Listeria monocytogenes, a Food-Borne Pathogen

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To my parents (W. R. I. Peiris and W. J. W. Peiris)

Abstract

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Listeria monocytogenes is a Gram positive, aerobic, facultative anaerobic and non-acid fast bacterium, which can cause the disease listeriosis in both human and animals. It is widely distributed thoroughout the environment and has been isolated from various plant and animal food products associated with listeriosis outbreaks.

Contaminated ready-to-eat food products such as gravad and cold-smoked salmon and rainbow trout have been associated with human listeriosis in Sweden. The aim of this study was to analyse the occurrence and level of *L. monocytogenes* in gravad and cold-smoked salmon (*Salmo salar*) products packed under vacuum or modified atmosphere from retail outlets in Sweden. Isolated strains were characterized by serotyping and the diversity of the strains within and between producers were determined with PFGE (Pulsed-field gel electrophoresis). The characterized fish isolates were compared with previously characterized human strains.

L. monocytogenes was isolated from 11 (three manufacturers) of 56 products analysed. This included gravad salmon products from three manufacturers and cold-smoked salmon from one manufacturer. The highest level of *L. monocytogenes* found was 1500 cfu/g from a cold-smoked salmon product but the level was low (<100 cfu/g) in most of the products. Serovar 1/2a was predominant, followed by 4b. Three products of gravad salmon harboured more than one serovar. PFGE typing of the 56 salmon isolates detected five *Asc* I types: four types were identical to human clinical strains with *Asc* I and one was identical and one was closely related to human clinical strains with *Apa* I.

Isolation of identical or closely related *L. monocytogenes* strains from human clinical cases of listeriosis and gravad and cold-smoked salmon suggested that these kinds of products are possible sources of listeriosis in Sweden. Therefore, these products should be considered risk products for human listeriosis.

Key Words: *L. monocytogenes*, gravad and cold-smoked *salmo salar*, Food, PFGE

Contents

Introduction

Listeria monocytogenes, a Gram positive, facultative anaerobic and non-acid fast bacterium, is a non-spore forming rod that expresses a typical tumbling motility at 20-25ºC. It is widely distributed throughout the environment and is considered hardy bacteria because of their ability to grow over a wide range of temperatures (0.5-45ºC), pH (4.7-9.2) and osmotic pressures, which allows them to survive longer under adverse conditions. *Listeria* species have been isolated from various plant and animal food products associated with many listeriosis outbreaks; therefore, contaminated food are considered a primary source of transmission of infection in sporadic cases as well as outbreaks (Farber and Peterkin, 1991).

L. monocytogenes causes several illnesses in humans and animals: the annual incidence of human listeriosis ranges from 1.6 - 6 cases per million people (Rocourt *et al*., 2000). Predominant clinical manifestations of human listeriosis are meningitis, encephalitis, cerebritis and septicaemia: mortality rate in this group is as high as 90% in untreated cases and 27-30% in patients receiving therapy (Calder, 1997). In addition, endocarditis, as a consequence of valve prosthesis and HIV infection (Nieman and Lorber 1980; Spyrou *et al*., 1997), and pericarditis, with a mortality rate up to 50%, have been documented (Manso *et al*., 1997). Local inflammatory reactions, such as pyogenic abscesses in different organs, for example, eye, heart, bone and peritoneum can occur in *L. monocytogenes* infected people (Schlech, 1991; Hof *et al*., 1992). Recent reports of food associated listeriosis outbreaks describe *L. monocytogenes* as a pathogen causing febrile gastroenteritis in previously healthy people (Dalton *et al.,* 1997; Miettinen *et al*., 1999; Salamina *et al.,* 1996; Aureli *et al.,* 2000; Sim *et al.,* 2002; Carrique-Mas *et al*., 2003).

The present review offers a brief introduction to the organism *L. monocytogenes*, pathogenesis of listeriosis associated with gastrointestinal symptoms, CNS manifestations and septicaemia and reported listeriosis outbreaks mainly associated with gastrointestinal symptoms.

History

In 1911, Professor G. Hülphers described a bacterium that caused necrosis of the liver in rabbits, and because of its characteristic affinity to liver, it was named *bacillus hepatis* (Hülphers, 1911). In 1926, Murray, Webb and Swann isolated the same bacterium in pure cultures during a spontaneous epidemic disease of rabbits and it was named *Bacterium monocytogenes* because of the large mononuclear leucocytosis it caused (Murray *et al*., 1926). Pirie (1927) isolated the same organism from gerbilles (*Tetra lobengulae*): the disease caused was termed 'Tiger River Disease' as it was discovered near Tiger River in South Africa. The causative Gram positive organism was named *Listerella hepatolytica* the generic name being dedicated in honour of a British Surgeon, Sir Joseph Lister. In 1940, Pirie changed the name into *Listeria monocytogenes*: in 1948, *L. monocytogenes* was inserted under the same name in the 6th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al*., 1948). The first reported isolation of

L. monocytogenes from sheep was done in 1929 (Gill, 1933) and the first reported human isolation was in 1929 by Nyfeldt (1929).

Until 1961, *L. monocytogenes* was the only recognized species of the genus *Listeria*. Currently, there are six species under *Listeria*: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and *L. ivanovii*. subsp. *ivanovii*, and *L. ivanovii* subsp. *londoniensis* (Boerlin *et al*., 1992). Characteristics of *Listeria* species are presented in Table 1.

Morphology

Listeria bacteria are aerobic and facultative 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 with peritrichous flagella and occur singly or in short chains often represented by a 'V' or 'Y' shape. Fresh cultures of *Listeria* display Gram-positive staining and regular short rods, but older cultures may stain Gram-negative, with coccoid forms. The bacteria express a typical tumbling motility that is prominent at 20-25ºC.

Cultural characteristics

When cultured on nutrient agar (after 24 hours of incubation) colonies are round, 0.5-1.5 mm in diameter, translucent and with a smooth glistening surface (S-form). During prolonged incubation for 3-7 days, colonies may appear rough (R-form) and 3-5 mm in size. When the colonies are exposed to obliquely transmitted light they appear blue-green and under normal illumination they appear bluish grey. *L. monocytogenes* shows haemolysis on sheep blood agar, which distinguishes *L. monocytogenes* from other *Listeria* species. A narrow zone of β-haemolysis around colonies is characteristic of *L. monocytogenes*, weak β-haemolysis is characteristic of *L. seeligeri*, and wide or multiple zones of β-haemolysis are produced by *L. ivanovii.*

Growth limits

Optimum temperature for *L. monocytogenes* is 30-37ºC but the growth temperature ranges from 0.5ºC (Juntilla *et al*., 1988) to 45ºC (Petron and Zottola, 1989). *L. monocytogenes* can grow at temperatures as low as -2ºC in laboratory media broth (Bajard *et al*., 1996). In vacuum-packed smoked blue cod, growth has been observed at -1.5ºC (Bell *et al*., 1995). *L. monocytogenes* in milk does not survive heating at 69ºC and above for 16.2s. In a majority of cases, heating at 70ºC for 2 min inactivates *L. monocytogenes* in meat. Exposure of milk and meat to sublethal temperatures of around 44-48ºC before the final temperature can enhance the thermotolerance of the cells. This implies that temperature fluctuation can enhance the thermotolerance of *L. monocytogenes*. (Farber and Peterkin, 1991).

L. monocytogenes can grow at very low levels of pH, as low as 4.3 when HCl is used as the acidulant (George *et al*., 1996). However, in a study conducted by Tienungoon *et al* (2000) it was found that *L. monocytogenes* Scott A and L5 were able to grow at pH levels of 4.23 and 4.25.

