *L. monocytogenes* and *L. ivanovii*, and on the fermentation of xylose. *L. monocytogenes* colonies are mostly PIPLC positive and xylose negative (Allerberger 2003).

2.1.4 Identification

Listeria cells are small (0.5 μm in diameter and 1-2 μm long) non-spore-forming regular Gram-positive rods with rounded ends, that are sometimes coccoid (Seeliger and Jones 1986). *Listeria* is facultatively anaerobic, catalase positive with few exceptions, and oxidase negative (Cepeda *et al.* 2006). When grown at room temperature (20-25˚C) in liquid media, cells are motile due to a few peritrichious flagella. The tumbling motility can be seen in hanging drop preparations. Growth occurs best at 37˚C under microaerofilic conditions. Normal growth limits are 1-45˚C at pH 4.4-9.6 (the optimum is at pH 7). Some strains can grow at 0.5˚C or even at -0.2˚C (Junttila *et al.* 1988, Walker *et al.* 1990). *Listeria* is able to survive in high salt concentrations and can grow in 12% (w/v) NaCl and at minimum a_w values of 0.90 (Lou and Yousef 1999).

After isolation, additional tests are required for the identification of different *Listeria* species. These tests include hemolysis, Gram staining, the catalase test, motility, and the fermentation of different sugars (Anonymous 1990, Anonymous 1996). Commercial test kits, such as the API *Listeria* test kit (Bio-Meriéux, Rhone, France), are widely used for confirmation (Bille *et al.* 1992, Fujisawa and Mori 1994). *Listeria* species can be distinguished by differences in hemolysis and acid production in fermentation of sugars such

as D-xylose, L-rhamnose, α-metyl-D-mannoside, mannitol, and ribose (Table 1) (Cotoni 1942, Seeliger and Jones 1986, McLauchin 1987, Farber and Peterkin 1991, Allerberger 2003).

2.1.5 Serotyping

Serotyping was the first typing method used for *L. monocytogenes.* The serotyping of *L. monocytogenes* is based on agglutination reactions using antisera specific to different antigens on the surface of the cells. Antisera are obtained from rabbits immunized with different *L. monocytogenes* serotypes. The serotyping scheme is based on somatic cell wall (O) and flagellar (H) antigens. O-antigens are different structures on the cell wall, such as lipoteichoic acids and membrane proteins, and H-antigens are different structures of flagellas. Patterson recognised O- and H-antigens in 1939 and subdivided *L. monocytogenes* into four serotypes: 1, 2, 3, and 4 (Paterson 1939, Paterson 1940). The current serotyping is based on studies by Donker-Voet and Seeliger and was modified and extended into four serogroups (1/2, 3, 4, and 7) (Donker-Voet 1966, Seeliger and Höhne 1979, Seeliger 1987). These are further subdivided into 13 serotypes (Table 2). Serotype 4bX is a variant of serotype 4b and was implicated in an outbreak in the UK that was traced to contaminated pâté (McLauchlin *et al.* 1989, McLauchlin *et al.* 1991).

It is equally important to identify both O- and H-antigens in determining the correct serotype. Serotyping is still used in epidemiological and case studies of *L. monocytogenes* even though its discriminating power is poorer than that of other typing methods, such as ribotyping and PFGE, (Farber 1996). Moreover, serotyping is usually of limited value since most food and clinical isolates belong to serotypes 1/2a, 1/2b or 4b (McLauchlin *et al.* 1989). The reason for its still common use is historical, since serotyping remains the easiest way to of roughly compare strains with older findings. The serotyping of O-antigens is also fairly easy and quick to perform. Another reason for the frequent use of serotyping is typeability. A WHO multicentre *L. monocytogenes* subtyping study was reported in 1996, and all 80 of the strains studied were typeable (Schönberg *et al.* 1996). However, problems occurred with the inter- and intralaboratory reproducibility of serotyping, varying from 64 to 95% and from 82 to 100%, respectively. The study emphasized the need for good-quality antisera withstandardized strains.

TABLE 1. Identification of *Listeria* species.

Species	β-hemolysis	D-Xylose	L-Rhamnose	α-Metylmannoside	Ribose	D-Mannitol
L. monocytogenes	+	−	+	+	−	−
L. innocua	−	−	v	+	−	−
L. ivanovii subsp. ivanovii	+[a]	+	−	−	−	+
L. ivanovii subsp. londoniensis	+[a]	+	−	−	−	−
L. seeligeri	+	+	−	−	−	−
L. welshimeri	−	+	v	+	−	−
L. grayi	−	−	v	+	+	+
L. marthii	−	NK	−	NK	NK	NK

+ = positive

− = negative

[a] = wider hemolysis zone

v = variable

NK =unknown

TABLE 2. The serotypes and antigens of *L. monocytogenes.*

Serotype	O-antigens	H-antigens
1/2a	I, II, (III)	A, B
1/2b	I, II, (III)	A, B, C
1/2c	I, II, (III)	B, D
3a	II, (III), IV	A, B
3b	II, (III), IV, (XII), (XIII)	A, B, C
3c	II, (III), IV, (XII), (XIII)	B, D
4a	(III), (V), VII, IX	A, B, C
4ab	(III), V, VI, VII, IX, X	A, B, C
4b	(III), V, VI,	A, B, C
4c	(III), V, VII,	A, B, C
4d	(III), (V), VI, VIII	A, B, C
4e	(III), V, VI, (VIII), (IX)	A, B, C
7	(III), XII, XIII	A, B, C

( ) = not always present

2.1.6 Genotyping

Several DNA-based typing methods are available which can be used for typing *L. monocytogenes* strains (Bille and Rocourt 1996). These methods can be PCR (polymerase chain reaction)-based methods, such as RAPD (random amplification of polymorphic DNA) (Lawrence *et al.* 1993) and AFLP (amplified fragment length polymorphism) (Fonnesbech Vogel *et al.* 2002, Autio *et al.* 2003, Keto-Timonen *et al.* 2003).

Other DNA-based methods for subtyping *L. monocytogenes* include ribotyping (Grimont and Grimont 1986), REA (restriction enzyme analysis) (Wesley and Ashton 1991, Ericsson *et al.* 1993), PFGE (pulsed-field gel electrophoresis) (Ojeniyi *et al.* 1996, Unnerstad *et al.* 1996, Autio *et al.* 1999, Giovannacci *et al.*1999, Senczek *et al.* 2000), and MLST (multilocus sequence typing) (Meinersmann *et al.* 2004, Revazishvili *et al.* 2004).

One or more genotyping methods can be combined with phenotyping methods. PFGE has been combined with serotyping (Chasseignaux *et al.* 2001), ribotyping (Autio *et al.* 1999, Lukinmaa *et al.* 2004a) multilocus enzyme electrophoresis (MEE) (Harvey and Gilmour 2001), and PCR-based methods (Giovannacci *et al.* 1999, Fonnesbech Vogel *et al.* 2002, Autio *et al.* 2003) in order to achieve more information for typing purposes.

PFGE typing

PFGE typing is a genotyping method developed by Schwartz and Cantor in 1984 to separate large yeast chromosomes (Schwartz and Cantor 1984). The need arose to develop a method to separate DNA molecules larger than 50 kilobase pairs (kb), since this is the upper limit for migration in conventional gel electrophoresis (Lai *et al.* 1989). In PFGE, this is overcome by using two alternating electric fields. The angle between the electric field is usually 120˚ in CHEF (clamped homologous electric field) or 180˚ in FIGE (field inversion gel electrophoresis). This technique enables the separation of large DNA up to about 1 000 kb (Lognonne 1993). DNA is usually obtained from cells in solution by digesting their cell walls and proteins. To overcome the mechanical breakage of DNA molecules in solution, the cells are embedded in agarose before lysis. After lysis and washing away the cell walls and proteins, intact DNA is obtained embedded in highly purified agarose. DNA plugs are subjected to low-frequency restriction enzymes. Low-frequency restriction enzymes usually recognize six to eight base pairs and cut the DNA at the restriction site. The cut DNA molecules are usually separated in 0.6-1.5% agarose gel in TBE (Tris-borate EDTA) buffer by PFGE. The running time is several hours, usually around 18-24 h. Known DNA markers are included in every gel to enable estimation of the sizes of the fragments and comparison between different runs.

After elongation, DNA migrates in the gel in an electric field. When the direction of the electric field is changed, the DNA must reorient before it can migrate in this new direction. Molecular weight is linked to the time it takes for reorientation. Thus lower molecular weight DNA fragments migrate further in the PFGE gel than do fragments of higher molecular weight. In a uniform electric field, large DNA molecules migrate at a rate independent of their size and they are not resolved by size (Lerman and Frisch 1982).

The basic instrumentation includes the gel box, a device for temperature control, a switching unit, and a power supply. The switching unit is programmed with the run parameters (i.e. voltage, pulse time switch interval times and total running time). When increasing the pulse time, larger molecules can be separated (Lognonne 1993). The migration of DNA is also temperature dependent and dependent on agarose strength.

