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Listeria monocytogenes with Special Reference to Food Products and Human Listeriosis

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av

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, SLU, Uppsala, fredagen den 29 maj, 1998, kl 9.15

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Abstract

Listeria monocytogenes has been the cause of numerous sporadic cases and outbreaks of human listeriosis worldwide. In most of these cases the source and route of infection were unknown. Foodborne transmission of listeriosis, however, is a common mode of infection. In **Papers I** and **II** we show that *L. monocytogenes* often could be isolated from soft cheeses, especially those made of raw milk, as well as from vacuum-packed smoked and gravad salmon and rainbow trout. Serotyping has shown that the majority of the isolates belong to serogroup 1/2. A change in serogroup distribution among Swedish human isolates has been observed. Serogroup 4 was predominant until 1987, and after that it was counterbalanced with serogroup 1/2. Changes in eating habits are a possible explanation of this alteration. Serotyping of isolates is not sufficient to estimate a suspected source of human listeriosis. Application of more discriminating methods such as molecular typing methods must be used. In **Paper III**, using pulsed-field gel electrophoresis (PFGE), we show that the same food product may harbour more than one *L. monocytogenes* clonal type. It is recommended to isolate several colonies from both the direct plating and enrichment procedures. Practical application of the approach mentioned has been applied in tracing a source of human listeriosis in **Paper IV**. Among 23 food items collected from a patient's refrigerator, two harboured *L. monocytogenes*. The isolated strains belonged to the same serovar. By use of PFGE, medwurst was found to be the source of illness. This is the first reported foodborne case of listeriosis in Sweden. To find out if two groups of frequently contaminated ready-to-eat food products, soft cheeses and gravad and cold smoked salmon and rainbow trout, are potential risk foods in Sweden, PFGE was used in **Paper V**. An association between human cases of listeriosis and consumption of salmon and rainbow trout is presented. However, a link to soft cheese was not found.

In **conclusion**, raw milk soft cheeses, gravad and cold smoked salmon and rainbow trout were often contaminated with *L. monocytogenes* and thus potential risk foods, especially for immuno-compromised persons, including pregnant women. In the case of routine investigation of food products, an enrichment method and characterization of one isolate could be sufficient. In investigating outbreaks and sporadic cases it might be necessary to use two different isolation methods and to characterize several isolates with more than one typing method, where at least one should be a molecular typing method. PFGE was shown to be a highly discriminative method for this use.

Key words: *Listeria monocytogenes*, food, human listeriosis, serotyping, phage-typing, PFGE.

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Are we aware of the methods leading to the results we are looking at? And this question has to be asked not only in the scientific field but also in daily life. If we are not aware, we could draw the wrong conclusions out of results, which are not wrong according to the method used, but don't show the whole reality. Therefore our picture of the world should be regarded as restricted and we always have to prove whether it is necessary to update this picture.

Dr. Christian Baumgartner
Geschäftsführer

To my parents and

Jasa, Nera & Ada

Abstract

Loncarevic, S. 1998. *Listeria monocytogenes with special reference to food products and human listeriosis.* Doctor's dissertation.
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Listeria monocytogenes has been the cause of numerous sporadic cases and outbreaks of human listeriosis worldwide. In most of these cases the source and route of infection were unknown. Foodborne transmission of listeriosis, however, is a common mode of infection. *L. monocytogenes* has also been isolated from various food items; fruits, vegetables, meat and meat products, milk and dairy products. In **Papers I** and **II** we show that *L. monocytogenes* often could be isolated from soft cheeses, especially those made of raw milk, as well as from smoked and gravad salmon and rainbow trout. Serotyping, as the most utilized characterization method for *Listeria*, has shown that the majority of investigated food isolates in Sweden belong to serogroup 1/2, which corresponds with findings in other countries. However, among Swedish human isolates, a change in serogroup distribution has been observed. Serogroup 4 was predominant until 1987, and after that it was counterbalanced with serogroup 1/2. Changes in eating habits are a possible explanation of this alteration. Serotyping of isolates is important, but not sufficient, to estimate a suspected source of human cases of listeriosis. Therefore, application of other, more discriminating methods such as molecular typing methods may yield additional information. In **Paper III**, using pulsed-field gel electrophoresis (PFGE), we show that the same food product may harbour more than one *L. monocytogenes* DNA clonal type. The direct plating method revealed more clonal types from the same food than an enrichment procedure. It is also recommended to isolate several colonies from both the direct plating and enrichment procedure. Practical application of the approach mentioned has been applied in tracing a source of human listeriosis in **Paper IV**. Among 23 food items collected from a patient's refrigerator, two harboured *L. monocytogenes*. The isolated strains belonged to the same serovar. However, by use of the PFGE method, medwurst was found to be the source of illness. This is the first reported foodborne case of listeriosis in Sweden. To find out if two groups of frequently contaminated ready-to-eat food products, soft cheeses and gravad and cold smoked salmon and rainbow trout, are potential risk foods in Sweden, the PFGE method was applied in **Paper V**. An association between human cases of listeriosis and consumption of salmon and rainbow trout is presented. However, a link to soft cheese was not found.

In **conclusion**, soft cheeses and gravad and cold smoked salmon and rainbow trout were often contaminated with *L. monocytogenes* and thus potential risk foods, especially for immunocompromised persons, including pregnant women. One of the first problems when investigating a route of contamination or infection is to isolate *L. monocytogenes*. Another problem is to apply adequate bacterial typing methods to obtain sufficient information about the source. In the case of routine investigation of food products for the presence of *L. monocytogenes*, an enrichment method and characterization of one isolate could be sufficient. In investigating outbreaks and sporadic cases it might be necessary to use two different isolation methods and to characterize several isolates with more than one typing method where at least one should be a molecular typing method. PFGE was shown to be a highly discriminative method for investigation of human listeriosis and in determination of risk foods for humans.

Preface

The thesis is based on the following papers which will be referred to in the text by their Roman numerals:

I. Loncarevic, S., M.-L. Danielsson-Tham and W. Tham. 1995: Occurrence of *Listeria monocytogenes* in soft and semi-soft cheeses in retail outlets in Sweden. International Journal of Food Microbiology, 26: 245-250.

II. Loncarevic, S., W. Tham and M.-L. Danielsson-Tham. 1996: Prevalence of *Listeria monocytogenes* and other *Listeria* spp. in smoked and "gravad" fish. Acta Veterinaria Scandinavica, 37: 13-18.

III. Loncarevic, S., W. Tham and M.-L. Danielsson-Tham. 1996: The clones of *Listeria monocytogenes* detected in food depend on the method used. Letters in Applied Microbiology, 22: 381-384.

IV. Loncarevic, S., M.-L. Danielsson-Tham, L. Mårtensson, Å. Ringnér, A. Runehagen and W. Tham. 1997: A case of foodborne listeriosis in Sweden. Letters in Applied Microbiology, 24: 65-68.

V. Loncarevic, S., M.-L. Danielsson-Tham, P. Gerner-Smidt, L. Sahlström and W. Tham. 1998: Potential sources of human listeriosis in Sweden. Food Microbiology, 15: 65-69.

Contents

Abbreviations

CAMP	Christie Atkins Munch-Peterson
CFU	colony forming units
DNA	deoxyribonucleic acid
DPP	direct plating procedure
ELISA	enzyme linked immunosorbent assay
FDA	Food and Drug Administration
IDF	International Milk and Dairy Federation
HACCP	hazards analyses and critical control points
L.	*Listeria*
LEB	*Listeria* enrichment broth
LPM	lithium chloride-phenylethanol-moxalactam
LSM	*Listeria* selective medium
MEE	multilocus enzyme electrophoresis
MMA	modified McBride agar
MOX	modified Oxford agar
NaCl	sodium chloride
NMKL	Nordic Committee on Food Analysis
OXA	Oxford agar
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	random amplification of polymorphic DNA
REA	restriction enzyme analysis
REDP	restriction endonuclease digestion profile
RNA	ribonucleic acid
SSCP	PCR-single-strand confirmation polymorphisms
USDA	United States Department of Agriculture

History

The first description of a bacterium causing necrotic spots on rabbit liver dates back to 1911, when Hülphers (1911) named the organism *bacillus hepatis*. Fifteen years later, Murray, Webb and Swann (1926) found a bacterium which seemed to be identical with *b. hepatis*. They gave a detailed description of the bacterium, that caused typical monocytosis in rabbits and guinea pigs and named it *Bacterium monocytogenes*. Pirie (1927) isolated the same bacterium from gerbilles (*Tatera lobengulae*) independently of previous investigations. He named it *Listerella hepatolytica* in honour of Sir Joseph Lister, an English surgeon and Sir Spencer Lister, his chief. By combination of the two latter bacterial names, a new name was established; *Listerella monocytogenes*. However, Pirie (1940) later changed the name into *Listeria monocytogenes*, and finally, under the same name, this bacteria was inserted in 1948 in the 6th edition of Bergey's Manual of Determinative Bacteriology (Breed et al., 1948). The first reported isolation from sheep was done in 1929 by Gill (1933) and from a human case of listeriosis in 1929 by Nyfeldt (1929).

Taxonomy

Based on similarities with lactic acid bacteria, the genus *Listeria* is placed into Section 14 in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986) in the family *Corynebacteriaceae*.