L. monocytogenes requires carbohydrate as the primary energy source for growth, with glucose being the preferred source (Pine *et al*., 1989; Premaratne *et al*., 1991). Metabolic end-products differ under aerobic and anaerobic conditions. Lactate and acetate is produced under both aerobic and anaerobic conditions, but acetoin is produced only under aerobic conditions (Romick *et al*., 1996). The organism can grow in the presence of CO_2 at low temperatures but CO_2 concentrations above 70% inhibit the growth of *L. monocytogenes* at temperatures <7ºC. Even at high CO_2 concentrations (70%) and in the presence of just 5% O_2, *L. monocytogenes* is able to grow (Wimpfheimer *et al*., 1990).

Table 1. Characteristics of the species included in the genus *Listeria* (Seeliger and Jones, 1986, Boerlin *et al*., 1992)

Charcteris-tics	**L. monocy-togenes**	**L. inno-cua**	**L. seeli-geri**	**L. welshi -meri**	**L. grayi**	**L. ivanovii subsp. ivanovi**	**L. ivanovii subsp. london-iensis**
Tumbling motility	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Haemolysis	+	-	+	-	-	+	+
CAMP-test (Staph. aureus)	+	-	+	-	-	-	-
CAMP-test (Rhodo-coccus equi)	-	-	-	-	-	+	+
L-rhamnose	+	d	-	d	-	-	-
D-xylose	-	-	+	+	-	+	+
Hippurate	+	+			-	+	+
Ribose					-	+	-
N-acetyl-B-D-mannos-amine					-	-	+
Pathogenici-ty for mice	+	-	-	-	-	+	+

Human listeriosis

Incubation period and infectious dose

Listeria monocytogenes is widely distributed throughout the environment and has been isolated from soil, plants, and sewage. Moreover, it has been isolated from faeces, milk, uterine discharges of healthy, sick, and convalescent humans and animals.

Contaminated food has been considered as the primary source of infection in epidemics (Table 2 and 3) as well as in sporadic cases; therefore, the gastrointestinal tract is the primary site of entry of the *L. monocytogenes* into the host. The main route of acquisition of *L. monocytogenes* is ingestion of contaminated food products, although, the development of disease depends on the size of the inoculum, virulence of the strain, and susceptibility of the individual. Incubation period for illness varies from days to weeks in reported outbreaks. In listeriosis outbreaks mainly associated with gastrointestinal symptoms, clinical course of infection has developed about 24 hours after the ingestion of contaminated food (Dalton *et al*., 1997; Aureli *et al*., 2000; Sim *et al*., 2002; Frye *et al*., 2002). In sporadic cases and outbreaks of listeriosis mainly associated with CNS manifestations and septicaemia, clinical symptoms has developed 24 hours after the ingestion of contaminated food (Juntilla *et al*., 1989; McLauchlin *et al*., 1990). Incubation period as long as 31-35 days have also been reported in an epidemic but with a wide range of 1-91 days (Linnan *et al*., 1988).

The minimum dose of bacteria required to cause clinical infection is influenced by many variables including: immune status of the host; virulence of the microorganism; type and amount of food consumed; and concentration of the pathogen in the food. The infectious dose for listeriosis in either immunocompetent or susceptible humans or animals has not yet been determined.

In documented outbreaks, contamination levels as low as 10^2-10^4 cells per gram of food have caused severe clinical disease (Linnan *et al*., 1988). In a case of listeriosis in a cancer patient, >1100 *L. monocytogenes* cells per gram were found in implicated food (Wenger *et al*., 1990). Out of the 12 reported listeriosis outbreaks mainly associated with gastrointestinal symptoms, the lowest level found in food was 1.9×10^5 cfu/g (Miettinen *et al*., 1999). In an experiment conducted on healthy *Cynomolgus* monkeys, only the monkeys receiving 10^9 cells developed noticeable signs of illness (Farber *et al*., 1991), suggesting a high infective dose. However, because of the elapsed time between diagnosis and the enumeration of *L. monocytogenes* in the food consumed, these numbers should be interpreted with care as the organism could have multiplied or died during that period (Vazquez-Boland *et al.*, 2001).

Serovars involved

Human listeriosis can be caused by all 13 serovars of *L. monocytogenes,* but serovars 1/2a, 1/2b and 4b account for more than 90% of both human and animal cases (Farber and Peterkin. 1991; Schuchat *et al*., 1991). In gastrointestinal outbreaks serovars 1/2a and 1/2b are predominantly involved in causing the illness (Table 2), whereas, serovar 4b is mainly involved in listeriosis outbreaks

associated with CNS manifestations, foetomaternal cases and septicaemia (Table 3).

Host risk factors

Host susceptibility has a major influence on disease acquisition and clinical outcome of the disease. The majority of exposed patients have an underlying condition (mainly a physiological or pathological defect) that affects cell-mediated immunity and predisposes to listeriosis. Risk factors include immunosuppressive therapy (steroids), diseases (HIV infection, malignancy), and physiological status (elderly adults, pregnancy). In addition splenectomy, antacid and cimetidine therapy, liver diseases particularly cirrhosis (Nieman and Lorber, 1980) have been associated with the acquisition of the disease. Even though listeriosis has been considered as a disease mainly occurring in patients with underlying conditions, there are reports of disease in healthy adults without any predisposing factors (Büla *et al.*, 1995; Goulet *et al*., 1998; Zuniga *et al*., 1992).

L. monocytogenes in the GI (gastrointestinal) tract

Colonization of the GI tract

L. monocytogenes, as well as the non-pathogenic *L. innocua* has the ability to colonize the GI tract of humans, which means the bacteria have to survive in the low pH in the stomach, elevated osmolarity and the presence of bile salts within the small intestine. The bacteria are shed in the faeces of 1-5% of normal, asymptomatic individuals (reviewed in Hof, 2001), which confirms the survival of bacteria in the GI tract. Different mechanisms that influence bacterial survival in low pH, elevated osmolarity, and the presence of bile in the GI tract have been reviewed by Gahan and Hill (2005).
In an animal experiment, reduction of gastric acidity by cimetidine treatment lowered the infective dose in rats (Schlech *et al*., 1993). The use of antacids and H_2 blocking agents also help *L. monocytogenes* to survive in the stomach (Shuchat *et al*., 1992; Ho *et al*., 1986). Recent studies of murine models (Briones *et al*., 1992; Hardy *et al*., 2004) demonstrate that bacteria replicate extracellularly within the lumen of gallbladder, which confirms bacterial survival in bile and the ability to resist high concentrations of bile.

Invasion of the GI tract

The major point of entry of *L. monocytogenes* in humans and the mechanism of translocation has not yet been fully elucidated. In an experiment conducted with guinea pigs (Rácz *et al*., 1972), animals were infected with *L. monocytogenes* intragastrically and developed gross changes in the GI tract as early as 9 hours after infection. *L. monocytogenes* was found throughout small intestinal epithelium, predominantly in the lower ileum where it multiplied before being phagocytized by macrophages. At 22 hours, the number of bacteria decreased in the apex and midvillous and most were in macrophages of the villous stroma, where even polynuclear leukocytes phagocytized them. Invasion of intestinal epithelium by *L. monocytogenes* is further demonstrated in an *in vitro* experiment conducted using

human enterocyte-like cell-line Caco-2 cells: *L. monocytogenes* entering the cells through the apical surface is presented (Karunasagar *et al*., 1994).
Colonization of *L. monocytogenes* in Payer's patches has been observed in mice (MacDonald *et al*., 1980; Marco *et al*., 1992; Marco *et al*., 1997). This suggests the penetration of M-cells, epithelium overlying Payer's patches, as a portal of entry, which has been confirmed by experiments conducted by Jensen *et al*., (1998).

Intracellular infectious cycle.