The reproducibility, discriminatory power, and ease of interpretation in PFGE are excellent (Farber 1996). In a WHO multicenter international typing study of *L. monocytogenes* with PFGE, the agreement data among the four participating laboratories varied from 79-90%, probably because all of the laboratories used slightly different run parameters (Brosch *et al.* 1996). Still, this study reconfirmed that PFGE is a very accurate and reproducible method for the fine structure comparison and molecular typing of *L. monocytogenes.*

Because *L. monocytogenes* is characterized by a low G+C DNA (39%) content, it is useful to choose restriction enzymes with recognition sequences that contain only G and C nucleotides in order to obtain a low-frequent cutting restriction enzyme (Glaser *et al.* 2001). Different rare-cutting restriction enzymes have proved useful for *L. monocytogenes.* Such enzymes are *Apa*I (GGGCC/C), *Asc*I (GG/CGCGCC), *Not*I (GC/GGCCGC) and SmaI (CCC/GGG), where "/" indicates the restriction site.

PFGE protocols for *L. monocytogenes* required usually four to seven days for DNA plug preparation, cell lysis, restriction enzyme digestion, and gel electrophoresis (Brosch *et al.* 1991, Maslow *et al.* 1993). A 30-h rapid protocol for *L. monocytogenes* where time-saving steps included the use of bacterial cells obtained directly from the culture plates instead of growing them first in liquid medium and harvesting the cells by centrifugation, a pre-lysis treatment with lysozyme, a shorter lysis time (2 h), reduced washing times (instead of washing for several hours, washing twice with 50-54˚C sterile distilled water for only 10 min, followed by four washes with TE buffer for only 15 min), and using minimum restriction enzyme digestion times (Graves and Swaminathan 2001). This protocol is standardized and used by the CDC PulseNet in the USA, which is a national network of public health and food regulatory laboratories established to detect clusters of foodborne disease. The participating laboratories exchange normalized DNA fingerprints via the internet.

Numerical analysis of restriction enzyme digestion patterns (REDPs)

The REDPs of different strains from PFGE gels can be visually compared to each other directly from the gel and then be saved by photographing. This is convenient and fast when only a few are compared. When large numbers of REDPs from strains are to be compared, saving the gel image in a data file enables computer-assisted numerical comparison. The REDPs bands are then normalized to each other with the help of the bands from the marker from each gel. The similarity between all pattern pairs can be calculated using the Dice coefficient. Large-scale grouping analysis such as UPGMA involves the creation of dendrograms or other means to reveal groups of related organisms.

2.2 *L. monocytogenes* in food processing

The fact that the populations in Western industrialized countries live longer lives and add to the proportion of immunocompromized individuals is a challenge for the food industry. The food industry needs to try to reduce *L. monocytogenes* levels in the foods they produce, especially in ready-to-eat foods. Guidelines are available on how to prevent foodstuffs from becoming contaminated in food processing plants and how to live with this pathogen in the plant (Tompkin *et al.* 1999, Anonymous 2000a).

The ecology of *L. monocytogenes* in food industry plants has been studied in order to trace the potential sources of contamination and the conditions where it can survive. Factors such as cross-contamination, the psychrotrophic nature of *L. monocytogenes,* its ability to adhere to various surfaces in the plant, biofilm formation, persistent contamination and inadequate cleaning and disinfection enable the persistent contamination of food processing plants (Unnerstad *et al.* 1996, Autio *et al.* 1999, Lundén *et al.* 2000, Lundén *et al.* 2002, Borucki *et al.* 2003, Lundén *et al.* 2003a, Lundén *et al.* 2003b, Peccio *et al.* 2003, Holah *et al.* 2004, Wulff *et al.* 2006, López *et al.* 2008b, Pappelbaum *et al.* 2008).

L. monocytogenes strains posses the variable ability of a glutamate decarboxylase (GAD) acid resistance system which increases the pH in the cell cytoplasm and which could explain why certain strains are more resistant to an acid environment and survive in glutamate-rich acid foods (Cotter *et al.* 2001a, Cotter *et al.* 2001b, Hill *et al.* 2002). Dykes &

Moorhead (2000) have shown that the acid stress response is needed during the infection process since all strains from clinical sources, though only 87% of strains from ready-to-eat meat products, were capable of surviving pH 2.4 for 2 h (Dykes and Moorhead 2000). This could also explain why not all *L. monocytogenes* strains encountered in the food processing environment have caused infections in humans (Lukinmaa *et al.* 2004a).

2.2.1 *L. monocytogenes* in dairy processing

Dairy processing includes the processing of raw milk and further processing into products such as butter, cheese, yoghurt and ice cream. The prevalence of *L. monocytogenes* in raw milk varies between 1-7% (Husu *et al.* 1990, Gaya *et al.* 1998, Waak *et al.* 2002, Meyer-Broseta *et al.* 2003, Muraoka *et al.* 2003). Raw milk is usually pasteurized, which is considered listericidal. Recontamination of dairy products could occur after heat-treatment.

In a study performed in 21 dairy plants, the overall prevalence of *L. monocytogenes* on equipment and in the environment was 9.3% (Pritchard *et al.* 1995). The prevalence was higher in the dairy environment (14.7%) than on the equipment (5.1%). During 1990-1999, the overall prevalence in the environment and product samples of Swiss cheese processing plants was 4.9% (Pak *et al.* 2002). The highest proportion of positive samples (9.5%) was observed in water samples used for cheese washing, followed by cheese surface swabs (5.0%). No positive samples were obtained from cream, ice cream, milk powder, yoghurt, or fresh cheese. In a French cheese plant examined for *L. monocytogenes* contamination, *L. monocytogenes* was isolated from four varieties of cheese, cheese brines, processing equipment, and the plant environment (Jacquet *et al.* 1993). No *L. monocytogenes* was isolated before the ripening and rind washing stages of the process. The contamination of cheeses probably took place during ripening since strains from cheeses and processing equipment (shelves) were of the same serotype and phagotype. All strains from this cheese plant were of the same ribotype, suggesting that they were all of clonal ancestry. A Scandinavian cheese-producing dairy was contaminated by a *L. monocytogenes* strain of serotype 3b for at least seven years (Unnerstad *et al.*1996). The same strain, which was characterized by three restriction enzymes (*Apa*I, *Asc*I and *Sma*I) with PFGE typing, was found in the wash water of the cheeses, in the cheese itself, and the production environment.

The prevalence of *L. monocytogenes* in ice cream is usually quite low (0-1.8%) (Busani *et al.* 2005, Cabedo *et al.* 2008). In a study in Hungary, the overall prevalence of *L. monocytogenes* in samples taken from food production and marketing companies during random inspections was 16.2% (18/613) and 1.8% in ice cream (2/43) (Kiss *et al.* 2006). The serotypes found in ice cream were 1/2a (14/18) and 4b (4/18), and those in butter were serotypes 1/2b and 4ab (one strain each). The prevalence of *L. monocytogenes* in butter was studied in 2004 in the UK on production, retail and catering premises during a two-month period. The prevalence was low, only 0.4% (13/3229) of these butter samples tested positive for *L. monocytogenes*, and the level was < 10 cfu/g in all samples (Anonymous 2005).

2.2.2 *L. monocytogenes* in poultry meat processing

Poultry meat was the first reported meat product to harbor *L. monocytogenes* (Gitter 1976). In a literature review, Jay (1996) reported an overall prevalence of 17% for fresh and frozen poultry meat during the period 1984-1994 in nine countries (Jay 1996). A similar figure (17.4%) was reported in a Danish study of raw turkey meat (Ojeniyi *et al.* 2000) (Gudbjornsdottir *et al.* 2004). A Swedish study followed the 11 largest broiler slaughterhouses in Sweden for one year (September 2002 to August 2003) and 254 carcass samples were taken (Lindblad and Lindqvist 2003). Of these, 29% tested positive for *L. monocytogenes*. The overall prevalence at the six largest broiler slaughterhouses was 21% varying between 0 and 55% with no seasonal variation. Others have reported higher prevalences (27-88%) in poultry meat (Skovgaard and Morgen 1988, Rorvik and Yndestad 1991, MacGowan *et al.* 1994, Uyttendaele *et al.* 1997, Samelis and Metaxopoulos 1999, Vitas *et al.* 2004, Praakle *et al.* 2006, Ceylan *et al.* 2008).

In July-October 2003 in Finland, 169 marinated broiler meat preparation samplesand 58 marinated turkey meat preparation samples from the retail level in the city of Helsinki were analyzed for *L. monocytogenes* (Pönkä *et al.* 2004). Altogether 38% of the marinated broiler meat preparations and 7% of the turkey meat preparations were contaminated with *L. monocytogenes.* The quantification analyses revealed that in 80% of these samples, the levels of *L. monocytogenes* were < 10 cfu/g. Only three samples had *L. monocytogenes* levels >100 cfu/g, and the highest finding was 240 cfu/g. The prevalence at the retail level for the five different manufacturers of these marinated poultry meat preparations varied between 0-

62% (Pönkä *et al.* 2004). In another Finnish study in 2002-2003, the overall prevalence of of *L. monocytogenes* in marinated broiler legs from retail stores was 39 % (Aarnisalo *et al.* 2008).

A French study found that the overall prevalence of *L. monocytogenes* in poultry processing environments was 18.3%, and in the equipment, 16.4 %, and in the finished raw products, 40% (Chasseignaux *et al.* 2001). In a Nordic study, the overall prevalence was 22% in both the poultry processing plants and the final raw product. Lopez *et al.* (2008a) found that the overall prevalence in a Spanish broiler abattoir was 31%. The French study examined the environmental factors associated with *L. monocytogenes* contamination on working and non-working surfaces (floors, walls, sewers) in raw poultry or pork processing plants (Chasseignaux *et al.* 2001). The authors identified a risk profile for *L. monocytogenes* contamination, where uneven (granular, stripped, or damaged) resin or plastic surfaces with organic residues, a near neutral pH (6-8), a low temperature (< 4-12ºC), and high humidity (70-80% or more) were associated with contamination. *L. monocytogenes* is apparently not a common airborne contaminant in poultry slaughtering environments, since a study of airborne microbes found none (Ellerbroek 1997, Gudbjornsdottir *et al.* 2004).