Until 1961, *L. monocytogenes* was the only known species of the *Listeria* genus. *L. denitrificans* was added to the genus in 1961 followed by *L. grayi* and *L. murrayi* in 1966 and 1971, respectively. All three species are capable of reducing nitrate. The difference between them is that *L. grayi* and *L. murrayi* are capable of fermenting mannitol while *L. denitrificans* is not. *L. denitrificans* was transferred to a new genus, *Jonesia,* in 1992 and the name was consequently changed to *Jonesia denitrificans* (Jones, 1992). This alteration was due to difference in moles % G + C content of the DNA (56%) related to *L. monocytogenes* (37 to 39%) (Stuart and Welshimer, 1974). *L. grayi* and *L. murrayi* were, on the basis of taxonomic studies including DNA composition and DNA-DNA hybridization (Stuart and Welshimer, 1974), first moved to the new genus *Murraya* as *M. grayi* subspecies *grayi* and *M. grayi* subspecies *murrayi*. Afterwards, the study of amino acid sequences of the cell wall showed that these subspecies have the same murein variation as *L. monocytogenes*. Thus, the two species were put back into genus *Listeria*. However, Rocourt et al. (1992) suggested that *L. grayi* and *L. murrayi* were brought together in one species; *L. grayi*. Seeliger introduced a new *Listeria* species in 1977. This species is characterized by not hemolysing erythrocytes, having no pathogenicity for

animals and human, no reactions in the CAMP test and, compared with *L. monocytogenes*, is serologically different. This new species took a place in genus *Listeria* as *L. innocua* (Seeliger, 1981). Other species that were described and added to the genus *Listeria* were *L. welshimeri*, *L. seeligeri* (Rocourt and Grimont, 1983) and *L. ivanovii* (First description by Ivanov, 1962; introduced as a genus by Seeliger et al., 1984). An analysis of 23 strains of *L. ivanovii* with multilocus enzyme electrophoresis resulted in separation of this species into two different genomic groups; *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*. DNA-DNA hybridization confirmed these findings (Boerlin et al., 1992).

Altogether, genus *Listeria* today includes six species, *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*.

Morphology

Listeria bacteria are aerobic and facultatively anaerobic, non spore- and non capsule-forming, regular, short rods with rounded ends (Seeliger and Jones, 1986). The rods are 0.4-0.5 µm in diameter and 0.5-2 µm in length, appearing singly, in short chains, or in V or Y forms. Gram positive characteristics could be lost, particularly in older cultures, and cells are stained Gram negative. A few peritrichous flagella are responsible for the typical tumbling motility, which is most prominent in the temperature range 20-25°C. On nutrition agar plate, after 24 hours of incubation, *Listeria* colonies are round, low convex, 0.5-1.5 mm in diameter with a smooth, glistening surface (S-forms). During prolonged incubation for 3-7 days they may alter into rough colonies (R-forms) and 3-5 mm in size. The bluish grey colour of colonies under normal illumination appears as a blue-green sheen under obliquely transmitted light. Regarding haemolysis on sheep blood agar, a narrow zone of ß-haemolysis around colonies characterizes *L. monocytogenes*, weak ß-hemolysis is characteristic of *L. seeligeri*, and wide or multiple zones of ß-hemolysis are usually produced by *L. ivanovii*. However, all *L. monocytogenes* strains are not hemolytic, e.g. type strain ATCC 15313.

Growth and biochemical properties

Temperature limits of growth are 1° and 45°C with optimum at 30° to 37°C. *L. monocytogenes* can grow between pH 6 and pH 9 and in concentration of NaCl up to 10% (Seeliger and Jones, 1986). Biochemical studies of the *Listeria* genus show that all species are catalase-positive, oxidase-negative, methyl red (MR) and Voges-Proskauer (VP)-positive and indol-negative. They utilize glucose and esculin with production of acid but not gas and do not hydrolyze urea. All species

display negative mannitol reaction and do not hydrolyze sodium hippurate, except *L. grayi*. Characteristics of *Listeria* species are shown in Table 1.

Table 1. Characteristics of *Listeria* species (Farber and Peterkin,1991; Benedict, 1990)

Characteristic	*monocytogenes*	*innocua*	*seeligeri*	*ivanovii*	*welshimeri*	*grayi*
Hemolytic	+	-	+	+	-	-
CAMP-*S.aureus* test	+	-	+	-	-	-
CAMP-*R.equi* test	-	-	-	+	-	-
Utilization of:						
Mannitol	-	-	-	-	-	+
Rhamnose	+	V	-	-	V	-
Ribose				+/-*		
Xylose	-	-	+	+	+	-
NO_3 reduction	-	-	-	-	-	-
Virulence to mice	+	-	-	+	-	-
(GC) % DNA	37-39	36-38	36	37-38	36	41-42

+ = positive; - = negative; V = variable; * *Listeria ivanovii* subsp. *ivanovii* / subsp. *londoniesis*.

Listeriosis in humans

The majority of human cases of listeriosis are sporadic with unknown source or route of infection. However, a few *Listeria* outbreaks appearing in Europa, North America and New Zealand (Table 2) have indicated that food-borne transmission is an important mode of infection. A characteristic of these outbreaks is the high mortality rate, approximately 30% (Table 2). In most cases, listeriosis occurs in individuals with decreased immune system caused by disease (malignancy, AIDS, diabetes mellitus), medication (corticosteroids), extremes of age (neonates and persons over 60 years) or pregnancy.

Malignancy has been found in 25% of adults with *Listeria* meningitis (Nieman and Lorber, 1980) and 7% of patients with listerial endocarditis (Carvajal and Frederiksen, 1988). Particularly hematogenic malignancy enfeebles cellular

immunity against *Listeria*. Medication with corticosteroids interferes with the production of T cells which are responsible for resistance to *Listeria* (Lane and Unanue, 1972; Mackaness, 1971). With increasing age (>60 years) the immunity is waning and the tendency for development of cancer is larger (Ciesielski et al., 1988).

Usually, *Listeria* infections in non-pregnant adults are asymptomatic, resulting in intestinal or cervical carriage (Boucher and Yonekura, 1986). Between 0.6% and 10% of healthy humans and convalescents shed *L. monocytogenes* in faeces (Kwantes and Isaac, 1975, Seeliger, 1990). The most common *Listeria* manifestations are infection of the central nervous system (CNS), bacteremia, endocarditis and pericarditis, gastrointestinal infection and focal infections.

CNS Listeria infection

L. monocytogenes has affinity to the CNS, thus meningitis and cerebritis are the most usual manifestations. Meningitis manifests as an acute form of illness and can be found in all age groups. Altogether 48% of all *Listeria* infections in non-pregnant adults reported in the USA between 1980 and 1982 gave rise to meningitis. Mortality rate could be as high as 90% in untreated and 27-30% in patients receiving therapy (Calder, 1997). The symptoms of illness are high fever, headache, vomiting and mobility disorders. Cerebritis is the second most common form of *Listeria* CNS manifestations of illness with 20% of all CNS cases (Nieman and Lorber, 1980), and is especially seen in renal transplatation patients (Stamm et al., 1982). Similar symptoms as in the meningitis form can be observed at the beginning of illness. However, phenomena of cranial nerve palsies and hemiparesis may also rapidly occur (Nieman and Lorber, 1980). Brain abscess is rather rare but is characterized by a high mortality rate (up to 57%) (Dee and Lorber, 1986). One-half of all documented cases with this form of listeriosis were leucemia or renal transplantation patients.

Septicemia / Bacteremia

Septicemia / bacteremia in the absence of documented listerial infection in CNS or any other site is another common outcome of listeriosis. Patients could be found in all groups of people with predisposing factors. The most common symptoms are fever and chills, sometimes followed by vomiting, diarrhea, nausea and abdominal pain.

Endocarditis and pericarditis

Increasing frequency of endocarditis caused by *L. monocytogenes* has been

observed as a consequence of valve prosthesis and HIV infection (Nieman and Lorber, 1980; Spyrou et al., 1997). Altogether, 58 reports of listerial endocarditis have been announced worldwide since 1955, the mean age of the patients being 53 years (Spyrou et al., 1997). The symptoms are similar to other subacute forms of endocarditis with high mortality rate (37%) (Spyrou et al., 1997). Another type of cardiovascular involvement is pericarditis, which has been documented eight times, with a mortality of up to 50% (review of cases between 1971 and 1997, Manso et al., 1997).

Gastrointestinal infection

Gastrointestinal infection as a primarily self-limited illness caused by *L. monocytogenes* has been recognized recently in two outbreaks due to consumption of rice salad and chocolate milk, respectively (Salamina et al., 1996; Anonymous, 1997; Dalton et al., 1997). In the rice salad outbreak, affecting immunocompetent adults, a *Listeria* gastroenteritis syndrome involved 14 of 18 patients with symptoms. In the second outbreak, 54 of 60 apparently otherwise healthy persons who drank the chocolate milk might have become ill. The most recognized symptoms of gastrointestinal illness are fever, diarrhoea, nausea, vomiting, myalgia, arthralgia and headache. Febrile gastroenteritis may be more common than is currently recognized since healthy carriers have been shown to excrete *L. monocytogenes* from 1 - 10% (Seeliger, 1990).

Focal Listeria infections

Non-foodborne transmission of *L. monocytogenes* is rarely reported. A few cases have been reported with skin infection and eye infection.