The cycle begins with adhesion to the host cell and subsequent penetration. Once the bacteria invade cells, macrophages or non-phagocytic cells, such as epithelial cells, fibroblasts, hepatocytes, endothelial cells and nerve cells, they undergo an intracellular life cycle. *L. monocytogenes* invades non-phagocytic cells by a zipper-type mechanism, in which the host cell engulfs the bacteria and the cell membrane surrounds the bacterial cell forming a phagocytic vacuole. After 30 min the phagosome membrane is lysed by the bacteria and a fine, fibrillar material composed of actin filaments immediately surrounds bacterial cell in the cytoplasm. These filaments form an actin tail up to 40 μm long at one pole of the bacterium that propels the bacterial cell into the cytoplasm at an average velocity of 10-15μm/min, but can be as fast as 36 μm/min. In contact with the cell membrane, a finger-like protrusion is formed with the bacterium at the tip. Uninfected neighbouring cells are penetrated by these protrusions, which are engulfed by phagocytosis forming a secondary phagosome. The bacteria are covered with two cell membranes, the donor cell forms an inner membrane and the newly infected cell forms an outer membrane. Bacteria rapidly dissolve these membranes (within 5 min) and initiate another round of intracellular proliferation in the cytoplasm, and thus, spread from cell to cell (Gouin *et al*., 2005; Cossart *et al*., 2003; Vázquez-Boland *et al*., 2001).

Virulence Factors

(reviewed by Vázquez-Boland *et al*., 2001; Portnoy *et al*., 1992; Cossart *et al*., 2004; Dussurget *et al*., 2004).

Virulence factors involved in adhesion and invasion

There are different proteins involved in adhesion and invasion of host cells. Internalin A (InlA) and B (InlB) were the first proteins to be identified and are expressed on the surface (Dramsi *et al*., 1995; Gaillard *et al*., 1991).

InlA
InlA is composed of 800 amino acids, promotes entry into epithelial cells and plays a major role in crossing the intestinal barrier. Its receptor is E-cadherin, which is a transmembrane glycoprotein, and the interaction between InlA and E-cadherin is species specific. Clinical strains of *L. monocytogenes* expresses a full length InlA far more frequently (96% of the cases) than strains recovered from food products (65% cases), suggesting that InlA has a critical role in pathogenesis of human listeriosis, and can be used as a marker of virulence in food assessments.

InlB
InlB is composed of 630 amino acids, promotes entry into many different cell lines, including hepatocytes and nonepithilial cells. Hepatocyte growth factor or Met has been recognized as the main receptor for InlB on target cells.

Ami
Ami is a protein with lytic activity on the *L. monocytogenes* cell wall and is involved in cell adhesion.

p60
p60 is a protein involved in cell wall hydrolase.

FbpA
FbpA is involved in efficient colonization of the bacteria in the spleen and liver in mouse. It modulates the levels of listeriolysin O, InlB and thereby prevents degradation of some virulent proteins.

Listeriolysin O (LLO)
LLO is a pore forming protein involved in helping *Listeria* escape from primary and secondary phagosomes. In addition, LLO triggers many cellular responses, such as interleukin-1 secretion in macrophages, apoptosis, cell adhesion, and protein expression in infected epithelial cells. Many of these functions are Ca^{+2} dependent.

ActA
ActA is a protein involved in attachment and entry into target cells.

Sortases
Sortases are transpeptidases involved in cell wall anchoring of surface proteins and they contribute to virulence.

Auto
Auto is a surface protein with autolytic activity and is involved in invasion of eukaryotic cells. Auto is involved in both early and late stages of the infection process, but is absent in *L. innocua* and in *L. monocytogenes* serovar 4b.

Bile Salt Hydrolase
An enzyme that deconjugates conjugated bile salts. Bile salt hydrolysis is a mechanism to protect *L. monocytogenes* from bile salt toxicity.

Virulence factors involved in intracellular life cycle

LLO and two phospholipases C
LLO, together with two phospholipases play a critical role in the bacterial escape from primary and secondary intracellular vacuole. The two phospholipases include phosphatidylinositol-specific phospholipase C (PI-PLC) and Phosphatidylecholine specific phospholipase C (PC-PLC).

ActA
ActA is a protein with 639 amino acid and it induces polymerisation of the actin filaments promoting movement of bacteria in the cytoplasm of infected cell and cell-to-cell spread.

Hexsose-phosphate transporter (Hpt)
L. monocytogenes uses glucose-1-phosphate, a phosphate sugar available in the cytoplasm. This process is enhanced by PrfA-dependent expression of a hexose phosphate transporter (Hpt). PrfA is a protein that regulates in particular, the *Listeria* virulent genes.

Metalloprotease
Metalloprotease is a zinc-dependent protein that activates phospholipases and host-cell cystein protease. Metalloprotease encoded by *mpl* aids maturation of PC-PLC, which is synthesised as a proenzyme.

L. monocytogenes in the blood and liver

L. monocytogenes crossing the intestinal barrier into the lymph or blood are transported to mesenteric lymph nodes, the spleen and the liver (Marco *et al*., 1992). *Listeria* principally replicates in the liver, which captures >90% of the inoculum, and spleen, which captures most of the remainder. Bacteria in the blood are rapidly cleared from the blood stream by resident tissue macrophages, kupffer cells, in the liver of experimentally infected mice (Conlan and North, 1991). Kupffer cells play a critical role in the development of immunity to infection (Gregory and Wing, 1990), and can promote the secretion of many factors including interlukin (IL-6, IL-1β), tumour necrosis factor (TNF)-α, and nitric oxide, which are capable of promoting inflammatory response and antimicrobial activity (Cousens and Wing, 2000).

Bacteria multiply in the hepatocytes and spread cell-to-cell forming infectious foci. This type of spread prevents the bacteria to coming into contact with the humoral immunity. Hepatocytes respond to *Listeria* infection by releasing neutrophil chemotactants: neutrophil aggregation kills bacteria, lyses infected host cells, stimulates hepatocyte apoptosis and secretes cytokines that suppress intracellular replication in hepatocytes. This type of neutrophil-mediated defence mechanism can reduce >90% of bacterial growth in the liver during the first 24 hours of infection (Conlan and North, 1991). *Listeria,* which are not killed by neutrophils, are internalised by hepatocytes and undergo intracellular replication. Migrating T-cells rapidly eliminate infected hepatocytes. The antilisterial activity of T-cells is assisted by TNF-α and interferon (IFN)-γ (Gregory and Liu, 2000). If *Listeria* still survive due to inadequate immune response, rapid proliferation of *L. monocytogenes* in the liver hepatocytes may result in the release of bacteria into the blood stream resulting in infection of a wide range of host tissues.

L. monocytogenes in the brain

Listeriosis associated with CNS (central nervous system) infection ranges from meningitis to meningoencephalitis. In ruminants, mainly the rhombencephalon is

affected: rhombencephalitis in ruminants is characterized by unilateral nerve paralysis leading to circling disease syndrome (J.C. Low and Donachie, 1997). However, the mechanism by which *L. monocytogenes* enters the CNS is poorly understood and various animal experiments provide evidence of different routes of infection.

Intravenous inoculation occasionally results in CNS infection in sheep, whereas, intracarotid inoculation consistently results in encephalitis, suggesting the blood as the route of infection (Cordy *et al*., 1959; Jungherr *et al*., 1937; Olson *et al*., 1950). Neurological signs developed in mice after infection via intranasal and conjunctival routes, suggest the bacteria enter the brain by migration along the cranial nerves (Asahi *et al*., 1957).

Experiments with rat dorsal root ganglia and hippocampal neurons, in culture (Dons *et al*., 1999) and clinical cases of ovine listerial encephalitis (Charlton, 1977) provide evidence of *L. monocytogenes* ability to infect axons and dorsal root ganglia nerve cell bodies and migrate in a retrograde as well as anterograde direction. Intra-axonal spread of *L monocytogenes* is demonstrated in mouse model (Otter and Blakmore, 1989). In an experiment, 6 out of 21 sheep developed neurological signs after injection of *L. monocytogenes* into dental pulp (Barlow and McGorum, 1985). In naturally infected humans and animals, *Listeria* have to cross the intestinal barrier and escape clearance mechanisms by the liver and spleen; therefore, invasion of the CNS is dependent on the concentration of bacteria in the blood and persistent bacteraemia (Berche, 1995; Blanot *et al*., 1997).