The ecology of *L. monocytogenes* in two French poultry and pork processing plants was studied with PFGE (Chasseignaux *et al.* 2001). This same French study found 50 combined genotypes and showed that some clones could survive for several months. The authors concluded that contamination may be due to contaminated raw materials, bacterial spread, and ineffective cleaning procedures. Another study used RAPD to characterize 113 isolates from a chicken processing plant and found one particular RAPD type in the evisceration area, the processing area, and in the final product (Martinez *et al.* 2003). The contamination lasted for at least eight years. One Spanish broiler abattoir was surveyed for 1.5 years, and 14 different pulsotypes were found of serotypes 1/2a and 1/2b; the strains found on the carcasses were also found in the evisceration area (Lopez *et al.* 2008a). One *L. monocytogenes* PFGE type found in marinated broiler legs from retail stores in Finland in 2002-2003 that were produced at three different broiler producers but most of the PFGE types were producer-specific (Aarnisalo *et al.* 2008).

2.2.3 *L. monocytogenes* in fish processing

The prevalence of *L. monocytogenes* in raw fish varies between 0-86% (Jemmi and Keusch 1994, MacGowan *et al.* 1994, Rørvik *et al.* 1995, Vaz-Velho *et al.* 1998, Nørrung *et al.* 1999, Dauphin *et al.* 2001, Fonnesbech Vogel *et al.* 2001, Hoffman *et al.* 2003, Medrala *et al.* 2003, El-Shenawy and El-Shenawy 2006, Klaeboe *et al.* 2005, Markkula *et al.* 2005, Miettinen and Wirtanen 2005, Chou *et al.* 2006, Soultos *et al.* 2007, Cruz *et al.* 2008, Parihar *et al.* 2008a). One Finnish study in which samples of gills, viscera, and skin were taken separately from unprocessed fresh rainbow trout from fish farms in lakes and sea areas found that rainbow trout was contaminated almost exclusively in the gills and only sporadically in the skin and viscera (Miettinen and Wirtanen 2005). The *L. monocytogenes* contamination of farmed fish is linked to the fish farm environment, such as brook waters, as well as to rainy weather periods (Miettinen and Wirtanen 2006).

The prevalence of *L. monocytogenes* in different fish products varies between 0-78% (Lyhs et *al.* 1998, Klaeboe *et al.* 2005, Medrala *et al.* 2003, Cruz *et al.* 2008). During 1992-2000, the overall prevalence of *L. monocytogenes* in gravad salmon imported to Switzerland was 38% (Jemmi *et al.* 2002). In a Swedish study, 7% (35/467) of fish products in 2001 tested positive for *L. monocytogenes* (Rosengren and Lindblad 2003). These fish products includeds cold-salted ("gravad"), cold-smoked, or hot-smoked fish products. The majority of the samples had levels < 10 cfu/g. Only 1% (6/46) had levels of *L. monocytogenes* > 100 cfu/g. The highest level was 6 200 cfu/g. "Gravad" fish products were the most contaminated, since 14% (n=126) tested *L. monocytogenes* positive. Cold-smoked fish products were less contaminated, since 8% (n=80) tested positive for *L. monocytogenes*. The prevalence was lowest in hot-smoked fish products, with 3% (n=129) in 2001 (Rosengren and Lindblad 2003). An interesting seasonal variation between 5-20% was observed in all fish products samples; more positive samples were found in autumn (September-November) than in the other seasons. This seasonality was especially noted in "gravad" fish products, with a 4% prevalence in summer (July-August) and a 38% prevalence in autumn (September-November) (Rosengren and Lindblad 2003). In Poland, 78% of vacuum-packed sliced salmon was contaminated with *L. monocytogenes* (Medrala *et al.* 2003). In 2001-2004 in France, the prevalence in cold-smoked salmon from nine producers was 10% (Beaufort *et al.* 2004). The prevalence varied between 0-41% among producers.

A survey of *L. monocytogenes* in vacuum-packed cold-smoked fish products originating from 37 producers in Finland in 2001 showed that 13% of 356 samples tested positive on the use-by date. In most of the samples, the level was < 100 cfu/g, and the highest detected level was 25 000 cfu/g (Hatakka *et al.* 2002). In 2004 in Finland, *L. monocytogenes* was isolated from 17% (9/279) of cold-smoked fish and from 14% (41/285) of "gravad" fish at retail (Anonymous 2005). In the same survey, levels exceeding 100 cfu/g were found in 3% (9/279) of cold-smoked fish and 2% (5/285) of "gravad" fish at retail. In 2008, the prevalence in a Finnish survey was 16% (10/63) for "gravad fish" and 8% (5/64) for cold-smoked fish (Åberg *et al.* 2008). Of the 15 positive samples, 14 had levels of < 10 cfu/g, and 1 had a level of 20 cfu/g. The majority of these samples were taken from retail stores (105/127) and analyzed on the use-by date.

Serotype 1/2a is the most common finding in fish and fish products; as much as 80% of isolates are of this serotype (Autio *et al.* 2002, Jemmi *et al.* 2002, Gudmundsdóttir *et al.* 2005, Corcoran *et al.* 2006). In a Polish study, however, 92% of isolates from fish products from one fish processing plant were of serotype 4 (Medrala *et al.* 2003).

Norton *et al.* (2001) used ribotyping to track possible contamination sources in three cold-smoked fish processing plants and found that raw materials (raw fish) and the processing environment were the potential contamination sources of finished products. Rørvik *et al.* (1995), using MEE as a method of characterization, found in a contamination study of a salmon slaughterhouse and smoked salmon processing plant that contamination of the final products occured during processing. However, the same MEE electrophoretic type (ET-6) was also isolated from an environmental sample in the salmon slaughterhouse, and the original site of contamination could have been there. Job rotation was the strongest risk factor for *L. monocytogenes* contamination at fish smokehouses (Rørvik *et al.* 1997).

A study of ten different Danish fish smokehouses, obtained 16 different RAPD profiles (Fonnesbech Vogel *et al.* 2002). Different RAPD types dominated in cold-smoked salmon products from different smokehouses, but some RAPD types could be isolated from several smokehouses. The authors concluded that there was a possible persistence of closely related strains of *L. monocytogenes*. Fonnesbech Vogel *et al.* (2001) studied two Danish fish smokehouses for *L. monocytogenes* contamination of cold-smoked rainbow trout, and the primary sites of contamination were the brining and slicing areas. Based on RAPD typing

results, contamination of the processing equipment and environment of one of the smokehouses from raw fish could not be excluded. The same RAPD type was found over a four-year period, indicating that an established in-house strain persisted and was not eliminated by routine hygienic procedures (Fonnesbech Vogel *et al.* 2001).

Autio *et al.* (1999) found two major *L. monocytogenes* contamination sites, the brining and slicing of rainbow trout fillets, in a cold-smoked rainbow trout processing plant. Only 1 sample in 60 raw fish samples tested positive for *L. monocytogenes*, harboring a PFGE type different from the strains isolated from the environment and product samples. PFGE types were found in the brining and slicing machines were similar to those found in ready-to eat products. Researchers in France studied *L. monocytogenes* contamination with PFGE and concluded that the cold-smoked salmon was contaminated from the processing environment (Dauphin *et al.* 2001). In Japan, the findings of a contamination study suggested that the contamination of cold-smoked fish occured during slicing in a processing plant (Nakamura *et al.* 2006).

The PFGE types of serotype 1/2a found in water and sludge samples from two rainbow trout farms in Finland were the same as those found in fish products on the retail market (Katzav *et al.* 2006). Markkula *et al.* (2005) found the same PFGE types in raw and processed rainbow trout from a fish processing plant. These results suggest that *L. monocytogenes* contamination in fish processing plants could originate from the influx of contaminated raw fish.

2.3 *L. monocytogenes* infections in humans

2.3.1 Listeriosis

Listeriosis is a severe invasive foodborne infection in humans caused by *L. monocytogenes* and that predominantly leads to sepsis or meningitis or both, and only seldom causes brain stem encephalitis (Antal *et al.* 2005). Additional clinical illnesses included are pneumonia, endocarditis, septic arthritis, and abscesses (Hof 2001, Schett *et al.* 2005). Pregnant women may suffer from flu-like symptoms and give birth prematurely. Immunocompromised individuals, such as recipients of organ transplants, are at higher risk for listeriosis (Girmenia

et al. 2000, Reek *et al.* 2000). The first reported cases of human listeriosis occured in 1929 in Denmark (Nyfelt 1929).

Healthy humans seldom carry *L. monocytogenes* in their feces or throats. Heir *et al.* (2004) studied samples from employees in three meat plants; no *L. monocytogenes* was found in 70 throat swab or 45 feces samples (Heir *et al.* 2004). An estimated 1-5% of the normal, asymptomatic population are believed to carry *L. monocytogenes* in their feces (Hof 2001).