Skin infection is usually manifested as cutaneous lesions such as nodulae, which may develop into pustulae. Veterinarians (Owen et al., 1960), slaughterhouse workers (Elischerova and Stupalova, 1972), farmers (Ödegaard et al., 1952) and laboratory staff working with *Listeria* (Anton, 1934) are the most usual victims of this form of listeriosis. Purulent conjuctivitis could be found in neonatal cases (Becroft et al., 1971).

Nosocomial infection

Nosocomial infection is rarely reported, probably due to the weak evidence of person-to-person transmission. One of the confirmed nosocomial outbreaks, however, was reported among newborn infants in a maternity ward caused by mineral oil containing *L. monocytogenes* (Schuchat et al., 1991). Two late-onset cases of listeriosis were acquired within a week as a result of tape measurements

of heads in a Swedish hospital. The measuring-tape had recently been used on an early-onset case of listeriosis in the same maternity ward (Higgins et al., 1988). In a hospital in Germany, eleven patients developed septicemia due to *L. monocytogenes* during a one-year period. All patients were immunocompromised (Elsner et al., 1997).

Perinatally acquired listeriosis

Due to appearance of disease this category could be divided into two groups (Anonymous, 1980; Spencer, 1987; Becroft et al., 1971).

The first group consists of children born ill or falling ill within a few hours or days (early onset) after delivery in a serious septical disease with high mortality. The foetus is often spontaneously aborted or will die in utero. The child may also be prematurely delivered, still-born, or die shortly after delivery. The foetus/child displays a syndrome called *granulomatosis infantiseptica* which includes respiratory difficulties, pneumonia, and widespread microabscesses. Infection is probably acquired in utero during the third trimester of pregnancy. In 40% of cases the pregnant women have had flu-like symptoms such as fever, headache, or myalgia due to bacteremia. It is likely that the foetus/child is infected during this time.

The second group includes children falling ill in meningitis during the second to fourth weeks of life (late onset). These children are usually in good health when born. With adequate therapy the prognosis is good and mortality low. The child is considered to be infected during passage of the birth channel, but there are also theories about horizontal spread, i.e. that infection is acquired from the environment. Thus, the very occasion of infection lies within the perinatal period, but the disease does not appear until later.

Listeria in food

L. monocytogenes has been isolated from a wide range of food products such as vegetables, milk and dairy products, and meat and meat products.

The presence of *L. monocytogenes* on vegetables is due to contamination from decaying vegetation, animal manure, soil, surface river and canal waters, or effluents from sewage treatment operations (Beuchat et al., 1990). Raw celery, tomatoes and lettuce served as salad were involved in a *Listeria* outbreak in USA in 1979 when altogether 20 cases were registered (Ho et al., 1986). In another outbreak caused by coleslaw in Canada, two years later, 41 cases of illness were reported (Schlech et al., 1983). *L. monocytogenes* can survive in plant vegetation

for 10 to 12 years and increase by 2 - 3 Log_{10} CFU/g in raw broccoli florets, cauliflower florets and asparagus stored at 15°C (Beuchat et al., 1990).

Particularly food of animal origin, e.g. raw and pasteurized milk and dairy products, raw and processed meats, poultry, fish and seafood, can become contaminated with *L. monocytogenes* during processing.

It is well documented that ruminants can be healthy carriers of *L. monocytogenes* (Kampelmacher and van Noorle Jansen, 1969) and also shed *L. monocytogenes* with faeces (Skovgaard and Morgen, 1988). Therefore, during the milking process there are many opportunities for contamination of the milk with *L. monocytogenes*. Incidences of 0% (Lovett et al., 1987) to 16% (Fenlon et al., 1995) in raw milk samples have been demonstrated. Also subclinical *Listeria* mastitis are reported (Errebo-Larsen and Jensen, 1977). Thus raw milk received by dairy plants may become a source of *L. monocytogenes* for dairy products. The highest *Listeria* incidence (50% of samples) in a cheese processing plant was found in the raw milk reception area (Franco-Abuin et al., 1996). The safest way to eradicate *Listeria* from raw milk is by the high-temperature, short-time (HTST) pasteurization process (71.7°C for 15 s) (Lovett et al., 1990). However, the process may fail and *L. monocytogenes* may be found in milk that has been inadequately heat-treated. This happened in the U.S. which caused the illness of 49 persons (Fleming et al., 1985).

Raw milk is a particularly important source of *L. monocytogenes* for raw milk cheese. **Paper I** in this thesis deals with the occurrence of this organism in soft raw milk cheeses. *L. monocytogenes* was detected in 42% of samples from raw milk cheeses in contrast to cheeses made from heat-treated milk, which were much less contaminated (2%). Such frequent contamination and the large numbers of this pathogen per g of raw milk cheese (up to 1×10^5) constitute a public health risk. Even a small number of *Listeria,* in such good substrate as soft cheese, may reach considerable levels if stored inadequately. Immunocompromized persons, including pregnant women, are strongly recommended not to eat soft cheeses made of raw milk.

Besides being contaminated by raw milk, cheeses may become contaminated through cheese ingredients (caseinate, spices), from the environment of the processing plant, during distribution, or during handling by the consumer (Terplan, 1988; Franco-Abuin et al., 1996). The environment of the processing plant, e.g. the surface of dairy equipment, condensation water, floor and floor drains, staff at the dairy plant, animals (insects, mice, rats) and air have to be considered as potential sources of *L. monocytogenes* (Terplan, 1988). It has been shown that *L. monocytogenes* can survive at least seven years in a processing plant as part of the domestic flora and thus be a menace to dairy products

(Unnerstad et al., 1996).

Distribution and/or handling, e.g., slicing or parcelling for retail stores, may contribute to contamination, and even such cheeses where the milk has undergone a listericidal procedure (HTST pasteurization process) therefore could become risk food products. Handling in retail stores is of great concern, especially with regard to refrigerator temperature, separation between wedges and the use of cutting machines.

Soft cheese is a very popular ready-to-eat dish in Sweden, as in many other countries. Three reported outbreaks (Table 2) attracted our attention towards soft cheeses as possible food risks. In a study of potential sources of human listeriosis in Sweden (**Paper V**), however, isolates from human cases and cheeses were not identical on the DNA level. Furthermore, no human case of listeriosis due to soft cheeses has yet been reported in Sweden.

Two outbreaks in England and France, associated with paté and pork tongue in aspic (McLauchlin et al., 1991; Goulet et al., 1993), showed that meat products are a potential source of listeriosis (Table 2). A few sporadic cases of listeriosis after consumption of sausages have been described (Anon., 1990; Parodi et al., 1990; **Paper IV**). High incidence of *Listeria* in raw meat, animal organs, raw meat products and ready-to-eat meat products is well documented (Johnson et al., 1990). Difficulties in isolating *L. monocytogenes* from raw meat may be due to compartmentalization of bacteria inside muscle fibres (for a review see Johnson et al., 1990). *L. monocytogenes* was isolated from 10% of raw beef and 8% of raw pig meat samples (Loncarevic et al., 1994a). Oversights common during slaughtering, e.g., when dehiding, scalding and evisceration, may contribute to cross-contamination of raw meat. Contamination during processing of meat products is considered to play an important role. *L. monocytogenes* was found in 95% of minced meat samples (Lowry and Tiong, 1988) and 33% of ready-to-eat meat products (Farber et al., 1988).

L. monocytogenes may become frequent in broilers after certain phases in the slaughtering procedure. Especially the chilling procedure may contribute to large numbers of contaminated broiler carcasses. Immediately after bleeding, for example, *Listeria* was not found, whereas after passing the chiller 50% of neck skin samples were positive for the presence of *L. monocytogenes* (Hudson and Mead, 1989). However, carcasses passing through chilling water with at least 20 ppm total chlorine can reduce the *Listeria* contamination (Loncarevic et al., 1994b). Prevalence of *L. monocytogenes* in raw broiler meat ranges from 0% to 64% (Ternström and Molin, 1987; Kwantes and Isaac, 1975). Altogether, 64% of 64 frozen and 50% of 38 fresh chickens, which reached the home kitchens in West Glamorgan (U.K.), contained *L. monocytogenes* (Kwantes and Isaac, 1975).

Table 2. Food-borne human outbreaks of listeriosis since 1975 worldwide

Country	Year	Food associated	Number of cases (lethality rate, %) Total	Adults/Materno-foetal	Serovar	Reference
USA	1979	Vegetables	20 (25)	20 (25) / 0 (0)	4b	Ho et al. (1986)
New Zealand	1980	Shellfish, raw fish	29 (17)	7 (0) / 22 (23)	1/2b	Lennon et al. (1984)
Canada	1981	Coleslaw	41 (34)	7 (29) / 34 (27)	4b	Schlech et al. (1983)
USA	1983	Past. milk	49 (29)	42 (29) / 7 (29)	4b	Fleming et al. (1985)
USA	1985	Cheese	142 (34)	49 (37) / 93 (32)	4b	James et al. (1985), Linnan et al. (1988)
Austria	1986	Raw milk, vegetables?	28 (18)	4 (25) / 24 (17)	1/2a, 4b	Allerberger and Guggenbichler (1989)
Switzerland	1983-87	Raw milk cheese	122 (27)	61 (NK) / 61 (NK)	4b	Bille (1990)
USA	1986-87	Ice cream, salami	36 (44)	32 (44) / 4 (50)	4b, 1/2a, 1/2b, 3b	Schwartz et al. (1989)
USA	1989	Shrimp	9 (11)	7 (0) / 2 (50)	4b	Riedo et al. (1990)
U.K.	1987-89	Paté	>300 (?)	NK	4b	McLauchlin et al. (1991)
France	1993	Pork tongue in aspic	279 (23)	(56 deaths) / (7 deaths)	4b	Goulet et al. (1993)
France	1993	Rillettes	38 (29)	7 (14) / 31 (32)	4b	Goulet et al. (1998)
Italy	1993	Rice salad	18 (0)	18 (0) / 0	1/2 b	Salamina et al. (1996)
France	1994	Raw milk cheese	20 (10)	9 (0) / 11 (18)	4b	Goulet et al. (1995)
Sweden	1994-95	Rainbow trout	8 (25)	5 (20) / 3 (33)	4b	Ericsson et al. (1997)
USA	1997	Past. chocolate milk	45 (0)	45 (0) / 0	1/2 b	Dalton et al. (1997)

NK = not known

Undercooking of such food and cross contamination of other food at home should be of concern. A study of undercooked chicken (chicken meat that was still pink) in the USA showed that the risk for sporadic listeriosis increased 3.2 times with consumption of such food (Schwartz et al., 1988). In the same study, it was shown that 6 of 80 cases of listeriosis had eaten undercooked chicken.