L. monocytogenes in the gravid uterus

L. monocytogenes has a unique predilection for pregnant women which may result in serious outcomes such as abortion, still birth, or severe neonatal infections. Depressed cell-mediated immunity during gestation plays an important role in the development of listeriosis (Weinberg 1984). In an experimental murine model of pregnancy-associated listeriosis (Abram *et al*., 2002), impaired maternal immune response facilitates bacterial multiplication in the liver, placenta and foetal tissue. Listeriosis may occur at all stages of gestation but predominantly during the third trimester. *L. monocytogenes* gains access to the foetus by a haematogenous route by penetrating the placental barrier. In a guinea pig model, tropism of *L. monocytogenes* to the placenta and invasion, growth, and cell-to-cell spread of trophoblasts and trophoblast-derived cell lines has been demonstrated (Bakardjiev *et al*., 2004).

The common clinical manifestations of infected pregnant women include, mild flu-like illness with fever, headache, myalgia, arthralgia and malaise and occasionally gastrointestinal symptoms including abdominal pain and diarrhoea. There are few case reports of *Listeria* meningitis and meningoencephalitis in pregnant women (Boucher and Yonekura, 1984; McLauchlin *et al*., 1990).

Table 2. **Reported listeriosis outbreaks mainly associated with gastrointestinal symptoms.**

Year	Place	No. of cases	Vehicle	Sero group	Illness duration	No. of (+) blood samples for L.m.	No.of stool samples (+)	Incubation period	Cfu/g food	Author
1979	USA	23	NR	4b	NR	18/20	NR	NR	NR	1
1989	USA	2	Shrimp	4b	NR	2 / 2	0 / 2	2 days	NR	2
1993	Italy	18	Rice salad	1/2b	NR	2 / 4	NR	18 hours (11-60h)	NR	3
1994	USA	45	Chocolate milk	1/2b	NR	3*/ 4	11 / 41	20 hours (9-31 h)	$1.2x10^9$ - 8.8×10^8	4
1996	Denmark	3	NR	4	NR	1 / 1	3/ 3	NR	NR	5
1996	Canada	2	Imitation crab meat	1/2b	NR	1 / 1	2/ 2	12-18 h	2.1×10^9	6
1997	Italy	1566	Maize and tuna salad	4b	3 days (1-7 days)	1/ 40	123 / 141	24 h (6-51 h)	10^6	7
1999	Finland	5	Smoked rainbow trout	1/2a	NR	NR	2/ 2	27 h	1.9×10^5	8
2000	New Zealand	30	Corned beef and ham	1/2	1.5 - 7 days	NR	30/ 30	24 h (12-101 h)	1.8×10^7	9
2001	Sweden	>120	Cheese	1/2a	NR	NR, one L. m. joint abscess.	27 / 32	31 h (10-240 h)	6.3×10^7	10
2001	USA	16	Turkey	1/2a	7 days (2-21 days)	NR	6 / 8	25 h (6-49 h)	10^7	11
2001	Japan	38	Cheese	1/2b	NR	NR	19 / 32	<24- >144 h	$9.3x\ 10^8$	12

1. Ho *et al.,* 1986; 2. Riedo *et al.,* 1994; 3. Salamina *et al.,* 1996; 4. Dalton *et al.,* 1997; 5. Heitmann *et a.,* 1997; 6. Farber *et al.,* 2000; 7. Aureli *et al.,* 2000; 8. Miettinen *et al.,* 1999; 9. Sim *et al.,* 2002; 10. Carrique-Mas *et al.,*2003; Danielsson-Tham *et al*., 2004; 11. Frye *et al.,* 2002; 12. Makino *et al*., 2005.
NR- Not reported, L. m. – *Listeria monocytogenes*. * - 3 additional patients found by surveillance not included in the picnic.

Table 3. **Reports of listeriosis outbreaks mainly associated with CNS manifestations and perinatal infection**.

Year	Place	No. of cases	Food associated	Sero group	Incubation period	cfu/g of food	Author	Remarks
1980	New Zealand	29	Shellfish and raw fish.	1b	NR	NR	1	22 perinatal infections, 5 foetal deaths: common symptoms were flu and urinary tract symptoms.
1981	Canada	41	Coleslaw	4b	NR	NR	2	34 perinatal listeriosis and 7 adults. 5 abortions, 4 stillbirths. No patient had evidence of any underlying immunosuppressive condition.
1983	USA	49	Pasteurised milk	4b	NR	NR	3	42 immunosuppressed adults and 7 foetuses or newborn involved. Case fatality rate - 29%
1985	USA	142	Cheese	4b	35 or 31 days	NR	4	Pregnant women, neonates or foetuses predominantly affected. 10 neonatal and 20 foetal deaths. 98% had predisposing factors.
1986	Austria	28	Raw milk, Vegetables	½ a 4b	NR	NR	5	Out of 28 patients, 24 were new born. 4 adults, only one showed compromised host defence.
1983-1987	Switzerland	122	Milk, soft cheese	4b	NR	NR	6	65 occurred in newborn infants and pregnant women, 57 in adults. Out of the 57, 24 had underlying condition and mortality rate was 32%.
1986-1987	USA	36	Ice cream or salami	4b, 1/2a ½ b, 3b	NR	NR	7	32 adults and 4 new born, one third of the patients had vomiting and diarrhoea.
1987-1989	U.K.	>300	Paté	4b	NR	NR	8	Thirteen of 15 patients had eaten paté within 3 weeks before onset of disease.
1993	France	38	Rillettes (Pork pate)	4b	21 days	<100cfu/g - 10^4	9	Case fatality rate 32%. 31 materno-neonatal case patients. 9 foetal deaths. 2 healthy adults with CNS form of the disease.
1994	Sweden	9	Rainbow trout fish	4b	NR	<100cfu/g - 6200cfu/g	10	All the patients were immunosuppresed.

1. Lennon *et al*., 1984; 2. Schlech *et al.*, 1983; 3. Fleming *et al*., 1985; 4. Linnan *et al*., 1988, James *et al*., 1985; 5. Allerberger & J.P. Guggenbichler. 1989; 6. Büla *et al*., 1995; 7. Schwartz *et al*., 1989; 8. J.McLauchlin *et al*., 1991; 9. Goulet *et al*., 1998; 10. Ericsson *et al*., 1997

L. monocytogenes as a causative agent of febrile gastroenteritis

Reported listeriosis outbreaks mainly associated with gastrointestinal symptoms are listed in Table 2. Ho (1986) describes an outbreak occurring in 1979, where 65% of people suffered from gastrointestinal symptoms and 40% from meningitis. Out of the 13 people with diarrhoea, 12 had septicaemia. Vomiting, abdominal pain, or diarrhoea occurred within 72 hours before onset of fever. Important features of this outbreak are the occurrence of disease in less immunosuppressed people and the association of antacid and cimetidine therapy in the acquisition of the disease.

In 1989, two cases of listeriosis occurred in pregnant women attending the same party (Riedo *et al.,* 1994): one had diarrhoea four days after eating the implicated food and the other had headache and myalgia three days after. *L. monocytogenes* was isolated from blood samples of both the patients after 23 and 29 days. Seventeen (47%) of 36 partygoers reported having at least one gastrointestinal symptom. Out of the 25 stool samples collected after six weeks, only one was positive for *L. monocytogenes*.

In an outbreak occurring in Italy in 1993 (Salamina *et al*., 1996), out of 39 persons who attended a dinner party, 18 developed gastrointestinal symptoms and two of the four hospitalised had bacteraemia.

In 1994, in an outbreak of febrile gastroenteritis (Dalton *et al*., 1997) 75% (45 of 60) attendees at a picnic developed symptoms after consuming contaminated milk. The strain isolated from the patients, is indistinguishable from the strains found in unopened cartons of milk, environmental specimens and the dairy supplying the milk. Serological studies conducted among 48 of the picnic attendees revealed the highest anti-listeriolysin O level, and the people with mild illness, asymptomatic people, and the control group had comparatively lower levels. During surveillance *L. monocytogenes* was isolated from the blood samples of a 2-year-old girl, 81-year-old man and from a cerebral abscess in a 72-year-old man; the latter having gastrointestinal symptoms three weeks before hospitalisation. All the isolates were indistinguishable from the strains identified in the picnic outbreak.