An estimated 2 500 *L. monocytogenes* infections occur in the USA each year with a mortality of 28% (Mead *et al.* 1999). In 2007, as many as 1 554 confirmed cases of listeriosis were reported from EU member states, which makes the reported incidence in these countries 0.3 cases per 100 000 population (Anonymous 2009). In Finland, between 18 and 46 human listeriosis cases were reported to the KTL (Finnish National Public Health Institute) or since 2009 to THL (the National Institute for Health and Welfare) to the National Infectious Disease Register between 1995 and August 2009, and the incidence varied from 0.34 to 1.03 cases per 100 000 population (www3.ktl.fi/, reported as of 28 August 2009) (Table 3). A seasonal trend seems evident in these reported human listeriosis cases in Finland between 1995 and August 2009 (Fig. 2). The number of reported human listeriosis cases began to rise in July and remained high until January.

2.3.2 Febrile gastroenteritis

The intestinal tract is the major port of entry for *L. monocytogenes* from contaminated food. Acute enteritis may be the only symptom of infection or may precede typical symptoms of listeriosis, such as sepsis. Febrile gastroentritis often goes undocumented, especially if it fails to progress to meningitis or sepsis, or is simply overlooked in the presence of a life-threatening infection of the central nervous system (Hof 2001). A case of febrile gastroenteritis from salted mushrooms followed by sepsis occurred in Finland (Junttila and Brander 1989). The acute onset of symptoms (severe abdominal pain, watery diarrhea, and fever) resulted from severe contamination of the mushrooms (3.5×10^6). *L. monocytogenes* was cultured from a blood sample from the patient.

TABLE 3. Cases of human listeriosis reported to the THL National Infectious Disease Register in Finland between 1995 and 2009.

Year	Human listeriosis cases	Incidence/100 000 population
1995	34	0.67
1996	29	0.57
1997	53	1.03
1999	46	0.89
1999	46	0.89
2000	18	0.35
2001	28	0.54
2002	20	0.34
2003	42	0.81
2004	35	0.67
2005	37	0.71
2006	46	0.88
2007	40	0.76
2008	40	0.75
2009	33	0.62

The literature describes foodborne *L. monocytogenes* febrile gastroenteritis cases with no progression to severe forms of listeriosis (Ooi and Lorber 2005). All kinds of foods have been implicated as vehicles (Table 4), most of which were severely contaminated with *L. monocytogenes* in the range of 10^6 to 10^8 cfu/g. A high concentration of *L. monocytogenes* may be needed to cause illness in healthy individuals, but the illness fails progress to an invasive form.

FIGURE 2. Cases of human listeriosis in Finland between 1995 and 2009 by month reported to the THL National Infectious Diseases Register (www3.ktl.fi/stat).

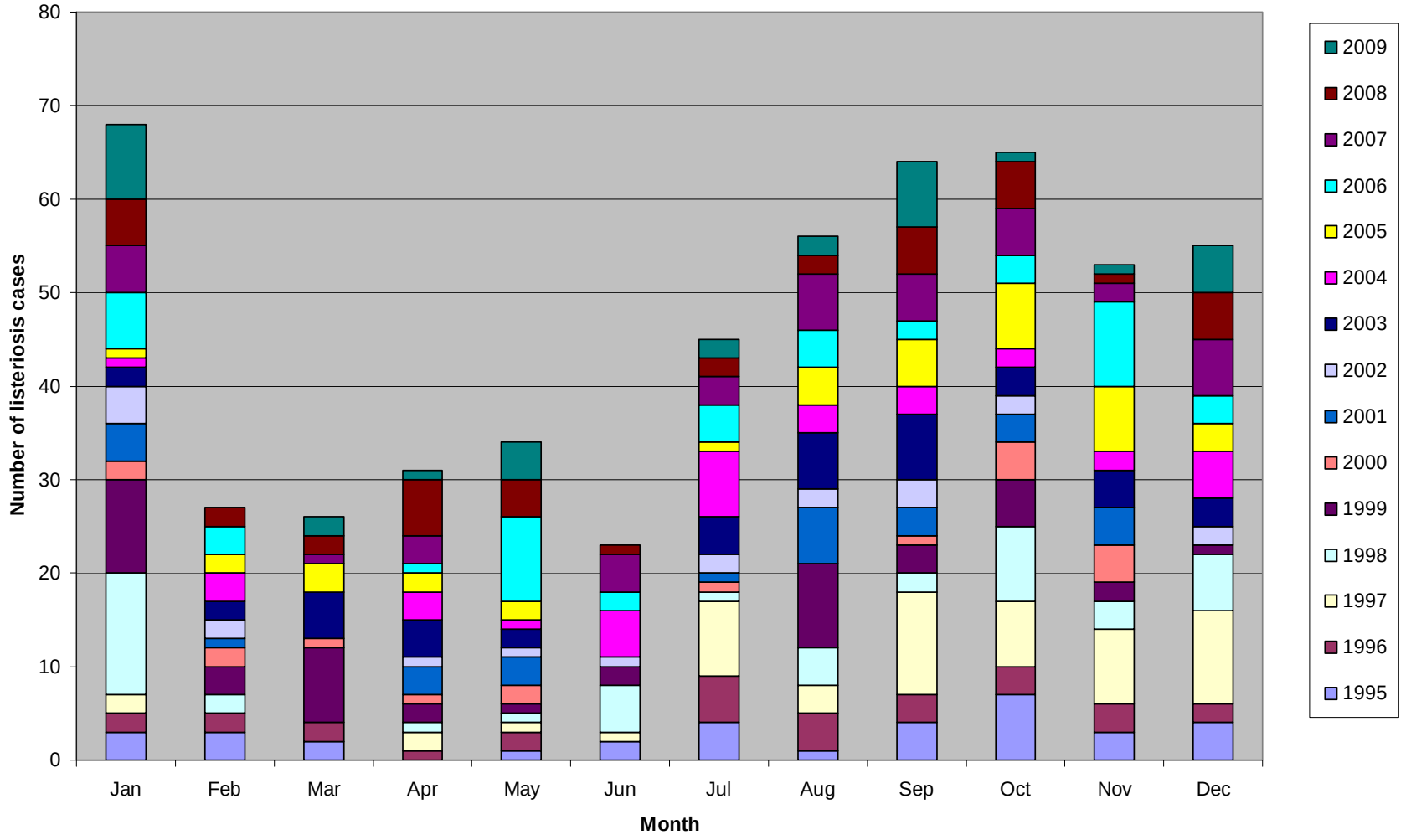

TABLE 4. Reported human *L. monocytogenes* gastroenteritis cases.

Country, year	Cases	Food, cfu/g, serotype	Reference
USA, 1989	10	Shrimp, NK, 4b	Riedo *et al.* 1994
Italy, 1993	8	Rice salad, NK, NK	Salamina *et al.* 1996
USA, 1994	48	Chocolate milk, 10^8-10^9 cfu/ml, 1/2b	Dalton *et al.* 1997
Canada, 1996	2	Imitation crab meat[a], 2.1×10^9 cfu/g,1/2b	Farber *et al.* 2000
Italy, 1997	1566	Corn and tuna salad, $>10^6$, 4b	Aureli *et al.* 2000
New Zealand, 2000	7	Ready-to-eat meat, 1.8×10^7 cfu/g, 1/2	Sim *et al.* 2002
New Zealand, 2000	21	Ready-to-eat meat, $>2.5\times10^9$ cfu/g, 1/2	Sim *et al.* 2002
USA, 2001	16	Precooked turkey, 1.6×10^9 cfu/g, 1/2a	Frye *et al.* 2002
Sweden, 2001	48	Soft cheese, 3.0×10^1-6.3×10^7 cfu/g, 1/2a	Carrique-Mas *et al.* 2003
Japan, 2001	28	Cheese, <3.0-4.6×10^7/g, 1/2b	Makino *et al.* 2005
Belgium, 2001	1	Ice cream cake, NK, 4b	Yde and Genicot 2004
Austria, 2008	12	Jellied pork, 3.2×10^2-3.0×10^4, 4b	Pichler *et al.* 2009

NK= unknown

[a] artificially flavored Alaskan pollock

2.3.3 Foodborne outbreaks

The first described large human foodborne outbreaks occurred in Germany in 1949-1957 after the consumption of contaminated raw milk and milk products (Seeliger 1961). In 1959 in Sweden, four cases of listeriosis occurred where poultry meat was the suspected vehicle, but no isolates were obtained (Olding and Philipson 1960). The first well-documented foodborne invasive infections caused by *L. monocytogenes* were reported in 1983 and suggested that the outbreak resulted from contaminated coleslaw (Schlech III *et al.* 1983). From 1983 onwards, several foodborne epidemics of human listeriosis have been reported in North America and Europe. Typical foods include soft cheeses and dairy products, pâtés, sausages, cold cuts, smoked fish and salads. Many of these food items are minimally processed ready-to-eat foods.