L. monocytogenes has often been isolated from fish and fish products and other seafoods (Jemmi, 1990; Farber, 1991; Ben Embarek, 1994, **Paper II**). The way of farming fish, producing fish products and storage conditions may contribute to the occurrence of *Listeria* in such products. The first time that the interest was focused on fish and seafoods as potential sources for human listeriosis was 1980 (Lennon et al., 1984). In this investigation of an outbreak including 19 cases of perinatal listeriosis in New Zealand it was shown that the actual mothers more often consumed raw fish and seafoods than the control persons did. To our knowledge, the first fully investigated and reported fish-borne outbreak happened in 1994/95 in Sweden. At least eight cases of listeriosis were considered due to consumption of "gravad" or cold-smoked rainbow trout (Ericsson et al., 1997). High prevalence of *L. monocytogenes* among fish products (**Paper II**) and the mentioned fish-borne outbreak, in fact the first outbreak of food-borne listeriosis ever reported in Sweden, have put gravad and cold smoked fish on the list of risk food products in Sweden.

In **Paper II** it is shown that gravad fish was most frequently contaminated with *L. monocytogenes* (20.7%) followed by cold smoked salmon (15.4%) and cold smoked rainbow trout (7.7%). Similar results have been reported by other authors (Jemmi, 1990; Jemmi et al., 1992). Treatment of raw salmon / rainbow trout with salt, sugar, pepper, dill (gravad fish) or with 2.5-3.5% NaCl and cold smoke at 25-30°C for 2-3 h (cold smoked fish) is not sufficient to kill *L. monocytogenes*. Producers and retailers endeavour to achieve a longer shelf life for these foods by packaging them in vacuum. This means long storage at refrigeration temperatures before consumption. Therefore, vacuum-packed and gravad or cold smoked fish which are initially contaminated with small number of *L. monocytogenes*, might harbour large numbers of bacteria at the day of consumption. Furthermore, fish products have high water activity, e.g., smoked salmon has an a_W value of about 0.97 (Dillon et al., 1992), thus the condition for multiplication of *L. monocytogenes* is good. Due to the reasons mentioned, the Swedish National Food Administration recommends that the shelf life of these vacuum-packed products is shortened from six weeks to three weeks and that they are stored at maximum +4°C.

Risk food products and general health problems

In the previous text, two ready-to-eat food products (soft cheeses and gravad and

cold smoked fish) are highlighted with special attention to *L. monocytogenes*. An outbreak of listeriosis due to rainbow trout and high incidence of *L. monocytogenes* in gravad and cold smoked salmon and rainbow trout are reasons to regard these food products as risk foods in Sweden. At the same time, consumption of ready-to-eat fish products such as cold smoked and gravad salmon and rainbow trout and soft cheese are high in Sweden (Figures 1 and 2) and consequently, the risk of these foods being involved in human listeriosis is higher. At the same time, however, this does not imply that they are an essential public health problem in other countries. Consumption of a certain type of food may differ from country to country. Thus in Sweden, consumption of ready-to-eat products such as pig tongue in aspic is low. On the other hand, this dish is popular in France, and, thus, may be regarded as risk food in France, particularly after the *Listeria* outbreak in 1993 (Goulet et al., 1993).

Outbreaks of listeriosis caused by cold smoked salmon and reports of occurrence of *L. monocytogenes* in the food products mentioned, were reported by the mass media, which may help in making the general public more aware of problems involving *L. monocytogenes* and in providing dietary recommendations among immunocompromised individuals. In some countries, persons with a high risk of listeriosis are individually informed about risk foods by their physician or by poster information in maternity wards.

"Zero-tolerance" policy

Decreased incidence of human listeriosis in the USA by 48% between 1986 and 1993 (Tappero et al., 1995) may be due to preventive measures such as "zero-tolerance", intensified clean-up programmes and dietary recommendations. Zero-tolerance (no *L. monocytogenes* detected per gram of food) was introduced in the USA by Food and Drug Administration (FDA) for commercial foods for consumption without further cooking. The recommendation refers to dairy products, but it has also been accepted by the meat and seafood industry. Regulations for the zero-tolerance policy in ready-to-eat food have been adopted by other countries such as New Zealand, South Korea, Switzerland and Hong Kong. However, the zero-tolerance is not accepted worldwide and there are numerous polemics about the effectiveness of this regulation. Authorities not supporting the zero-tolerance claim that not all *L. monocytogenes* strains are pathogenic and that no sampling plan can completely eliminate *Listeria* from food and the food industry. Thus, allowing up to 100 *L. monocytogenes* organisms per gram food on the sell-by date is thought to be more acceptable. Incorporating HACCP - based preventive measures, education of workers in the food industry, consumer education, and shortening of shelf-life of risk food products, is suggested to be a more realistic and effective means of protection against *L. monocytogenes*. Implementation of the measures mentioned in the USA and

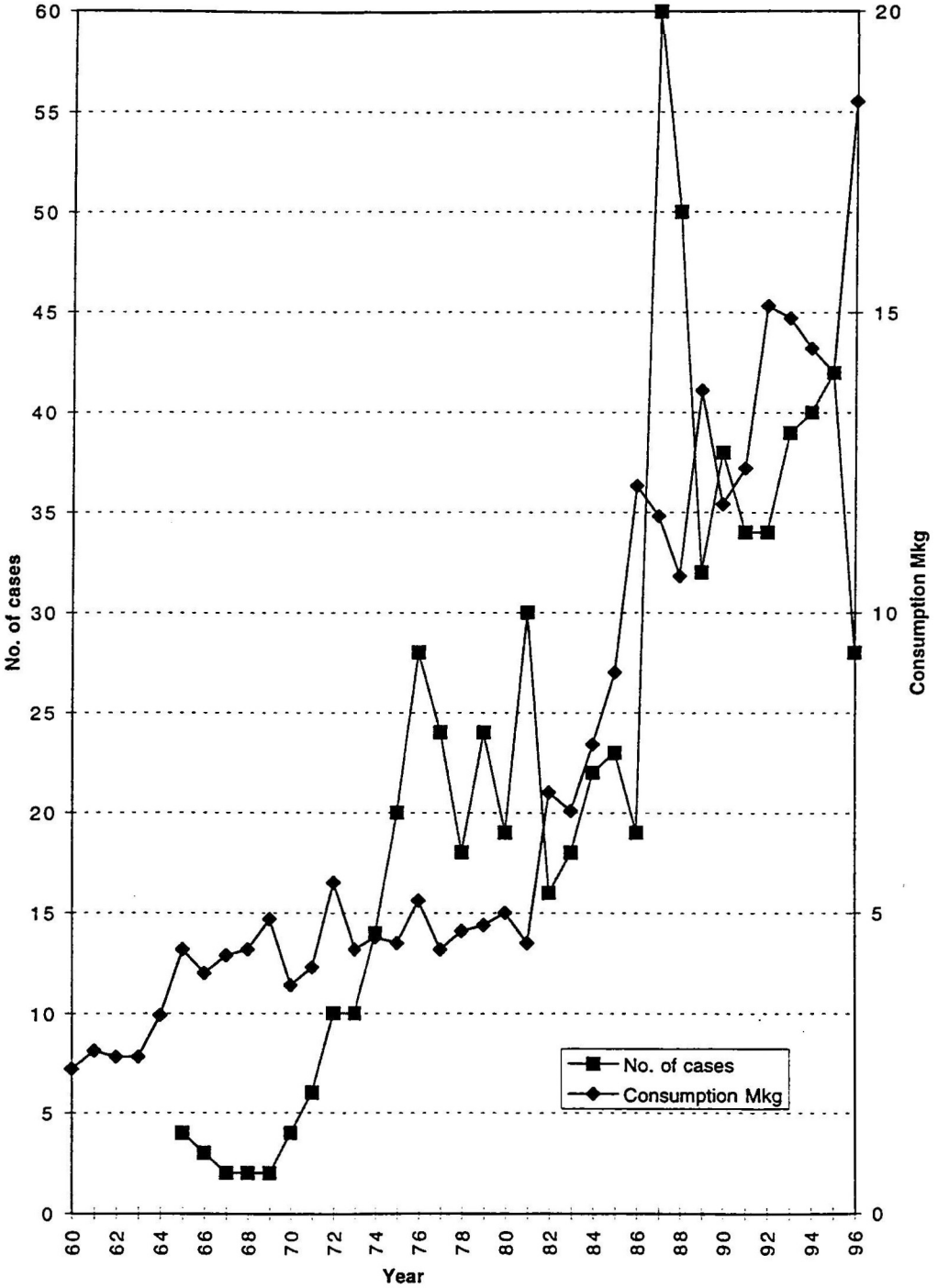


Figure 1. Consumption of salmon and rainbow trout and number of human cases of listeriosis in Sweden (Statens Jordbruksverk, 1996; Personal communication, Malin Arneborn)