In 1996, two small outbreaks associated with gastrointestinal symptoms occurred in Denmark and Canada: in the Danish outbreak (Heitmann *et al*. 1997), three-stool samples and one blood sample collected from the three diseased were positive for *L. monocytogenes*. The Canadian outbreak was due to the consumption of imitation crabmeat, and both the blood samples taken from the hospitalised person and the stool samples from both patients were positive for *L. monocytogenes* (Farber *et al*., 2000).

The largest outbreak of febrile gastrointestinal listeriosis was reported in 1997 from Italy (Aureli *et al.,* 2000): 1566 students and staff members of two primary schools had eaten at cafeterias served by the same caterer, 292 were hospitalised. One of the 40 blood cultures and 123 of the 141 stool specimens were positive for *L. monocytogenes*: the strains isolated from the contaminated food (maize and tuna salad), from hospitalised patients and from the environment were indistinguishable. In 1999, another small outbreak of febrile gastroenteritis, five persons, occurred in Finland after the consumption of cold-smoked rainbow trout. Two stool samples

collected a week after the onset of symptoms, were positive*: L. monocytogenes* isolates from the stool samples and cold-smoked rainbow trout were indistinguishable (Miettinen *et al*., 1999).

A series of incidents of febrile gastroenteritis, linked to the consumption of corned beef and ham, occurred in New Zealand in 2000. All strains isolated from the suspected food and the cases were indistinguishable, suggesting that all cases were part of series of incidents where ready-to-eat meat products were the cause (Sim *et al*., 2002).

In 2001, an outbreak of febrile gastroenteritis occurred in at least 120 people after the consumption of on-farm manufactured dairy products from a summer farm (Carrique-Mas *et al*., 2003; Danielsson-Tham *et al*., 2004). The affected group included three pregnant women and a 64-year-old woman with chronic arthritis and undergoing immunosuppressive therapy with corticosteroids. Only one of the pregnant women developed symptoms. All three women were treated with amoxicillin and gave birth normally. The 64-year-old woman developed diarrhoea as well as a septic *L. monocytogenes* joint abscess. Twenty-seven of 32 stool samples from the case patients yielded *L. monocytogenes*, compared to 9 of 11 non-cases. All the isolates from the human cases, food products involved, dairy animals, environment and equipment shared the same serovar and PFGE profiles (Danielsson-Tham *et al*., 2004).

Another outbreak of acute febrile gastroenteritis occurred among 16 of 44 attendees of a catered party in Los Angeles (Frye *et al*., 2002). Six of the eight stool samples collected, median seven days after the onset of symptoms, tested positive for *L. monocytogenes*. Of the two cases with negative stool samples, one had begun receiving empirical treatment with antibiotics before stool collection.

Makino *et al*. (2005) reported the first documented food-borne listeriosis outbreak in Japan. Thirty-eight people who consumed the implicated cheese developed gastroenteritis. Isolates from those patients exhibited the same serotype (1/2b), DNA fingerprinting patterns and PCR amplification patterns.

Important features of listeriosis associated with febrile gastroenteritis

- Contaminated food appears to be the source of infection. In most of the outbreaks *L. monocytogenes* isolates from the patients were indistinguishable from the strain identified from the associated food.
- Mainly previously healthy people are affected.
- In most outbreaks, >70% of patients had suffered from gastrointestinal symptoms such as diarrhoea, vomiting, nausea, and abdominal pain. Other symptoms reported were fever, muscle pains and headache.
- The incubation period is usually short, 24 hours or less, ranging from 6 hours to 10 days (Table 3).
- High infectious doses. The lowest number of bacteria found in the food that have caused febrile gastroenteritis was 1.9×10^5 cfu/g where as the highest number was 2.1×10^9 cfu/g.
- Eight out of 12 outbreaks were due to serogroup 1/2 and four due to serogroup 4.

As most of the gastrointestinal listeriosis outbreaks reported have been in immunocompetent people, it can be suggested that the mild illness in this group is the initial phase of infection, which can progress into more severe clinical manifestations in individuals with altered immunity (Riedo *et al*., 1994). Sometimes gastroenteritis goes undocumented when the disease does not progress into severe clinical manifestations, or may be overlooked when another more life threatening infection is present (Hof, 2001). However, gastrointestinal symptoms before the onset of CNS manifestations and septicaemia have been reported (Ho *et al.*1986; Juntilla *et al*., 1989; Schwartz *et al*., 1989).

When routine stool cultures fail to identify the cause of febrile gastroenteritis, *L. monocytogenes* should be considered as a possible agent (Ooi and Lorber, 2005). Bacteria are cleared from the blood within minutes after intravenous inoculation and are eliminated from the infected tissues without subsequent bacteraemia, except for high bacterial challenges. This may be the reason why *L. monocytogenes* is rarely isolated from the blood of people with gastrointestinal listeriosis and why CNS infections remain rare in immunocompetent people. Moreover, during listeriosis epidemics, the number of human cases remains few compared to the number of persons exposed to the same highly contaminated food products (Farber and Peterkin, 1991).

In a study conducted on strains from gastroenteric listeriosis and invasive listeriosis, no significant difference in the virulent related molecules, such as PrfA, PlcA, *hly*, *mpl*, PlcB, inlA, and inlB, were detected. Moreover, all the strains were equally pathogenic following intra-peritoneal inoculation of mice, suggesting that the strains were comparable in terms of mean oral infectivity. However, this information is not sufficient to conclude that strains of gastrointestinal listeriosis and invasive listeriosis are identical because more genes essential for the virulence of *L. monocytogenes* continue to be identified (Franciosa *et al*., 2005).

It could be questioned if listeriosis mainly associated with febrile gastroeneteritis is a non-invasive disease, or whether it is more invasive than supposed. Gastrointestinal listeriosis should not be underestimated: stool culturing as well as blood culturing should be performed as the diagnosis of the presence of *L. monocytogenes* can help prevent progression into more severe form of illness with appropriate therapy initiated in the case of bacteraemia.

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Research Report

Gravad and Cold-Smoked Salmon, a Potential Source of Listeriosis.

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Abstract

Gravad or smoked (hot or cold-smoked) salmon and rainbow trout have been associated with listeriosis in Sweden. The aim of this study was to analyse the occurrence and level of *L. monocytogenes* in gravad and cold-smoked salmon (*Salmo salar*) products packed under vacuum or modified atmosphere from retail outlets in Sweden. Isolated strains were characterized by serotyping and the diversity of the strains within and between producers were determined with PFGE (Pulsed-field gel electrophoresis). The characterized fish isolates were compared with previously characterized human strains.

L. monocytogenes was isolated from 11 (three manufacturers) of 56 products analysed. This included gravad salmon products from three manufacturers and cold-smoked salmon from one manufacturer. The highest level of *L. monocytogenes* found was 1500 cfu/g from a cold-smoked salmon product but the level was low (<100 cfu/g) in most of the products. Serovar 1/2a was predominant, followed by 4b. Three products of gravad salmon harboured more than one serovar. PFGE typing of the 56 salmon isolates detected five *Asc* I types: four types were identical to human clinical strains with *Asc* I and one was identical and one was closely related to human clinical strains with *Apa* I.

Isolation of identical or closely related *L. monocytogenes* strains from human clinical cases of listeriosis and gravad and cold-smoked salmon suggested that these kinds of products are possible sources of listeriosis in Sweden. Therefore, these products should be considered risk products for human listeriosis.