The first well-documented case of human listeriosis from poultry meat occurred in England in 1988, where cooked chicken served as the vehicle (Kerr *et al.* 1988). The first documented case in the USA from a poultry meat product was a cancer patient who contracted the disease from a turkey frank. The level of contamination exceeded 1100 cfu/g from an opened package in the patient's home (Wenger *et al*. 1990). Other contaminated meat products, such as pâté, rillettes, frankfurters, or cold cuts, have been incriminated in epidemics since the 1990s (McLauchlin *et al.* 1991, Goulet *et al.* 1998, Anonymous 2000b, Attaran *et al.* 2008). Fish and fish products have also been recognized as causative agents of listeriosis (Ericsson *et al.* 1997, Farber *et al.*, 2000, Tham *et al.* 2000). Milk and milk products, such as soft cheese, have been implicated several times as sources of infection (Fleming *et al.* 1985, Bille 1990, Cook *et al.* 2004, Lundén *et al.* 2004). Frozen ice cream cakes and butter belong to more unusual milk products that have been reported as sources of human listeriosis (Cook *et al.* 2004, Yde and Genicot 2004).

2.3.4 Tracing *L. monocytogenes* outbreaks

Characterizing *L. monocytogenes* strains from sporadic or epidemic outbreaks of listeriosis is necessary because it is an essential part of the epidemiological investigation. Typing food isolates and creating an electronic library of different genotypes enables later comparison with human strains in the event of a suspected listeriosis outbreak. A national Finnish electronic network for the comparison of PFGE profiles of *L. monocytogenes* has

been established in order to enable the faster investigation of possible foodborne outbreaks (Rantala *et al.* 2001). To be effective in tracing a particular isolate incriminated in an outbreak, it is useful to combine both phenotypic and genotypic characterization methods or to use several genotyping methods (Sauders *et al.* 2001, Jaradat *et al.* 2002).

PulseNet is a network of public health and regulatory laboratories in which all 50 US state and Canadian public-health laboratories participate (Graves and Swaminathan 2001, Gerner-Smidt *et al.* 2006, Pagotto *et al.* 2006). PFGE patterns from clinical specimens or food products are transmitted electronically to the participating laboratories. The detection of indistinguishable patterns alerts the public health system to the possibility of an outbreak, which is unintentional, but could also be deliberate as part of a biological terrorist attack on the food supply (Sobel *et al.* 2002). A feasibility study of PulseNet Europe aimed to identify European laboratories willing to participate in the surcveillance (Martin *et al.* 2006).

Before characterization can be performed, one must first isolate *L. monocytogenes* from different samples. Ideally food samples should be analyzed with both direct plating and after an enrichment method. In an ideal setting, many isolates should be confirmed from one sample for further characterization, since in some cases different strains will be isolated from the same sample; some reports describe this when PFGE typing has been used (Danielsson-Tham *et al.* 1993, Loncarevic *et al.* 1996, Ericsson *et al.* 1997, Autio *et al.* 1999, Autio *et al.* 2000, Dauphin *et al.* 2001). The enrichment method itself may favor some *L. monocytogenes* strains and *L. innocua*, whereas other strains in the sample remain undetected. This has been reported for the University of Vermont medium where strains of serotype 1/2a outgrew strains of serotypes 1/2b and 4b (Bruhn *et al.* 2005). Moreover, *L. innocua* overgrowing *L. monocytogenes* during enrichment makes isolation of *L. monocytogenes* difficult (Johansson 1998, Bruhn *et al.* 2005).

One report described two different *L. monocytogenes* strains from a patient with fatal listeriosis, where different PFGE types were found from the blood and meninges (Tham *et al.* 2002). Recovering two different PFGE types from a single blood sample from a listeriosis patient is also possible (Tham *et al.* 2007).

3 AIMS OF THE STUDY

This thesis focused on the use of serotyping and PFGE typing of *L. monocytogenes* isolated from food processing plants and human foodborne infections. The main objectives were the following:

1. to study with serotyping and PFGE typing *L. monocytogenes* contamination in two types of food processing plants: an ice cream plant and two broiler abattoirs (I, II);

2. to characterize with serotyping and PFGE typing isolates from Finnish human invasive *L. monocytogenes* infections from 1990-2001 (III);

3. to study with serotyping and PFGE typing human foodborne *L. monocytogenes* outbreaks of febrile gastroenteritis or listeriosis (IV-V);

4. to evaluate the use of serotyping and PFGE typing in identifying human foodborne *L. monocytogenes* outbreaks (III-V).

4 MATERIALS AND METHODS

4.1 Sampling (I-V)

Sampling for *L. monocytogenes* in study I was carried out in an ice cream factory during the years 1990-1997. During these eight years, a total of 2 544 samples were taken from the production environment and from the ice-cream produced. Of these 2 545 samples, 1 320 were taken from the production environment or equipment and 1 225 from either the non-pasteurised raw materials, such as chocolates or nuts, or the ice cream. Samples from the environment and equipment were taken from the floors and floor drains, the outer and inner surfaces of the whipping, filling, molding and packaging machines, the conveyor belts, and freezing tunnels. With the exception of the floor drains, all sampling sites in the production environment and equipment were swabbed with several cotton swabs or non-toxic sterilized sponges moistened with 0.1% peptone-0.85% saline. After sampling, the swabs or sponges were soaked in 0.1% peptone-0.85 % saline. Samples taken from the floor drain consisted of 100 ml of water taken from the drains before sanitation. Product samples were taken from the finished products and transported frozen to the laboratory. Of the product samples, 39 were pooled from 3 or 5 subsamples.

Sampling in study II was performed at two broiler abattoirs and at two associated broiler processing plants, which were located separately from the abattoirs. The sites were sampled three times in 1996 at either one- or two-week intervals, resulting in 498 samples. The process was assessed throughout, beginning with dirt from the broiler transportation crates and concluding with the ready products: either raw broiler pieces from the abattoirs or raw macerated broiler meat and cooked ready-to-eat broiler products from the broiler processing plants.

Samples from the broiler abattoir environment in study II were taken with gauze sponges moistened with *Listeria* Enrichment Broth (Oxoid, Basingstoke, UK). The area sampled was 20 x 20 cm. When possible, organic material was included in the sample. The gauze sponges were placed into sterile plastic bags. Water samples were collected in sterile plastic jars. Neck skin samples were taken with sterile scissors and put into a sterile bag.

Gloves from workers at the packaging area were rinsed with sterile water into a sterile plastic bag.

Some of the 498 samples in study II were pooled together and a total of 127 analyses were carried out for *L. monocytogenes*. Before analysis, 30 packaged raw broiler meat samples taken from the broiler abattoirs and 25 packaged ready-to-eat broiler product samples taken from the broiler processing plant were kept until the use-by date in order to reveal the prevalence of *Listeria* contamination in them prior to cooking and consumption. Small pieces of the raw or processed broiler products were cut from the surface with sterile scissors to make a 25-g sample.

The retail level was studied in 1997 and 1998 by taking 61 sample pieces of raw broiler from retail stores (II). Analyses for *L. monocytogenes* of the raw broiler pieces in study II began the purchase day for samples which had been sold unpackaged, or on the sell-by date for packaged samples. Each broiler piece sample was placed in a sterile plastic bag, to which was added 50 ml of peptone (0.1%)-saline (0.85%). The broiler part in the plastic bag was then macerated by hand; 25 ml of this peptone-saline solution was then used for the enrichment procedure.

During 1990-2001, clinical microbiology laboratories isolated 314 *L. monocytogenes* strains from human clinical infections and submitted them to KTL (now THL) (Helsinki, Finland) for verification and serotyping of O-antigens (III). Of these isolates, 25 were from outbreak-associated human *L. monocytogenes* infection cases associated with butter, from June 1998 to April 1999 (V).

In 1997, five healthy persons fell ill with gastroenteritis, experiencing nausea, abdominal cramps, and diarrhea within 27 h after eating a meal containing vacuum-packed cold-smoked rainbow trout. Based on patient interviews and a questionnaire, the consumed fish product seemed a very likely vehicle of food poisoning. A vacuum-packed cold-smoked rainbow trout sample from the same production lot was obtained from the same retail store from where the incriminated fish had been purchased. Stool samples were taken on the day after the onset of symptoms. Two additional stool samples were studied from two patients involved in the outbreak of febrile gastroenteritis (IV).

In study V 13 samples of butter from the hospital kitchen and 139 samples of butter from the dairy and wholesale store were analyzed for *L. monocytogenes*, as were an additional 430 environmental samples from the dairy.

4.2 Isolation of *L. monocytogenes* from food processing plants (I-II), stool samples (IV), and food (I-II, IV-V)

Altogether 25 ml of buffer (including the swabs or sponges) and 25 ml of the drain water sample or 25 g of the sample (excluding the environmental gauze sponges, which were not weighed in study II) were put in 225 ml of Listeria Enrichment Broth (Oxoid, Basingstoke, UK or LabM, Bury, UK) and blended (I, II). The isolation method of the Nordic Committee on Food Analysis (NCFA) was followed according to the single stage enrichment procedure (Anonymous 1990) (II). The stool sample swabs were dipped in 9 ml of Listeria Enrichment Broth (IV). The *Listeria* Enrichment Broth was incubated at 30°C for 48 h. In study V, samples of 1 or 25 g of butter were added to half Fraser broth in a proportion of 1:9, and after incubation at 30˚C for 24 h, an aliquot of 0.1 ml was transferred to the Fraser broth and incubated at 37˚C for 24 h according to the ISO method (Anonymous 1996).