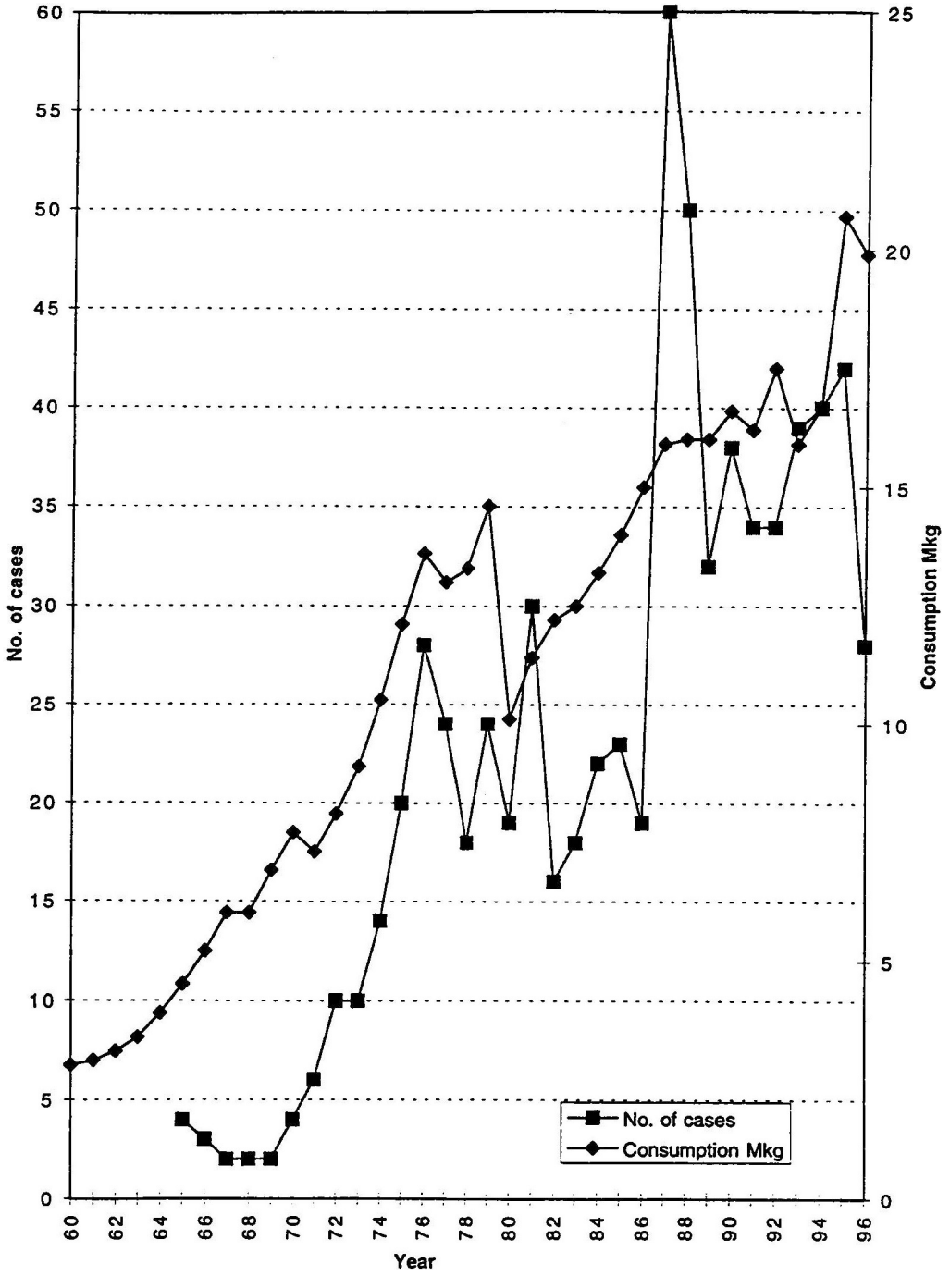


Figure 2. Consumption of soft cheese and number of cases of listeriosis in Sweden (Statens Jordbruksverk, 1996; Personal communication, Malin Arneborn).

England, Wales and Northern Ireland will substantially decrease the number of reported cases (Klima and Montville, 1995; Gilbert, 1995).

Isolation of *Listeria* from foods

Conventional methods for detection of L. monocytogenes

Significant progress has been done in development of isolation methods for *Listeria* in food, particularly during the last ten years. The main improvements have been on greater sensitivity and shortening the time required for analysis. The very first methods were based on direct plating of samples onto agar media. In the case of large numbers of *Listeria* bacteria present in the sample, and in the absence of competitive microorganisms, this method was reliable. However, already in 1939 a new method was described based on a cold enrichment procedure; better results were obtained by incubation of a sample in an enrichment broth at refrigeration temperatures (Biester and Schwarte, 1939). Afterwards, a cold enrichment procedure with prolonged incubation time at 4°C was described (Gray et al., 1948). The incubated homogenized enriched sample (in a broth or water) was directly streaked on tryptose or McBride agar plates at regular time intervals. The incubation time required, sometimes up to a few months, was the major disadvantage. Newer methods are based on the use of one or more different selective agents supplemented to enrichment broth and agar media. Furthermore, the time and temperature of the enrichment broth procedure have been changed by shortening the time to 48 hours and increasing the incubation temperature to 30° - 37°C. Many factors, e.g., food type, number and injury status of *Listeria* bacteria, and other microorganisms present affect the choice of eligible enrichment broth and plating media. A major problem is how to get recovery of all *L. monocytogenes* cells. It is well known that *Listeria* isolation media may inhibit the bacteria (including *Listeria*) in the food. Also the type of food plays a role for successful detection. No medium has been shown to be suitable in all situations. The plating media most often used are Lithium Chloride-Phenylethanol-Moxalactam Medium (LPM), Oxford agar (OXA), Modified Oxford agar (MOX), PALCAM agar and Haemolytic Ceftazidime Lithium Chloride agar (HCLA). USDA (United States Department of Agriculture) recommended their own method for detection of *Listeria* spp. from meat, poultry and egg products; FDA (Food and Drug Administration) for dairy products, seafood and vegetables. While the media of choice in the FDA protocol (Lovett, 1988) are LPM and Oxford agar, the USDA protocol (McClain and Lee, 1988) includes MOX agar which is the one used most in the United States. PALCAM and Oxford agar are preferred in Europe. In experiments examining the effectiveness of different media, it was observed that LPM and Oxford medium

were more successful in recovery of *L. monocytogenes* than modified McBride agar (MMA) (Brackett and Beuchat, 1989; Heisick et al., 1989; Northolt, 1989). PALCAM agar is effective in suppressing competitive microorganisms, but, on the other hand, it is not good at detecting low numbers of *Listeria* bacteria (Gunasinghe et al., 1994). The International Dairy Federation, IDF (1995) recommends a specific method for detection of *L. monocytogenes* in milk and milk products by a one-step enrichment broth procedure and plating onto OXA. A method intended for use in examination especially of meat and cheese products was developed by Nordic Committee on Food Analysis, NMKL (1990). The method is based on two enrichment broth steps, or one enrichment step with a longer incubation time. Plating is performed on OXA plates.

All available *Listeria* broth and solid media contain ingredients that more or less inhibit growth of microorganisms. The selective agents used most frequently are acriflavine, cycloheximide and nalidixic acid, followed by lithium chloride, cefotetan, fosfomycin and colistin sulfate. Cycloheximide inhibits growth of fungi, nalidixic acid prevents growth of Gram negative microorganisms and moulds, and acriflavine suppresses growth of Gram positive cocci. At low pH value <5.8, there is an increased interaction of acriflavine with food, and at the same time, increased growth of *L. monocytogenes* (Beumer et al., 1996). Media with various acriflavine concentrations are recommended for specific food types, e.g. the USDA method recommends two different concentrations of acriflavine, 12 mg for primary and 25 mg for secondary enrichment broth, while for LEB in the FDA method the concentration is 15 mg. The NMKL method has also been formulated with higher concentrations of acriflavine in the secondary LEB. Enrichment broth of the IDF method contains 23 mg of acriflavine.

It has been shown that different enrichment and plating media could more or less favour or suppress certain types of *L. monocytogenes* and consequently give false information. **Papers III** and **IV** demonstrate that also the clones of *L. monocytogenes* detected in food depend on the medium and the method used. In **Paper III** as well as in Danielsson-Tham et al. (1993), it is shown that the best overview of the *Listeria* population is after direct plating.

Rapid methods for detection of L. monocytogenes

To reduce time and labour in detection of *L. monocytogenes*, methods have been developed for rapid mass screening.