Key Words: *L. monocytogenes*, gravad and cold-smoked *salmo salar*, Food, PFGE

Introduction

Since the 1980s, *Listeria monocytogenes* has been recognized as a food-borne pathogen that causes listeriosis in human beings. Even though human listeriosis is relatively rare, with an incidence ranging from 1.6 - 6 cases per million of population (J. Rocourt *et al*., 2000), fatality rate of listeriosis is as high as 20-30%. *L. monocytogenes* is of major concern for the food industry due to its ability to adapt and tolerate a wide range of temperatures and pH and to form biofilms (Jeong and Frank, 1994; Bansal, 1996; Tello *et al*., 1997; Lundén *et al*., 2000).

Food-borne outbreaks of listeriosis in humans are associated with a wide variety of food products, such as milk products (Fleming *et al*., 1985; Dalton *et al*., 1997; Linnan *et al*., 1988; Danielsson-Tham *et al*., 2004), vegetables (Schlech *et al*., 1983), meat products (McLauchlin *et al*., 1991; Goulet *et al*., 1998) and fish products (Brett *et al*., 1998; Ericsson *et al*., 1997; Miettinnen *et al*., 1999; Farber *et al*., 2000). Reported studies have identified ready-to-eat food, stored at refrigeration temperature for a long period as food at high risk, as it promotes the growth of *Listeria*. Furthermore, products that are vacuum-packed, ensuring long shelf life, also allow *L. monocytogenes* to multiply to hazardous levels during storage (Guyer and Jemmi, 1991; Loncarevic *et al*., 1996).

Gravad or smoked (hot or cold-smoked) salmon and rainbow trout are popular ready-to-eat dishes in Sweden. They can be packed under vacuum or modified atmosphere and are consumed without prior heat treatment. These food types have been associated with human listeriosis in Sweden (Ericsson *et al*., 1997; Loncarevic *et al*., 1998). Gravad fish is prepared from raw salmon, fillets are rubbed with a mixture of sugar, salt and pepper covered with dill and stored in the refrigerator for two days in a plastic bag. Fillets are then packaged sliced or whole under vacuum or modified atmosphere. In the preparation of cold-smoked fish, fillets are rubbed with salt or the cure is injected with multiple needles into the fillet. It is then smoked at 25-30ºC for 2-3 hours and packed sliced or whole under vacuum or modified atmosphere. These products are generally stored up to 3-4 weeks at 4ºC.

The aim of this study was to analyse the occurrence and level of *L. monocytogenes* in gravad and cold-smoked salmon (*Salmo salar*) products packed under vacuum or modified atmosphere from retail outlets in Sweden. Isolated strains were characterized by serotyping and the diversity of the strains within and between producers were determined with PFGE (Pulsed-field gel electrophoresis). The characterized fish isolates were compared with previously characterized human strains.

Materials and Methods

Fifty-three salmon (*Salmo salor*) products were purchased between June and September 2005 from retail stores in Sweden, and three were bought in Germany.

The purchases were from 16 manufacturers and included 30 vacuum-packed gravad, 1 modified-atmosphere gravad, 19 vacuum-packed cold-smoked and 6 modified-atmosphere cold-smoked products. All products purchased in Sweden were kept under refrigeration (4ºC) and analysed on the recommended best before day. The German products were frozen for 16 hours and kept at room temperature for 10 hours during transportation to Sweden, and refrigerated until analysis on the recommended best before day.

Enrichment and culture

Enrichment and cultural procedures for isolation and detection of *L. monocytogenes* were according to the Nordic method (NMKL, 2004), with some modifications. From each product, 25g was transferred to a stomacher bag and macerated with the help of a rolling pin. Enrichment broth I was added and the suspension was incubated at 30ºC. After 24 hours, a second enrichment was performed and incubated at 30ºC for 24 hours. From the second enrichment, 0.1ml was inoculated onto the surface of Palcam and Oxford agar. Plates were incubated at 37ºC and observed for growth after 24-48 hours.

Quantification

All products to be analysed were stored in the freezer (-20ºC) awaiting the quantification procedure. Only samples that tested positive for *L. monocytogenes* after enrichment were quantified according to the following procedure. A 10g of fish sample was macerated in a stomacher bag with the help of a rolling pin and 100ml of peptone water was then added. Ten fold serial dilutions of 1ml of the macerate were made and each dilution was surface-plated onto Oxford, Palcam and horse-blood agar plates. All plates were incubated at 37ºC for 48 hours and presumptive *L. monocytogenes* colonies were counted.

When five or less colonies were present after both quantification and enrichment, all colonies were picked from each plate and streaked onto blood agar for purity and presence of haemolytic activity. In the absence of well-isolated colonies, a 2 cm streak was taken with a loop from the growth, which was re-streaked on a horse-blood agar plate. Five isolated colonies were taken from this blood agar plate and once again streaked onto a blood agar plate. All strains were tested for Gram reaction and motility (20ºC for 10 hours in Brain Heart Infusion). Species were identified with rhamnose and xylose fermentation tests.

Serotyping

One isolate from each sample was serotyped with *Listeria* O Antiserum types I/II, I, IV, V/VI, VI, VII, VIII, IX and H Antiserum A, AB, C, D (Mast Diagnostics, Mast house, Derby Road, Bootle, L 20 1EA) according to manufacturer's instructions, with some modifications. A fresh dense culture was prepared on a horse-blood agar plate, and 3ml of 0.2% saline was added. This suspension was transferred to a tube and kept in a 100ºC water bath for 1h, allowed to cool and then centrifuged at 3000rpm for 20 min to obtain a pellet. The pellet was

resuspended in a small amount of 0.2% saline and a slide agglutination test was conducted with the O antiserum.
To determine the H antigens cultures of isolate, strains were passaged three times through nutrient agar medium before serotyping. Plates were incubated at 30ºC for 24 hours after each passage. These cultures were inoculated into Brain Heart Infusion Broth and kept at 30ºC overnight, after which equal volumes of 1% formal saline were added. The tube agglutination test was conducted with H antiserum with the prepared cell suspension.

PFGE (Pulsed-field gel electrophoresis)

All isolates were characterized by restriction enzyme analysis (REA) using *Asc* I and *Apa* I followed by PFGE. The PulseNet standardized protocol by Graves and Swaminathan (2001) was used with some modifications. Altogether, 56 isolates were analysed with *Asc* I and 11 of 56 with *Apa* I.

Preparation of genomic DNA.

L. monocytogenes strains were cultured onto Blood agar and incubated at 37ºC for 24 hours. One isolated colony was inoculated into 5ml Brain Heart Infusion Broth (Oxoid CM 225), which was then incubated at 37ºC for 24 hours. The suspension was cooled (4-8 ºC), centrifuged (Wifug, 5500rpm) for 5 min and the supernatant was discarded. The pellet was suspended in 5ml of TN buffer (10mM Tris HCl, pH 8.0, 5M NaCl), cooled, centrifuged again and the supernatant was discarded. The pellet was suspended in 0.9ml of lysozyme solution (1mg lysozyme/ ml TN buffer) and incubated at 37ºC for 45 min.
Fifteen ml SeaKem Gold agarose (1.2%) supplemented with 1.67ml ESP (1g N-lauroylsarcosin, Merck + 100ml 0.5 EDTA, pH 8 + 200mg pronase, Roche) was prepared and maintained at 55ºC and 1.2ml of the solution was added to each culture with lysozyme and kept at 55ºC for 30min. The mixture was poured into plug moulds (Gene Navigator Pharmacia-Biotech, USA) and allowed to cool for 10 min before being transferred to Eppendorf tubes with 1ml of ESP. The ESP was renewed twice during 2h and the plugs were stored at 55ºC for 48 h.

Digestion of genomic DNA.

The plugs were cut into half longitudinally and transferred to Eppendorf tubes containing 0.5 ml PEFA (3.5 mg PEFA block in 10 ml TE solution [10ml 1M Tris HCl, pH 8.0 and aqua dest. water up to 1000ml]) and incubated at 37ºC. After 40-60 min PEFA was replaced with the same amount of fresh PEFA and incubated again 37ºC for 40-60 min. PEFA was replaced by 1ml of TE twice during 1 hour and incubated at 55ºC. Finally TE was removed and restriction solution was added.