After 24-48 h of incubation, the enrichment broth was streaked with a sterile cotton tip onto *Listeria*-selective Palcam agar plates (Merck, Darmstadt, Germany or LabM) (I,V), Oxford plates (Oxoid) (II, IV) or LMBA plates (trypticase soy agar base [Difco, Detroit, Mich.], 10 g/l of lithium chloride, 10 mg/l of polymyxine B sulfate [Sigma Chemicals, St. Louis, Mo.], 20 mg/l of ceftazidime [Abtek Biologicals Ltd, Liverpool, England], 5% sterile defibrinated sheep blood) and incubated for 48 h at 37°C. After 24-48 h of incubation at 37°C, the plates were examined for typical *Listeria* colonies of which one to ten were cultured on horse or sheep blood agar plates, and in study I, on TSA (tryptone soya agar, LabM).

Colonies which were hemolytic on LMBA (V) or blood agar (I, II) or showed a typical bluish sheen on TSA (I) were confirmed by Gram stain, catalase test, and motility at 25°C by growing them overnight in BHI (brain heart infusion) broth (Oxoid) (II). In study I, these colonies were tested for the fermentation of mannitol, salicin, xylose, α-methyl D-mannoside, and rhamnose. In study II, IV, and V, API-*Listeria* kits (Bio-Meriéux; Rhone, France) were used for *Listeria* species identification.

4.3 Serotyping of *L. monocytogenes*

The serotyping scheme followed was that of Seeliger and Höhne (1979). Serotyping was performed using commercial *Listeria* antisera (Denka Seiken, Tokyo, Japan) as described by the manufacturer (Anonymous 1995) (V) or with the modification of incubating the strains at 26°C instead of 30°C in semi-liquid 0.2 % BHI agar in Craigie's tubes before determining the H-antigens (I-V) or when determining the O-antigens by heating the bacterial suspension in NaCl at 100°C for 1 h, instead of 121°C for 30 min (III). All isolates were serotyped in studies I and III-V, and one isolate representing each PFGE type was serotyped in study II.

4.4 PFGE typing of *L. monocytogenes*

4.4.1 DNA isolation (I-V)

In studies I-III, pure cultures were grown on horse blood agars for 24 h at 37°C. A single colony was inoculated into 5 ml of BHI broth (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. DNA isolation was performed as described by Maslow *et al.* (1993) with the modifications described by Björkroth *et al.* (1996). Briefly, cells from overnight BHI broth (Oxoid) cultures were mixed with an equal volume of 1.2% (V) or 2% (I-IV) (w/v) low melting temperature agarose (InCert agarose (I-IV) or Sea Plaque (V), FMC Bioproducts, Rockland, ME, USA). Instead of using insert molds, as in study V, GelSyringe dispensers (New England Biolabs, Beverly, Mass., USA) were used according to the manufacturer's instructions (I-IV). In order to obtain complete cell lysis 10 U/ml of mutanolysin (Sigma, St. Louis, USA) were added to the lysing solution (I-IV). The inactivation of proteinase K (Sigma) and restriction endonuclease digestion of the agarose-embedded DNA was performed as described by New England Biolabs.

4.4.2 Macrorestriction analysis with PFGE (I-V)

Three rare-cutting enzymes, *Apa*I (I, II, V), *Asc*I (I-V) and *Sma*I (I, III) (New England Biolabs, Beverly, USA), previously found suitable for *L. monocytogenes* characterization

were used for cleaving of the DNA (Brosch *et al.* 1991, Buchrieser *et al.* 1991, Howard *et al.* 1992, Buchrieser *et al.* 1993, Brosch *et al.* 1994, Moore and Datta 1994 and Brosch *et al.* 1996). The samples were electrophoresed through 1.0% (I-IV) or 1.2% (w/v) (IV) agarose gel (SeaKem Gold, FMC Bioproducts) (I-IV) or through Pronadisa D-5 (Hispanlab, Madrid, Spain) (V) in 0.5 x TBE (45 mM Tris, 4.5 mM boric acid, pH 8.3, and 1 mM sodium EDTA) at 200 V at 10ºC using a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden) (I-IV) or a CHEF-DR III Variable Angle Pulsed-field Electrophoresis System (Bio-Rad Laboratories, Richmond, CA, USA) at a 120º angle (IV). In study V, CHEF Mapper™ or CHEF-DR systems (Bio-Rad Laboratories) were used for electrophoreses. *Apa*I and *Asc*I restriction fragments were resolved with pulse times linearly ramping from 0.5 to 29.5 s over 20 h (I, II, IV) or from 1 to 35 s over 18 h (III) or, as in study IV, *Asc*I 1 to 35 s for 18 h and *Apa*I 1 to 15 s for 18 h, and in study V, *Asc*I 1 to 28 s for 10 h followed by 28 to 38 s for 10 h. *Sma*I digests were linearly ramped from 0.5 to 18 s over 20 h (I) or from 1 to 18 s over 18 h (III), respectively. Mid-Range PFG marker I (I, II, III, IV), Lambda Ladder PFG marker (I, II, IV), and Low-range PFG marker (III-V) (New England Biolabs) were used as fragment size markers.

4.5 Numerical analysis of macromolecular banding patterns (V)

The PFGE gels in study V were photographed with AlphaImager™ 1220 (Alpha Innotec, San Leandro, CA) after visualization on a UV transilluminator. The tiff images were analyzed using BioNumerics software (Applied Maths, Kortrijk, Belgium) and normalized by using the Low-range PFG marker standards on each gel. The similarity between all pattern pairs was calculated with the Dice coefficent converted for convenience to a percentage value. UPGMA clustering was used for the construction of the dendrogram. One-band differences were considered sufficient to distinguish different PFGE types.

5 RESULTS

5.1 *L. monocytogenes* contamination in an ice cream plant (I)

The overall prevalence of *L. monocytogenes* in the ice cream plant environment, equipment, raw material, unfinished product, and ice cream samples during 1990-1997 was 2.8% (71/2 545). The 41 strains available for typing were divided into two serotypes: 1/2b (37 strains) and 4b (4 strains) (Table 5). The flagellar H-antigens of one serotype 1/2 could not be determined because this strain was immotile. Six REDPs were generated with restriction enzyme *Apa*I, whereas *Asc*I and *Sma*I generated eight and seven, respectively. When combining these results, 12 different PFGE types were distinguished. A single *L. monocytogenes* strain of serotype 1/2b of PFGE type II dominated, since 63% (26/41) were of this type. This strain was found on different equipment, especially the packaging machine, in the environment and in the ice cream. The 37 strains of serotype 1/2b were divided into 11 PFGE types, showing differences of one to five bands, and seemed to represent the same clonal lineage.

5.2 *L. monocytogenes* contamination at two broiler abattoirs and prevalence in retail raw broiler meat (II)

The level of *L. monocytogenes* contamination in the environment and products at two broiler abattoirs (X and Y) as well as at the adjoining processing plants varied from 1 to 11%. Sampling sites that tested positive for *L. monocytogenes* include the air chiller, the skin removing machine (in both broiler abattoirs), the conveyor belt leading to the packaging area, and the packaged raw broiler itself. The raw macerated broiler mass tested positive for *L. monocytogenes* in one broiler processing plant.

Of the raw broiler meat samples (legs, drumsticks, breast and wings) that originated from three different poultry abattoirs (X, Y and Z), 62% tested positive for *L. monocytogenes* at the retail level. The prevalence of *L. monocytogenes* varied depending on the poultry abattoir from which it originated from: poultry abattoir X 56% (5/9), Y 78% (27/36) and Z

TABLE 5. Results from serotyping and PFGE typing in studies I-V.

Study	Serotype	Number of REDP			Number of PFGE types /serotype
		*Apa*I	*Asc*I	*Sma*I	
I Ice cream plant	1/2b	5	7	6	10
	1/2*	1	1	1	1
	4b	1	1	1	1
II Broiler processing	1/2a	9	10	ND	10
	1/2c	2	2	ND	3
	4b	1	1	ND	1
III Human invasive listeriosis	1/2a	ND	49[a]	ND	49
1990-2001	1/2b	ND	10	ND	10
	1/2c	ND	2	ND	2
	3a	ND	5[b]	ND	5
	4b	ND	18	ND	18
IV Febrile gastroenteritis from vacuum-packed cold-smoked rainbow trout	1/2a	ND	1[a]	1	1
V Butter outbreak	3a	1	1[b]	ND	1

* H-antigens could not be determined due to poor growth in the BHI motility agar

ND = not done

[a] One identical *Asc*I REDP in studies III and IV

[b] One identical *Asc*I REDP in studies III and V

31% (5/16). The overall prevalence for *L. monocytogenes* in raw broiler at the retail level meat was 62 %.

One hundred strains were further characterized with serotyping and PFGE typing. Restriction enzymes *Apa*I and *Asc*I were used in the PFGE typing of these strains. *Apa*I yielded 12 unique patterns, and *Asc*I, 13 patterns. When combining these patterns, we obtained 14 different PFGE types (Table 5). One strain from every PFGE type was serotyped, and three different serotypes were predicted based upon these results: 1/2a (65%), 1/2c (25%) and 4b (10%).

Altogether six different PFGE types were found in these two broiler abattoirs. The same PFGE type of serotype 1/2a was found in abattoir X in the skin-removing machine, and two years later in raw broiler meat originating from this broiler abattoir at retail level. Three closely related PFGE types all of serotype 1/2a were also found in these raw broiler meat samples.