Immunological antibody-based tests, e.g. enzyme-linked immunosorbent assays (ELISAs) and nucleic acid (DNA and RNA)-based tests are rapid tests used for detection of *L. monocytogenes*.

ELISA tests are based on antibody specificity for the target antigen. These tests usually require 10^5 to 10^6 bacterial cells per ml enrichment broth to be reliable. Disadvantages of ELISA tests, such as the long time for performing the test, low sensitivity, high cost per test, special equipment, need of well trained staff and confirmation of presumptive positive samples with conventional methods, are reasons for the lack of enthusiasm for utilising these tests. However, by automatisation of the ELISA system with, for example, VIDAS or mini VIDAS (bioMérieux-Vitek, Inc.), the disadvantages mentioned have been reduced, which has made ELISAs more useful in routine testing.

Nucleic acid based tests usually require 10^4 to 10^5 *Listeria* cells per ml enrichment broth or growth on agar plates. Specificity depends on the target nucleic acid (DNA or RNA) sequence. A new, rapid and highly-specific DNA-probe identification assay for *L. monocytogenes* based on specificity for the 16S rRNA target by use of the VIDAS automatic analyzer has been introduced by bioMérieux-Vitek, Inc. The procedure is based on releasing of target nucleic acid by a quick chemical lysis of the bacterial culture. Non-radioactive hybridisation reaction of extract loaded into the VIDAS automatic analyzer takes two hours. Mabilat et al. (1996) showed that 68 out of 69 *L. monocytogenes* isolates could be recognised by means of this method without cross-reactions to other *Listeria* (103 strains) or other bacteria species (15 strains).

Polymerase Chain Reaction (PCR) technique is based on enzymatic synthesis of selected DNA sequences (Taylor, 1991). Reaction starts with denaturation of template DNA by heating to 95°C followed by cooling to 37°C. During the cooling, two oligonucleotide primers hybridize to the two opposite strands of the target DNA and bind to the region of current interest. Starting from the primer's binding region, heat-stable DNA polymerase (Taq DNA) extends complimentary DNA strands during the next heating step of PCR reaction (72°C). In each repetitive cycle of heating at 95°C (denaturation), cooling at 37°C (primer annealing) and heating at 72°C (extension), two double DNA strands are synthezised. The amount of specific DNA sequence increases exponentially until one of the reaction components is expended or the enzyme is unable to synthesise new DNA fast enough. During 25 repeated cycles of denaturation, annealing and extension, the number of DNA copies can reach over one million. The method is extremely specific, sensitive and fast. However, use of this method in routine food analyses relies upon overcoming certain difficulties, e.g. inhibition of PCR reaction by food components (high levels of fat, proteins and calcium); extreme sensitivity, and specificity (PCR does not differentiate between DNA from viable and non-viable cells). By overcoming these problems, the method may be useful for detection of *L. monocytogenes* in food.

Typing of *Listeria*

Typing of *L. monocytogenes* is an indispensability in investigations of outbreaks or sporadic cases of human listeriosis, in clinical and ecological studies, as well as in HACCP work in food processing plants. Typing methods for *L. monocytogenes* have been rapidly developed, especially during the 1980s when a variety of techniques were introduced for DNA analysis (Figure 3). The typing methods can be divided into phenotypic and genotypic. Phenotypic methods, although with limited capacity in discrimination and typeability, are still the first step in establishing relationships among *L. monocytogenes* strains. Genotypic methods (Molecular typing methods) offer possibilities to study clonal relationship among the strains.

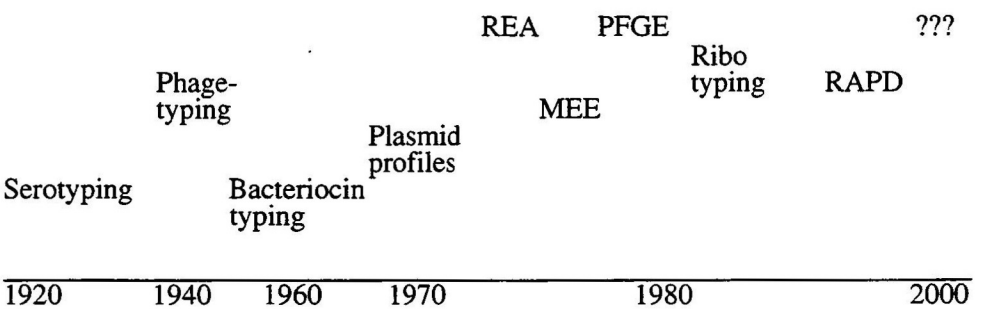


Figure 3. Evolution of typing methods *(Swaminathan, 1997).

* From a lecture given 23th October, 1997 at Amersham Pharmacia Biotech, Boländerna, Uppsala, Sweden.

Phenotypic typing methods

Phenotypic typing comprises serotyping, biotyping, phage- typing and bacteriocin typing methods. The most widely used methods are serotyping and phage-typing.

Serotypning of L. monocytogenes

Serotyping is based on slide agglutination reactions of antibodies against 15 different O (somatic) and five H (flagella) antigens of various *Listeria* strains (Seeliger, 1958; Donker-Voet, 1972). Sixteen serovars within five *Listeria* spp. can be identified (Table 3). *L. monocytogenes* can be divided into four serogroups, 1/2, 3, 4 and 7, by use of somatic antigens. Subdivision by agglutination of two or three flagellar factors A, B, C for serogroup 4, and A, B,

C, D for serogroups 1/2 and 3, gives 13 serovars of *L. monocytogenes*.

The majority of *L. monocytogenes* strains isolated from food and human cases of listeriosis belong to the two serogroups 1/2 and 4. Serogroup 1/2 is more

Table 3. Serovars of *Listeria* spp.

Listeria	Serovars*	O antigens	H antigens
monocytogenes	1/2a	I, II, (III)[a]	A, B
monocytogenes, seeligeri	1/2b	I, II, (III)	A, B, C
monocytogenes	1/2c	I, II, (III)	B, D
monocytogenes	3a	II, (III), IV	A, B
monocytogenes	3b	(III), IV, (XII, XIII)	A, B, C
monocytogenes	3c	(III), IV, (XII, XIII)	B, D
monocytogenes	4a	(III), (V), VII, IX	A, B, C
monocytogenes, innocua	4ab	(III), V, VI, VII, IX, X	A, B, C
monocytogenes	4b	(III), V, VI	A, B, C
monocytogenes, seeligeri	4c	(III), V, VI	A, B, C
monocytogenes, seeligeri	4d	(III), V, VI, VIII	A, B, C
monocytogenes	4e	(III), V, VI, (VIII, IX)	A, B, C
ivanovii	5	(III, V), VI, (VIII), X	A, B, C
monocytogenes	7	(III), XII, XIII	A, B, C
innocua,welshimeri	6a	(III), V, (VI, VII, IX), XV	A, B, C
innocua, welshimeri, seeligeri	6b	(III, V, VI, VII), IX, X, XI	
grayi		(III), XII, XIV	E
innocua, seeligeri	undesignated		

*Designation of Seeliger (1958) and Donker-Voet (1972)
[a]= not always present.

predominant among food isolates, whereas human isolates more often belong to serogroup 4 (Farber and Peterkin, 1991). **Papers I** and **II** show high predominance of serogroup 1/2 among fish and cheese isolates (71% of fish and 90% of cheese isolates). Serogroup 1/2 strains dominate in other food studies as well. Thus, of 737 strains isolated from different foodstuffs in Belgium, serogroup 1/2 was shared by 75.5% and serovar 4b by 22% (Gilot et al., 1996). In a review

study of serogroup distribution from human cases of listeriosis worldwide, out of 2344 human isolates serogroup 4 accounted for 68%, and serogroup 1/2 for 30% (Farber and Peterkin, 1991). Furthermore, McLauchlin (1990) found that 64% of the isolates from 1363 cases of human listeriosis belonged to serovar 4b, 15% to serovar 1/2a, 10% to serovar 1/2b and 4% to serovar 1/2c. Although the food-borne route is well-established, there is an inexplicable discrepancy between the serovar distribution in human and food isolates, respectively. One explanation might be that human beings are frequently exposed to serogroup 1/2 strains and thus will gain immunity (Gilot et al., 1996). In contrast, serogroup 4 strains, rarely encountered in food, may cause human listeriosis due to deficient immunity. McLauchlin (1990) suggested an association between serotype and virulence of *L. monocytogenes* as an explanation of the predominance of serovar 4b among human isolates. Although, still unconfirmed, serovar 4b should, thus, be more virulent than serovars 1/2a, 1/2b and 1/2c. On the other hand, serogroup 3, which is rare in human cases of listeriosis, was shown to have higher LD_{50} value in experimental infection of mice than serogroups 4 and 1/2 (Wirsing von Koenig et al., 1983).

Changes in distribution of serogroups 4 and 1/2 strains among human cases of listeriosis have recently been noticed in some countries, e.g. in Denmark (Gerner-Smidt et al., 1995), in the UK (McLauchlin and Newton, 1995), as well as in Sweden. The increase of human cases of listeriosis due to serogroup 1/2 in the UK, led McLauchlin and Newton to suggest that future resources should be directed to improve the ability to subtype within serogroup 1/2. The changes in serogroup distribution among human cases in Sweden reported in **Paper V** might be explained by changes in eating habits. Increased consumption of food that is frequently contaminated with *L. monocytogenes* serogroup 1/2 in Sweden (Figures 1 and 2) might be an explanation of the large number of serogroup 1/2 strains among humans isolates since 1986.