Restriction solution

Asc I
Aqua dest. water - 870µl
NE 4 buffer - 108µlºC
(10x conc., New England Biolabs, Beverly, MA, US
BSA -10µl
(Bovine Serum Albumin 10mg/ ml, Promega).
Asc I -12µl
(10 units/ml, New England Bio-labs)

Apa I
Aqua dest. Water - 870µl
Buffer A - 98µl
(10x conc., Boeringer Mannheim)
BSA - 10µl
Apa I - 22µl
(10 units/ml, Boeringer Mannheim)

Restriction solution (160µl) was added to each tube and incubated at 37ºC with *Asc* I and 30ºC with *Apa* I overnight. The restriction solution was removed and the plugs were washed with 200µl of 0.5x TBE (9ml aqua dest. And 1ml 5xTBE [54g 0.45M Tribase (Amersham Biosciences), 27.5g 0.45M Boric acid, 20ml 0.5M EDTA, pH 8.0 and aqua dest. water up to 1000ml]). Samples were incubated at room temperature for more than 30 min.

Electrophoresis and staining

The plugs restricted with *Asc* I were separated by electrophoresis through 1.17% SeaKem Gold Agarose gel in 0.5x TBE at 8ºC in a Pharmacia Gene Navigator (Pharmacia, Sweden). The electrophoretic parameters used were: initial pulse 4.0s, final pulse 40.0s, run time 24h. The plugs restricted with *Apa* I were separated by electrophoresis through 0.99% SeaKem Gold agarose gel in 0.5 x TBE at 14ºC in CHEF MAPPER XA (BIO-RAD) run time being 20h with initial pulse time of 1.0s and final pulse time of 15.0s. After electrophoresis, the gels were stained for 20-30 min in one litre of 0.5 x TBE containing 100µl of ethidium bromide (1µg/ ml) and then washed for 1h in another litre of 0.5 X TBE. The gels were photographed over a 312nm transilluminator.

Analysis

The photographs were analysed visually and the DNA restricted fragments were sized against Lambda ladder PFG Marker No 340 S (New England Bio-Labs, Inc., Beverly, MA, USA).

The different types obtained by restriction with *Asc* I were compared visually with previously characterized human strains with both *Asc* I and *Apa* I. *Asc* I types were designated as follows, The first figure refers to the serogroup and the second figure refers to the consecutive number of human clinical isolates within that particular *Asc* I type, starting from year 2000. Indistinguishable patterns with both enzymes were considered to belong to the same clonal type, whereas two to three fragment difference with one or both enzymes were considered closely related, a four to six fragment difference were possibly related and seven or more fragment differences were unrelated (Tenover *et al*., 1995).

Results

Table 1. Occurrence and characteristics of *L. monocytogenes* strains isolated from gravad and cold-smoked fish.

Manu-facturer	Number of (+) Samples	Product No.	Salmon type	SLU number	Serovar	Quantification Cfu/g	PFGE *Asc* I
A	7/8	28	Cold-smoked	6588	1/2a	<100	1/2:49
				6589	1/2a		1/2:49
				6590	4b		4:2
				6591	1/2a		1/2:49
				6592	1/2a		1/2:49
		32	Cold-smoked	6576-6580	1/2a	<100	1/2:49
		33	Cold-smoked	6581-6585	4b	200	4:2
				6586*	1/2a		1/2:49
				6587*	1/2a		1/2:49
		36	Cold-smoked	6593-6597	4b	100	4:2
		40[a]	Cold-smoked	6522-6526	1/2a	<100	1/2:49
		41[a]	Gravad	6527-6531	1/2a	<100	1/2:New one
		42[a]	Cold-smoked	6532-6536	1/2a	100	1/2:49
				6537*	1/2a		1/2:49
		47	Cold-smoked	6598*	1/2a	1500	1/2:49
				6599*	1/2a		1/2:49
				6610*	1/2a		1/2:49
				6611*	1/2a		1/2:49
				6612*	4b		4:2
				6613*	4b		4:2
				6614*	1/2a		1/2:49
B	2/3	45	Gravad	6538	1/2a	100	1/2:48
				6539			
		46	Gravad	6540-6542	1/2a	<100	1/2:48
C	1/1	48	Gravad	6600-6604	1/2a	100	1/2:93
				6615*	1/2a		1/2:93

* - Strains isolated from quantification.
a – Samples bought in Germany.

Occurrence and strains of *L. monocytogenes*.

L. monocytogenes was isolated from 11 (three manufacturers A, B and C) of 56 products analysed (Table 1). This included gravad salmon products from three manufacturers and cold-smoked salmon from one manufacturer. The level of *L. monocytogenes* in gravad salmon ranged from <100 – 100 cfu/g and in cold-smoked salmon it was <100 – 1500 cfu/g.

The *L. monocytogenes* isolates belonged to serovar 1/2a and 4b. From 56 isolates analysed, 43 (76.8%) belonged to serovar 1/2a and 13 (23.2%) belonged to serovar 4b. Isolates from product no. 28 obtained by enrichment, yielded two serovars. Isolates of product no. 33, obtained by enrichment, yielded serovar 4b; whereas, the isolates from quantification yielded serovar 1/2a (Table 1). Seven isolates obtained from the quantification of product 47 yielded five isolates belonging to serovar 1/2a and two isolates belonging to serovar 4b: each serovar with a unique PFGE profiles (Figure 1).

PFGE analysis

PFGE typing of the 56 salmon isolates detected five *Asc* I types (Table 1). Four of five *Asc* I types from salmon strains were found among human clinical strains (Figure 2). Salmon strains with *Asc* I type, 1/2:48 were identical with *Apa* I, whereas, strains with *Asc* I type 4:2 were closely related to the human clinical strains with *Apa* I (Figure 3). Remaining two were unrelated.

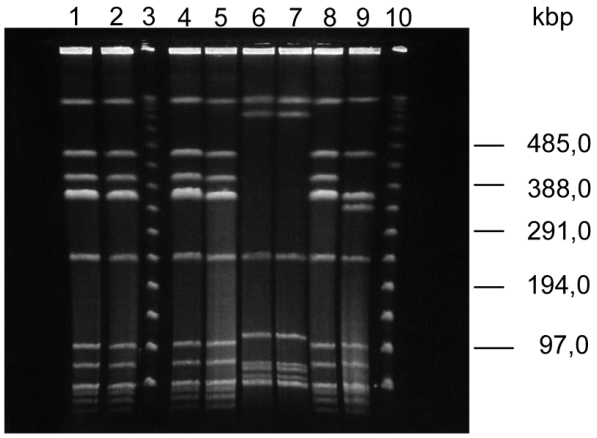

Figure 1. *Asc* I profiles of *L. monocytogenes* strains isolated by quantification of product no. 47 and 48. Lane 1 (strain no. 6598); lane 2 (strain no. 6599); lane 4 (strain no. 6610); lane no. 5 (strain no. 6611); lane 6 (strain no. 6612); lane no. 7 (strain no. 6613); lane no. 8 (strain no. 6614) from product 47. Lane 9 (strain no. 6615) from product 48. Lane 3 and 10 lambda ladder PFG marker.