Five different PFGE types were found in poultry abattoir Y and in macerated raw broiler meat mass in the adjoining poultry product plant. Three of these PFGE types (VIIa, VIIb, and VII), two of which were of serotype 1/2c and one of serotype 4b, could still be found one year later in raw broiler meat originating from this poultry abattoir. Both PFGE types, one of serotype 1/2c and the other of serotype 4b, found on the air chiller in poultry abattoir Y were also found in the raw poultry meat mass, and still one year later in raw broiler meat at the retail level. PFGE type VIIa of serotype 1/2c found on the skin removing machine was also found in packaged raw broiler meat, raw macerated poultry meat mass in the broiler product plant, and in raw poultry meat at the retail level even one year later.

5.3 Variation of serotypes and genotypes of human *L. monocytogenes* invasive infections in Finland during 1990-2001 (III)

Among 314 human isolates from invasive *L. monocytogenes* infections during 1990-2001 in Finland were found 5 different serotypes. Serotype 1/2a was the most common (53%), followed by serotypes 4b (27%), 3a (11%), 1/2b (6%), and 1/2c (3%). Strains of serotype 3a have been rare (except in 1997-1999) which can be attributed, at least in part, to the butter outbreak. The most common serotype has changed every year from 1990, when 61% (11/18) of the strains were of serotype 4b to 2001 when 67% (18/21) were of serotype 1/2a.

From the 314 clinical isolates with restriction enzyme *Asc*I, we distinguished 81 different PFGE types. Of these PFGE types, 28 were found on only one occasion. Each PFGE type was associated with one serotype, except for three PFGE types which were of either serotype 1/2a or 3a. The five most common PFGE types accounted for 42% of the strains. Only 11 of the 81 PFGE types contained five or more strains. Of these 11 PFGE types, 8 had closely related PFGE types among other PFGE types, and groups could be formed. The most common group included seven closely related PFGE types of serotype 1/2a and accounted for 23 % (71/314) of the strains. This group has been detected in cluster analysis every year since 1993. One specific PFGE type 1 (37/314 isolates) from this group has caused since 1994 2 to 11 listeriosis cases per year. This PFGE type 1 was identical to the strain that caused an oubreak of febrile gastroenteritis (IV).

5.4 Human febrile *L. monocytogenes* gastroenteritis outbreak caused by vacuum-packed cold-smoked rainbow trout (IV) and listeriosis caused by butter (V)

The vacuum-packed cold-smoked rainbow trout sample from the same incriminated production lot was tested positive for *L. monocytogenes* by selective enrichment and quantification. The sample contained 1.9×10^5 cfu/g of *L. monocytogenes.*

Stool samples taken on the day after the onset of symptoms and analyzed for *Salmonella, Shigella, Campylobacter* and *Yersinia* tested negative. Two additional stool swabs obtained from two patients were analyzed for *L. monocytogenes* by selective enrichment; both tested positive. Serotyping and PFGE typing was performed for isolates from the fish product and the two stool samples. These isolates were of serotype 1/2a and had REDPs identical to those of with restriction enzymes *Asc*I and *Sma*I.

An outbreak of human listeriosis was associated with a *L. monocytogenes* strain of serotype 3a in butter. This outbreak was initially detected as part of study III. At the time of the outbreak, the strains were serotyped for O-antigens at the Laboratory of Enteric Pathogens at KTL after obtaining them from the clinical laboratories. PFGE typing and complete serotyping for H-antigens was then performed at the Department of Food and Environmental Hygiene. PFGE typing showed that the REDPs were identical. These findings initiated epidemiological studies, which revealed the presence of an outbreak. The same strain, as identified by the PFGE of restriction enzymes *Apa*I and *Asc*I, was identified not only in 13

butter samples served at a hospital where 15 outbreak-associated patients had stayed, but also in the dairy, that manufactured the implicated butter. This same strain was also detected in several lots of 7-, 10-, and 500-g packages of butter from the dairy and the wholesaler. In the dairy environment, the strain was isolated from the packing machines for both small and 500-g packages, the screw conveyor of the butter wagon, and from two floor drains beneath the butter wagon of the small packaging line.

6 DISCUSSION

6.1 *Listeria monocytogenes* contamination in an ice cream plant (I)

One dominant *L. monocytogenes* strain of serotype 1/2b and of identical PFGE type was found in the environment, on the equipment, and in the ice cream of an ice cream plant. This strain had persisted in this plant for at least seven years and had probably adapted to the processing environment or the environment was suitable for survival and growth. Similar strains with a one- or two-band difference were encountered, suggesting that these are possibly clonally related. None of these similar strains persisted in the processing environment. One other strain of a different serotype 4b was found three times in four years. A Swedish study described the persistence of a strain of serotype 3b for seven years in the environment of a dairy plant that manufactured blue-veined cheese was (Unnerstad *et al.* 1996).

6.2 *L. monocytogenes* contamination at two broiler abattoirs and prevalence at retail level in raw broiler meat (II)

Of the samples taken from two broiler abattoirs and from broiler product processing plants, 1-11% tested positive for *L. monocytogenes*. This is in line with the results of a Danish study in which 0.3-18.8% tested positive for *L. monocytogenes* (Ojeniyi *et al.* 1996). In France, researchers found that 18% of samples taken at a poultry processing plant tested positive for *L. monocytogenes* (Chasseignaux *et al.* 2001), as did 24% of samples during processing at a turkey abattoir in Denmark (Ojeniyi *et al.* 2000). In our study, contamination of the broiler carcasses in the broiler abattoirs probably occurred during or after the chilling step in the skin-removing machine, since this was the first site in the broiler slaughtering process where *L. monocytogenes* was found; a similar finding from a turkey abattoir suggests the same (Clouser *et al.* 1995). In the Danish turkey processing study, the same PFGE types were found in all parts of the processing line as were found in raw and ready-to-eat, heat-treated turkey meat. The researchers had found no *L. monocytogenes* onthe turkey farms from which the slaughtered birds originated and concluded that the turkey meat became contaminated during processing, even though they could not pinpoint the exact location

(Ojeniyi *et al.* 2000). According to a Danish study in which 2% (1/48) of fecal droppings tested positive (Petersen and Madsen 2000), housed broilers may carry *L. monocytogenes* in their feces. In a survey in Spain, 27% of free-range poultry farms tested positive for *L. monocytogenes* when analyses of poultry droppings showed that even free-range poultry could play a role in bringing *L. monocytogenes* into poultry abattoirs (Esteban *et al.* 2008). Still, special attention should be focus on cleaning in broiler abattoirs to pinpoint contamination steps such as air chillers, skin removing machines, and conveyor belts.

PFGE typing found that the same PFGE type present in the skin-removing machine was present in packaged raw broiler meat at the retail level two years later. This finding suggests that this strain may have survived at the broiler abattoir for at least two years, during which time it continued to contaminate broiler meat. Another PFGE type was recovered from the air-chiller at the other broiler abattoir and, one year later was the most common PFGE type in raw broiler meat at the retail level from this broiler abattoir. One study that used PFGE with restriction enzyme *Asc*I to compare *L. monocytogenes* isolates from Estonian, Danish, Hungarian, Finnish, and Swedish poultry meat found identical PFGE types (Praakle-Amin *et al.* 2006). This could mean that some strains may have special characteristics enabling them to survive in poultry processing plants.

The prevalence of *L. monocytogenes* in raw broiler meat at the retail level was surprisingly high (62%). This may be due to cross-contamination at the retail level, since the majority were bought unpackaged on display. Because raw broiler meat may be contaminated with *L. monocytogenes,* it is important to realize that when using it as a raw material in broiler product processing plants, raw broiler meat may be a source of post-processing contamination. Special care is needed at the home and retail levels to avoid the cross-contamination of *L. monocytogenes* from raw broiler meat to other foodstuffs, especially ready-to-eat foods.

The most common predicted serotype was 1/2c (65%), followed by 1/2a (25%) and 4b (10%), and all serotypes were found in broiler meat at the retail level in 1997. A Finnish study of marinated broiler legs from retail stores in 2002-2003 found these same serotypes (Aarnisalo *et al.* 2008). Unfortunately, the study did not report the exact serotype distribution. This pattern differs from findings in poultry in the USA, where 1/2b (64%) was the most common serotype, followed by 1/2c (18%), 3b (6%) and incomplete (12%) (Bailey *et al.*

1989). In Denmark, the serotype distribution in poultry was 98% for serotype 1 and only 2% for serotype 4 (Ojeniyi *et al.* 1996). In Estonia, 91% (65/71) of broiler strains were of serotype 1/2a, 7% (5/71) of serotype 1/2b, and only 1% (1/71) of serotype 4b (Praakle-Amin *et al.* 2006). In a Spanish survey, the serotype distribution in raw poultry meat at retail was 37% for 1/2b (21/57), 26% for 1/2a (15/57), 16% for 1/2c (9/57) and 21% for 4b or 4bx (12/57) (Vitas *et al.* 2004). At one Spanish abattoir, only serotypes 1/2b (89%) and 1/2a (11%) were found (Lopez *et al.* 2008a).