Phage-typing of L. monocytogenes

Bacteriophages specific for *Listeria* were described for the first time in 1945. Since then, a method has been developed for species and strain differentation in epidemiological studies. The *Listeria* phages belong to either one of two groups: *Siphoviridae* (noncontractile tails) and *Myoviridae* (contractile tails). The International phage-typing system - the most common used - was introduced by WHO Phage Typing Centre at the Institut Pasteur, Paris. A number of modifications of this set, e.g. the Danish and German *L. monocytogenes* phage-typing systems, were developed by including additional phages. Twenty-six phages from the International phage set allow identification of a specific host. More than 78% of 823 strains of *L. monocytogenes* investigated in France, were phage-typeable by use of a set of 20 phages (Audurier and Martin, 1989). Strains

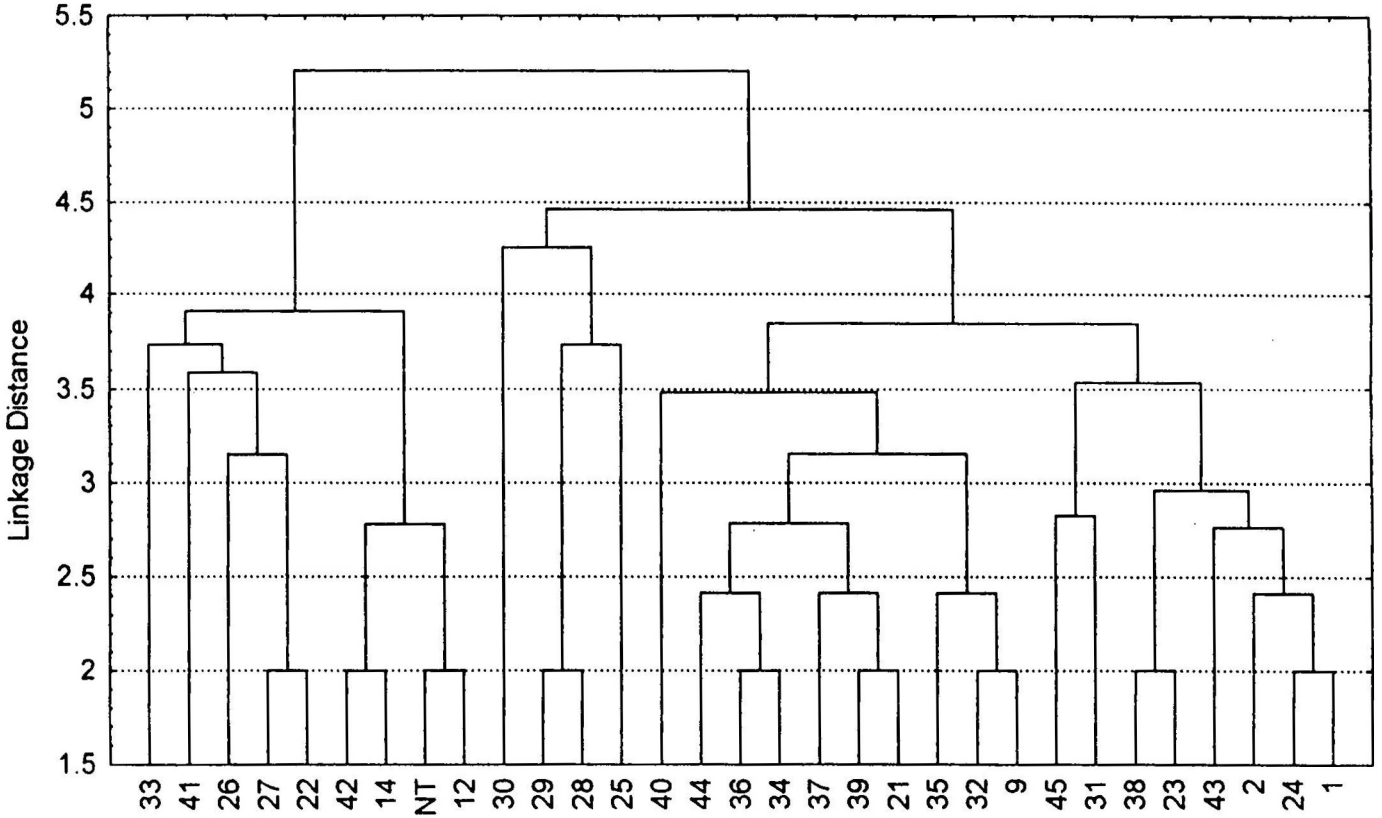


Figure 4. UPGMA clustering dendogram of the 31 phagovars of 68 *L. monocytogenes* isolates. Similarity calculated as Euclidean distances (By Gerner-Smidt, 1997).

belonging to serotype 4 were more often typeable (88%) than strains of serotype 1/2 (57%). Audurier and Martin (1989), furthermore, showed that 84% of 826 strains of *L. monocytogenes* serotype 4 and 49% of 1644 serovar 1/2 strains were typeable by using 35 phages. The lack of sensitivity to phages among many serogroup 1/2 strains and all serogroup 3 and 7 strains tested is the main disadvantage of this method (Gerner-Smidt et al., 1993; Loessner and Busse, 1990). However, typeability within serogroup 1/2 could be increased by use of additional phages. In a study of 180 non-phage typeable *L. monocytogenes* strains by use of the international set, it was shown that 78% were lysed with the additional broad-host-range virulent phage A511. Typeability of 130 serogroup 1/2 strains was 72% and 180 serogroup 4 strains 79% (Marquet van den Mee et al., 1997). A new phage-typing set in Denmark, which includes three phages from the international set and nine experimental phages, was better in typing *L. monocytogenes* than previous typing using only the international set. Typeability of serogroup 1/2 strains was 92% with a reproducibility of >90% (Gerner-Smidt et al., 1993). In **Paper V**, this phage-typing set was applied on 68 strains of *L. monocytogenes* belonging to serovar 1/2a. Typeability of these strains was 94%. This study showed that 23 isolates belonged to one phagovar (type 1) and the other isolates to 29 different phagovars (Figure 4). In this and other studies, however, phage typing is shown not to be sufficiently discriminating in that 23 isolates clustered in the same phage type, **Paper V**. Therefore, it is important to include more discriminating characterization methods, e.g., DNA fingerprinting, in addition to classical methods.

Molecular typing methods

Molecular (genotypic) methods are based on the characterization of nucleic acids target such as DNA and RNA. Ribotyping, Multilocus Enzyme Electrophoresis (MEE), Random Amplification of Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE) are the most utilized methods for molecular typing of *L. monocytogenes*. Single-Strand Confirmation Polymorphism (SSCP) is a newly introduced typing method.

Ribotyping

This method is based on characterization of *Listeria* by ribosomal RNA patterns. Altogether 45 unique ribotypes of *L. monocytogenes* have been identified among 93 different ribotypes of genus *Listeria*. After loading of samples into the main instrument, Riboprinter^{TM} Microbial Characterization System, all steps are fully automated and results can be obtained within 6 to 7 hours. Five steps follow each other during the automatic operation; DNA preparation process (lysis, deproteinisation, digestion), separation and transfer process (size-separation of

DNA fragments by electrophoresis and immobilisation on nylon membrane), membrane processing (chemically treated membranes will yield chemiluminescent DNA fragments), detection procedure (DNA pattern will be reproduced by photography and the image is stored in the database) and analysis (comparison of image pattern with recognition library) (Ryser, 1995).

Multilocus Enzyme Electrophoresis (MEE)

Genomic relationship between *L. monocytogenes* isolates by determination of electrophoretic mobilities of a set of water soluble cellular enzymes is obtained by means of this method. Genetic and allelic differentation between strains results in electrophoretic mobility variations. After the bacterial cells have been lysed and cellular debris eliminated by centrifugation, supernatant containing enzymes is placed in a gel and electrophoresed. Slices of cut electrophoresed gel are stained with corresponding solution to the specific enzyme until clear bands appear. A single band appears for each isolate and enzyme staining (electromorph). The combination of electromorphs for one strain is called electrophoretic type (ET). For genomic relationship of *L. monocytogenes* isolates it is recommended to use between 20 to 25 polymorphic enzymes (Boerlin, 1995).

Random Amplification of Polymorphic DNA (RAPD)

RAPD technique employs PCR to establish genomic polymorphism among closely related strains of *L. monocytogenes*. After lysis of cells, DNA is mixed with a short arbitrary primer and PCR mixture. However, it is shown that use of multiple arbitrary primers, instead of one single primer, may reveal more genetic differences between closely related *Listeria* strains (Wagner et al., 1996). Low stringency conditions during the PCR process allows annealing of primers at several locations on either strand of the genomic DNA and generation of array of strain specific fragments. Gel electrophoresis and staining make it possible to analyze the DNA fragments.