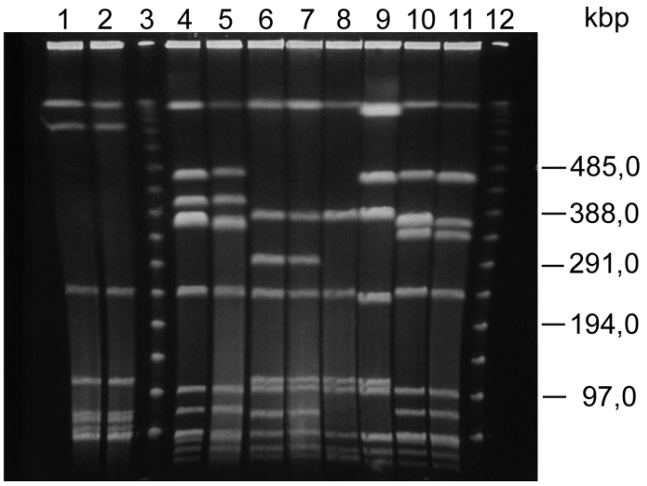

Figure 2. *Asc* I profiles of *L. monocytogenes* salmon strains and human strains. Lane 1.(salmon strain no. 6584); lane 2 (human strain no. 3291); lane 4 (salmon strain no. 6579); lane 5 (human strain no.6382); lane 6 (salmon strain no. 6538); lane 7 (human strain no. 6390); lane 8 (salmon strain no. 6530); lane 9 (human strain no. 4761); lane 10 (salmon strain no. 6604); lane 11 (human strain no. 2153). Lane 3 and 12, lambda ladder PFG marker.

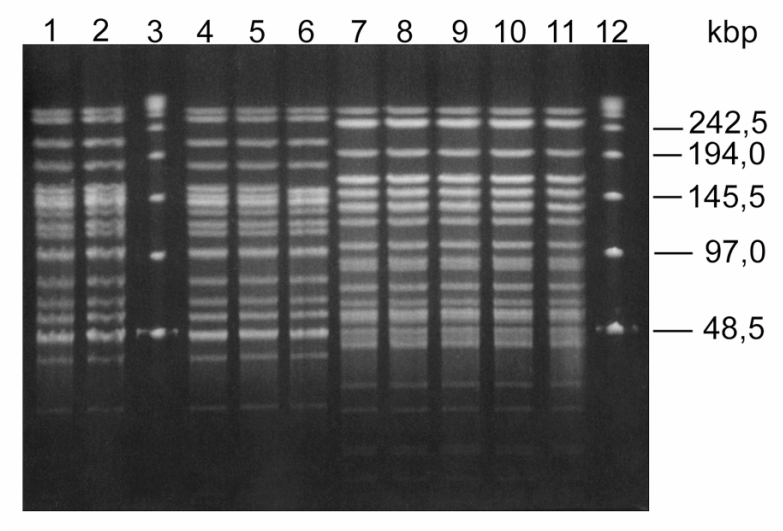

Figure 3. *Apa* I profiles of *L. monocytogenes* salmon strains and human strains. Lane 1, 2, 4 and 5 (salmon strain no. 6590, 6593, 6585, 6584); lane 6 (human strain no. 3291); lane 7, 8, 9 and 10 (salmon strain no. 6541, 6540, 6539, 6538); lane 11 (human strain no. 6390); lane 3 and 12, lambda ladder PFG marker.

Discussion

In the present study, *L. monocytogenes* was isolated from gravad salmon (Three manufacturers, A, B and C) and cold-smoked salmon (One manufacturer, A). Prevalence of *L. monocytogenes* in gravad fish has been reported to be as high as 20.7% (Jemmi, 1990) and 26.9% (Loncarevic *et al*., 1996), whereas in cold-smoked fish, overall prevalence is approximately 10% with a contamination rate of between 0% and 75% (Ben Embarek, 1994). As sea food is associated with human listeriosis (Lennon *et al*., 1994; Riedo *et al*., 1984; Ericsson *et al*., 1997; Loncarevic *et al*., 1998; Miettinen *et al*., 1999; Rørvik *et al*., 2000; Farber *et al*., 2000) prevalence studies are important in the identification of health hazards (Rørvik and Yndestad, 1991, Lonacarevic *et al*., 1996; Johansson, 1999; Dauphin *et al*., 2001; Vitas *et al*., 2004; Nakamura *et al*., 2004; Gudmundsdottir *et al*., 2005).

L. monocytogenes strains isolated from salmon manufacture A and B were identical and closely related, respectively to previously isolated human clinical strains (Figure 2 and 3). This suggests an association between human cases of listeriosis and the consumption of gravad or cold-smoked salmon.

The highest level of *L. monocytogenes* contamination found in the present study was 1500 cfu/g from cold-smoked salmon, but the level was low (<100 cfu/g) in most of the products. Loncarevic *et al*. (1996) reported 25 400 cfu/g of contamination level in cold-smoked fish whereas the contamination level of up to 132 000 cfu/g was found in hot-smoked rainbow trout. In an outbreak of listeriosis in Sweden associated with gravad and cold-smoked rainbow trout, the levels ranged from <100 cfu/g to 25 000 cfu/g (Ericssson *et al*., 1997). The high contamination level found in the above two studies may have been due to the longer storage time for these products 10 years ago *i. e*. up to 42 days. In a gastrointestinal listeriosis outbreak associated with cold-smoked rainbow trout, 190 000 cfu/g of *L. monocytogenes* was found (Miettinen *et al*., 1999). Even though the contamination levels are high in food responsible for epidemics and sporadic food-borne cases, low contamination levels (<100 cfu/g) cannot be excluded as non-infective (Rocourt *et al*., 2000).

In the present study, isolates of *L. monocytogenes* were mainly serovar 1/2a (76.8%) and the remainder were serovar 4b. Previous investigations have demonstrated serogroup 1/2 as the most predominant in gravad and hot and cold-smoked fish (Rørvik *et al*., 1995; Loncarevic *et al*., 1996; Johansson *et al*., 1999): the majority being serovar 1/2a (Dauphin *et al*., 2001; Vitas *et al*., 2004; Nakamura *et al*., 2004; Gudmundsdottir *et al*., 2005). However, the *L. monocytogenes* strain responsible for the listeriosis outbreak in Sweden associated with rainbow trout belonged to serovar 4b (Ericsson *et al*., 1997).

Seventy percent of human listeriosis strains in Sweden during 1976–1985 belonged to serogroup 4, but during the period of 1986–1997, strains were equally distributed between serogroups 1/2a and 4 (Tham *et al*., 1998). Even though reported overall incidence of listeriosis associated with serotype 4b is high, (Kathariou, 2002) pathogenicity of different serotypes cannot be distinguished.

Therefore, all strains of *L. monocytogenes* should be considered as pathogenic (McLauchlin *et al*., 2004).

L. monocytogenes isolated from cold-smoked fish of manufacturer A harboured two serovars (1/2a and 4b) each with unique PFGE profiles (Figure 1), implying that serotyping and genotyping of several isolates from each food sample is important (Loncarevic *et al*., 1996; Ericsson *et al*., 1997; Autio *et al*., 1999; Dauphin *et al*., 2001). Furthermore, products of the same manufacturer (A) purchased in both Germany and Sweden contained *L. monocytogenes* strains with same PFGE profiles with *Asc* I and *Apa* I (Table 1), thus indicating a common source of contamination, which could be the raw fish, employees or the environment in the processing plant. As *L. monocytogenes* has the ability to attach to surfaces and produce biofilms (Blackman and Franks, 1996; Bansal, 1996; Tello *et al*., 1997; Lundén *et al*., 2000), it can colonize, multiply and persist in the food-processing environment (Rørvik *et al*., 1995; Lawrence and Gilmour, 1995; Unnerstad *et al*., 1996; Boerlin *et al*., 1997; Vogel *et al*., 2001; Medrala *et al*., 2003).

Products of manufacturer A were processed in another European country and distributed to other European countries *e.g*. Sweden and Germany, which suggests that one processing plant can distribute and introduce pathogenic *L. monocytogenes* strains into many countries. Previous studies have confirmed the introduction of pathogenic strains of *L. monocytogenes* to the human population through processing environment (Loncarevic *et al*., 1998; Rørvik *et al*., 2000).

Conclusion

Isolation of identical or closely related *L. monocytogenes* strains from human clinical cases of listeriosis and gravad and cold-smoked salmon suggest that these types of product are possible sources of listeriosis in Sweden. Therefore, these products should be considered risk products for human listeriosis.

Prevalence studies are important and allow products contaminated with *L. monocytogenes* to be identified and recalled thus preventing human infections.

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