6.3 Variation of serotypes and genotypes of human *L. monocytogenes* invasive infections in Finland during 1990-2001 (III)

The most common serotype from 1990-2001 in human invasive *L. monocytogenes* infections in Finland was 1/2a, which accounted for 53% of the isolates studied. The second most common serotype was 4b, which accounted for 27%. The percentage of serotype 1/2a increased from 22% in 1990 to 67% in 2001, and that of serotype 4b decreased from 61% in 1991 to 26% in 2001. This change in serotype distribution is similar to that in Switzerland where the most common serogroup (60%) since 1994 has been 1/2 rather than serogroup 4 (40%) (Pak *et al.* 2002). Similar results reported from Sweden, the UK, Denmark, Iceland and Canada suggest that in many countries serotype 1/2a is replacing 4b in human infections (McLauchlin and Newton 1995, Gerner-Smidt *et al.* 1995, Loncarevic *et al.* 1997, Hjaltested *et al.* 2002, Parihar *et al.* 2008b, Clark *et al.* 2009). In 1994-2003 in Denmark, 59% belonged to serogroup 1/2 and 40% to serogroup 4 (Gerner-Smidt, 2005). One explanation for this serotype shift could be that serotype 1/2a and 1/2b infections are more common in blood stream infections than in meningoencephalitis, and the likelihood of detecting a blood stream infection has increased, because blood culturing systems have become more sensitive, and the indications for drawing a blood culture have become broader (Swaminathan and Gerner-Smidt 2007). However, in 1976-1995 in the Netherlands, 65% of human listeriosis cases were of serotype 4b (Aouaj *et al.* 2002), and in 1997-2000 in Austria, 61% of 41 human listeriosis infections were caused by serotype 4b, and 29% by serotype 1/2a (Wagner and Allerberger 2003). Moreover, in 2001 in Belgium, 54% of 48 strains were of serotype 4b, and 37,5 % of serotype 1/2a (Yde and Genicot 2004).

From November 1996 to June 2000 in the New York, USA, 74 PFGE types with restriction enzyme *Asc*I were detected in 131 human listeriosis isolates (Sauders *et al.* 2003). Of these, each of 50 PFGE types was found only once. The authors concluded, that while most human listeriosis cases were considered sporadic, PFGE typing indicated that 13 to 31% of cases may have represented single-source clusters. In 2000 and 2001, 42 cases of human listeriosis were recorded in Maryland and California in the USA (Gilbreth *et al.* 2005). Of these 42 strains, 35 different PFGE types were found with restriction enzyme *Asc*I. From these PFGE types 13 were identical to just a few of the PFGE types found in ready-to-eat foods in the same time period and area. In our study, 65% (53/81) of the different PFGE types distinguished from the 314 clinical isolates with restriction enzyme *Asc*I were encountered more than once.

The most common PFGE type (37/314 isolates, 12%) of serotype 1/2a was identical to the strain that caused an outbreak of febrile gastroenteritis in study II. This strain was recovered during 1994-2001 from 2 to 11 human invasive *L. monocytogenes* infection cases annually. A similar phenomenon occured in New Zealand, where 30% of human invasive *L. monocytogenes* cases were caused by the same PFGE type that caused a series of incidents of non-invasive gastroenteritis from ready-to-eat meats (Sim *et al.* 2002).

6.4 Human outbreaks of febrile *L. monocytogenes* gastroenteritis caused by vacuum-packed cold-smoked rainbow trout (IV) and listeriosis from butter (V)

Isolates of serotype 1/2a and those sharing identical PFGE types were recovered from two patient stool samples as well as from a vacuum-packed cold-smoked rainbow trout from the same production lot as the incriminated fish product. If the same *L. monocytogenes* enrichment method which was used for the fish product had not been used for the stool samples, the diagnosis would have been missed. This outbreak investigation added further evidence for previous findings that *L. monocytogenes* may cause non-invasive febrile gastroenteritis in previously healthy persons (Table 4).

An outbreak among 25 patients was traced to butter containing *L. monocytogenes* of serotype 3a. This was the first time this serotype had caused an outbreak, and the vehicle of infection was also unusual. The use of the two restriction enzymes with PFGE failed to

distinguish the outbreak strain. The rare serotype and the clustering of cases in a patient population spending long periods together at a hospital with an ongoing PFGE typing project of human listeriosis cases facilitated the recognition of the outbreak. This was the first time a listeriosis epidemic was identified and confirmed using genotyping methods.

6.5 Evaluation of the use of serotyping and PFGE typing for *L. monocytogenes* in food processing and human infections

Serotyping is a basic phenotypical typing method with limited discrimination power, but it enables rough comparison between strains in different laboratories without the need to exchange strains or electronic data. Serotyping requires the use of expensive antisera, is fairly cumbersome, and the interpretation of agglutination reactions can occasionally be a challenge. Still, in combination with a genotyping method such as PFGE, serotyping yields valuable information, since strains of identical REDPs are sometimes of different serotype (Chou and Wang 2006).

Overall, the restriction enzyme *Asc*I created REDPs, which were the easiest to compare visually. If only one instead of three restriction enzymes had been used different in study I, only six to eight different PFGE types would have been encountered instead of twelve. The combination of any of these two restriction enzymes would have created between nine and twelve PFGE types. If only one of these two restriction enzymes had been used in study II in PFGE typing, twelve or thirteen PFGE types would have resulted instead of fourteen. Combining these results with the serotyping results achieved good discrimination between these isolates.

PFGE in combination with serotyping proved to be very useful tools in the two foodborne outbreak investigations (IV-V). For regular human listeriosis PFGE typing surveillance, the use of one restriction enzyme is sufficient, but in the case of contamination studies or outbreaks, the use of at least two restriction enzymes is recommended in order to increase the discrimination power of the method (Barret *et al* 2006). Another approach would be to use, in addition to PFGE, serotyping and/or another genotyping method, such as ribotyping, RAPD or AFLP, in order to raise more confidence in the typing results. Corcoran

et al. (2006) also supported this approach of combining typing methods in typing *L. monocytogenes*.

7 CONCLUSIONS

1. PFGE typing and serotyping proved to be very useful methods in two food processing contamination studies, thus enabling the identification of potentially important contamination sites and possible prolonged contamination. The overall prevalence of *L. monocytogenes* isolated from all samples taken from the production environment, processing equipment or ice cream of an ice-cream plant during 1990-1997 was 2.8%. Two serotypes were detected (1/2b and 4b) and twelve different PFGE types were created using three restriction enzymes (*Apa*I, *Asc*I and *Sma*I). One PFGE type of serotype 1/2b was the most common PFGE type 63% (26/41) as well as the only PFGE type found in the production environment, processing equipment - especially on the packaging equipment – and in the ice cream. This persistent strain had survived in this ice cream plant for at least seven years.

The overall prevalence of *L. monocytogenes* at two broiler abattoirs and processing plants varied from 1 to 11%. The prevalence of *L. monocytogenes* in raw broilers at the retail level was 62%. Three serotypes were detected (1/2a, 1/2c and 4b) and fourteen PFGE types were created with two restriction enzymes (*Apa*I and *Asc*I). The sampling sites that tested positive for *L. monocytogenes* at the broiler abattoirs were the air chiller, the skin removing machine and the conveyor belt leading meat to the packaging area. The skin removing machine was a sampling site that tested positive at both broiler abattoirs studied, indicating an important contamination point of *L. monocytogenes.* Four different PFGE types of serotypes 1/2a, 1/2c, and 4b that may have persisted for at least one or two years in the broiler abattoirs were identified.

2. PFGE typing in combination with serotyping proved very useful in characterizing *L. monocytogenes* strains yhat cause human invasive infections in Finland. Human *L. monocytogenes* infections from 1990-2001 in Finland were caused by the following five serotypes: 1/2a (165 isolates, 53%), 4b (85 isolates, 27%), 3a (36 isolates, 11%), 1/2b (18 isolates, 6%), and 1/2c (10 isolates, 3%). These serotypes were further divided with PFGE employing restriction enzyme *Asc*I into 82 PFGE types. The most common PFGE type (37/314 isolates, 12%) of serotype 1/2a was identical to the strain that caused an oubreak of febrile gastroenteritis from vacuum-packed cold-smoked rainbow trout in study II. This strain was recovered during 1994-2001 from two to eleven human invasive *L. monocytogenes*

infection cases annually. The second most common PFGE type (32/314 isolates, 11%) of serotype 3a was identical to the strain that caused an outbreak of human listeriosis from butter. The percentage of serotype 1/2a increased from 22% in 1990 to 67% in 2001, and serotype 4b decreased from 61% in 1991 to 26% in 2001.

3. PFGE typing in combination with serotyping proved successful in human foodboorne outbreak investigations because the food vehicle could be identified. In fact, the human listeriosis outbreak from butter was initially identified using these methods. *L. monocytogenes* of serotype 1/2a in vacuum-packed cold-smoked rainbow trout was found to be the likely causative agent in an outbreak of human febrile gastroenteritis. Indistinguishable REDPs were created with restriction enzymes *Asc*I and *Sma*I for *L. monocytogenes* isolates from patients and fish from the same production lot that the patients had eaten.

L. monocytogenes of serotype 3a in butter was the causative agent in a listeriosis outbreak, which was identified due to the PFGE typing of human listeriosis strains. Identical REDPs were created with *Asc*I and *Apa*I for *L. monocytogenes* isolates from patients and butter of the same brand that the patients had eaten, as well as from the butter production environment, the packing machines, the screw conveyor of the butter wagon, and from the floor drains.

4. For regular typing surveillance of *L. monocytogenes* with PFGE, the use of one restriction enzyme is sufficient, but in the case of outbreaks, the use of at least two restriction enzymes is recommended in order to increase the discrimination power of the method. Another approach would be to use serotyping and/or another genotyping method, such as ribotyping or AFLP, to yield greater discrimination typing power.

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