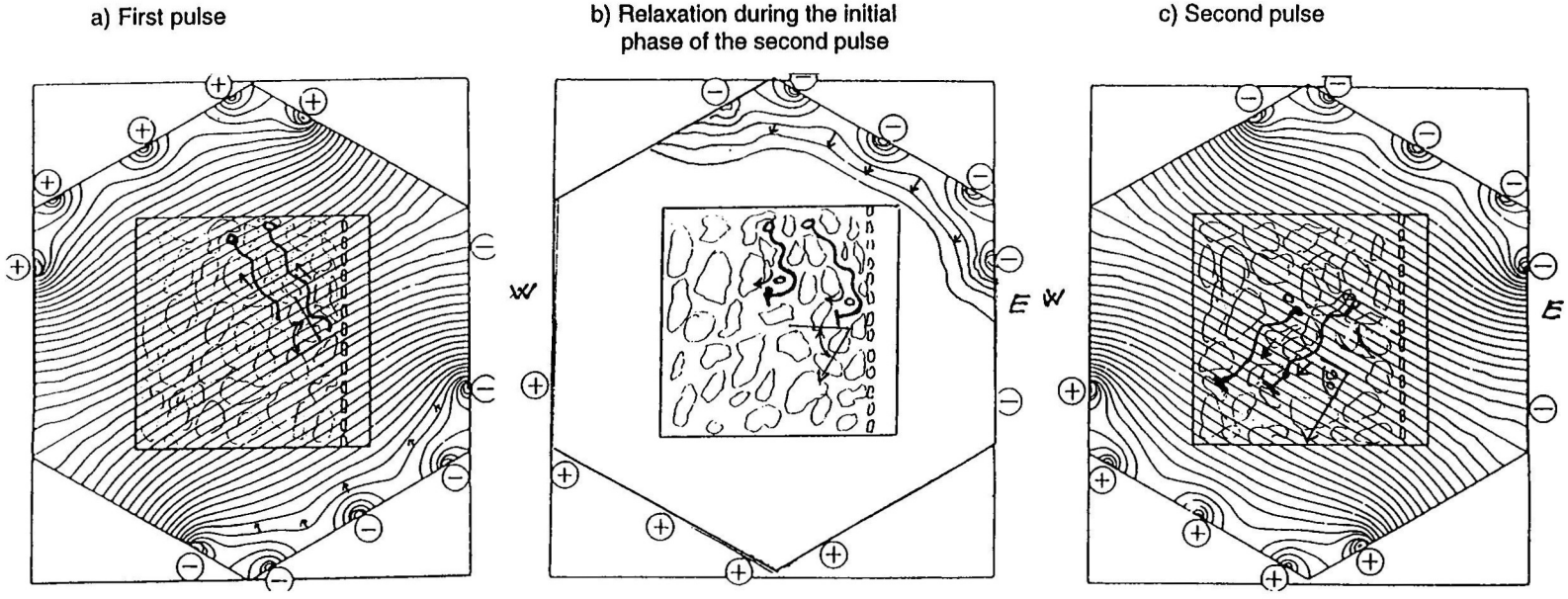
Pulsed-Field Gel Electrophoresis (PFGE)

Since 1987, when the first commercial PFGE was introduced by Pharmacia-LKB (Uppsala, Sweden) the method has been extensively applied for discrimination of a variety of pathogenic bacteria.. The main advantage of PFGE (Southern and Elder, 1995) in comparison with conventional electrophoresis is the possibility of resolution of genomic DNA in sizes from a few kb to over 10 mb, visualized as fingerprinting patterns. Bacteria to be tested are embedded in agarose plugs. To degrade cell walls, plugs are treated with lysozyme and then incubated with proteinase K to remove all proteins and RNA. In order to inactivate the very

stabile proteinase K, which can destroy added restriction enzymes, the plugs are treated with phenylmethylsulphonyl fluoride (PMSF) or 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (PEFA®SC). Cut plug portions are then treated with an appropriate restriction enzyme. The enzyme of choice should have recognition sites with lengths of six or eight bp. During enzyme digestion, DNA molecules are cut into a number of large fragments in the 20 to 2000 kb size range. For *L. monocytogenes* cleavage the most convenient enzymes are low-frequent cutters such as *Apa* I, *Sma* I, *Not* I and *Asc* I. After enzyme treatment, the plugs are placed into 1 to 1.5% agarose gel and run in a PFGE horizontal submarine gel box. To keep a constant temperature of buffer loaded in the box (12° - 15°C), recirculation is obtained by use of a cooling bath set at 8°C. The box has two independent perpendicular arrays of electrodes named north (N), south (S), east (E) and west (W). Alternate switching of the PFGE electrical current produces pulses between N-S and E-W for a chosen time (one second to more than five minutes), enabling migration of DNA through gel first to the south and then to the east. Electron microscopy of gel structure displays bundles of fibres with non-uniform voids in between, giving the appearance of a bath-tub sponge. When moving through the gel, DNA molecules must be stretched because their length is bigger than the gel pores. The end of each pulse will follow stretching of the molecule to the left along the orientation of the field. The first movement of stretched DNA molecules is by loops to the left of the field at an angle greater than 90° (Figure 5). By changing the field, these front loops will be trapped with the result that the next movement of the molecule will start with a free physical end - tail. Between each pulse change, the DNA spends part of the following pulse time in relaxing. Relaxation is proportional to DNA size and during that time the molecule changes shape and orientation. DNA molecules that spend most time in relaxation and least time in movement, obtain optimum separation as a result of being independent of molecular size and movement. During a relaxation time, smaller molecules will have enough time to change position and continue moving with the arrival of a new pulse. However, larger molecules will not get enough time to finish orientation, and thus the consequence will be less migration. When two DNA molecules of different length move through gel after the first pulse, the front loops have the same position. However, after the second pulse longer molecules will be distanced by shorter ones in proportion to their differences in length. When relaxation time becomes longer than pulse time, the larger DNA molecules will not react to the electrical field any more (Figure 5).

After PFGE separation of DNA molecules, fragments are visualized by staining with ethidium bromide. Photographing under UV light is followed by interpretation of PFGE patterns.

The method is highly effective in studying genetic relationships between bacterial isolates in epidemiological investigations, e.g. tracing a source of food



Long and short DNA fragments moving; the front end is trapped.

Reorientated DNA fragments held back. Movement led by back ends. Longer DNA needs more time for reorientation.

Movement by back end through the nearest pore. Longer DNA distanced by shorter ones in proportion to their differences in length.

Figure 5. Schematic illustration of DNA movement through gel under PFGE pulses.

contamination or a case of human listeriosis. In **Paper III** we could, by means of PFGE, show that direct plating from a food samples reveals more *L. monocytogenes* clonal types than revealed by an enrichment procedure. In **Paper IV**, PFGE helped us to find the source of illness among the food items contaminated with *Listeria*; i.e. medwurst collected from the patient's refrigerator. In the study of potential sources of human listeriosis in Sweden in **Paper V**, the PFGE method was used for characterisation and comparison of human and food isolates of *L. monocytogenes*. The results support the theory that there could be a connection between human cases of listeriosis and consumption of salmon and rainbow trout.

PCR-Single-Strand Confirmation Polymorphism (SSCP)

SSCP is a PCR-based method applied for determination of DNA point mutations for species- or subtype-screening. Sets of PCR primers are used to amplify short fragments within 16S rRNA for species-screening (Widjojoatmodjo et al., 1994) and within coding and non-coding regions of *hly*A, *mpl* and *prf* A locus for subtype-screening of *L. monocytogenes* (Lehner et al., 1997; Loncarevic et al., 1997). These three genes are coding for virulence factors and are clustered in the *L. monocytogenes* chromosome in the order *prf*A, *plc*A, *hly*, *mpl*, *act*A and *plc*B (Portnoy et al., 1992). After amplification of variable regions of 16S rRNA or a virulence gene by means of PCR primer sets, the product is denaturated into single stranded DNA with formamide and heating. Separation on a polyacrylamide gel is performed by electrophoresis. Generation of correct-sized single stranded products is detected by silver staining. By means of PCR-SSCP it was possible to discriminate 40 different species of bacteria, among other *Listeria* spp. (Widjojoatmodjo et al., 1994). Furthermore, this method was capable of discriminating between seven serovars among *L. monocytogenes* strains and also to identify additional diversity in strains of the same serovar (Loncarevic et al., 1997).

Concluding remarks

L. monocytogenes is a bacterium that can survive and also grow within a wide range of conditions. Optimal growth temperature is 30-37°C, but the bacteria can grow down to 1°C and up to 45°C. It can survive a pH value in the range 6 - 9. In addition, *L. monocytogenes* may be present in many raw foods, thus, the bacterium must be taken in earnest when discussing food safety. Atention must specially be drawn to foodstuffs which have been kept in refrigerators for weeks and are eaten without further heating.

In spite of frequent occurrence of *L. monocytogenes* in different food items, listeriosis is a rare human disease. However, after some large outbreaks with a high mortality rate *L. monocytogenes* has been classified as an "emerging foodborne pathogen". This thesis and also other studies show that soft cheese, particularly if they are made from raw milk often contain (large numbers of) *L. monocytogenes*. However, in Sweden vacuum-packaged gravad or cold smoked fish seem to be the most risky food. There has also been an outbreak of listeriosis due to gravad and cold-smoked rainbow trout in Sweden. To minimize the presence of *Listeria* in food, different control measures have been established. Utilization of HACCP, reduction of storage time and temperature, and freeze storage of certain ready-to-eat food items are effective methods for controlling *L. monocytogenes*.

In the case of routine investigation of food products for the presence of *L. monocytogenes*, an enrichment method and characterization of one isolate could be sufficient. In investigating of a human case of listeriosis it might be necessary to use two different isolation methods and to characterize several isolates with more than one typing method where at least one should be a molecular typing method. PFGE was shown to be a highly discriminative method for this use.

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