Human Listeriosis
Sources and Routes

a) *I am servant of that "GOD" to whom foolish people call human.*

b) *"I am the thread that runs through all these pearls," and each pearl is a religion. Such are the different pearls, and God is the thread that runs through all of them; most people, however, are entirely unconscious of it.*

Swami Vivekananda

Örebro Studies in Medicine 19

Vishal Singh Parihar

Human Listeriosis

Sources and Routes

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Vishal Singh Parihar

Human Listeriosis
Sources and Routes

Örebro University,
School of Health
and Medical Sciences
&
Department of Restaurant
and Culinary Arts
(Food Microbiology Section)

Indian Council of Agricultural
Research Complex for Goa
(Division of Animal Science)

Dedicated to

Dr. Apurva Sarin
National Center for Biological Sciences, Bangalore, India.
(Tata Institute of Fundamental Research)

Dr. John Young
IAH (Institute For Animal Health), Compton, UK

Abstract

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The bacterium *Listeria monocytogenes* can cause the disease listeriosis in both humans and animals. For the epidemiological investigation of listeriosis detection and characterisation of the organism are important steps.

Paper I. There are few reports on the incidence of *L. monocytogenes* in clinical samples from humans in India. Therefore, we investigated 144 samples from immunocompromised patients. *L. monocytogenes* was isolated from two placental bits from women with poor obstetric history, one patient with renal failure and three other patients. Five isolates were positive for the virulence genes *hlyA, actA* and *iap*. The sixth isolate was positive for *hlyA* and *actA* genes.

Paper II. Characterisation of 601 human *L. monocytogenes* isolates causing invasive listeriosis during the period 1986 to 2007 in Sweden reveals a decrease in serovar 4b strains. Since 1996, serovar 1/2a has become the predominant serovar causing human listeriosis: PFGE analysis revealed two clusters including different serovars suggesting that we need more studies on genetic relatedness among clinical isolates.

Paper III. The incidence of *Listeria* species in seafood from markets in Goa, India was studied. One hundred and fifteen raw/fresh seafoods bought at the fish markets were sampled and tested for presence of *Listeria* spp. *L. monocytogenes* was detected in 10 samples. *L. monocytogenes* in raw seafood may pose a health risk in kitchen if contaminating ready-to-eat food.

Paper IV. Gravad and cold-smoked salmon are associated with human listeriosis in Sweden. *L. monocytogenes* was isolated from 11 of 56 products. Serovar 1/2a was predominant, followed by 4b. REA/PFGE typing of the isolates identified five types of *L. monocytogenes*. One type was identical to a human type, two other were closely related. These findings suggest that gravad and cold-smoked salmon are still possible sources of listeriosis in Sweden.

Paper V. Many outbreaks of listeriosis have been related to consumption of dairy-associated products. Therefore, 123 farm bulk milk samples in India and 20 cervico-vaginal samples from dairy cows with reproductive disorders were investigated for *L. monocytogenes*. *L. monocytogenes* was isolated from 17.9% of bulk milk samples and from 10% of cervico-vaginal swabs. The virulence gene *hlyA* was detected in all isolates. These findings represent a public health risk where unpasteurised milk and milk products are largely consumed.

Paper VI. Isolates of *L. monocytogenes* (n=177) from 22 animal species were characterized and compared with human strains isolated between 1986-2006 in Sweden. Although many animals and humans shared pulsovars, they did not appear at the same time or with the same proportion of strains. The pulsovars shared by both animals and humans may indicate that there is an exchange of *L. monocytogenes* strains between these two groups due to either direct or indirect transmission.

Keywords: Listeria monocytogenes, listeriosis, zoonoses, PFGE, food safety, incidence, salmon, seafood, milk, serotyping, food-borne pathogens, PCR.

Vishal Singh Parihar, Örebro University, Department of Restaurant and Culinary Arts, Sörälgsvägen 2, SE-712 60 Grythyttan, Sweden *or* ICAR Research Complex for Goa, Ela, Old Goa - 403402, Goa. Phone: 0832-2284678 / 79; Fax: 0832-2285649
E-mail: drvishu1241@yahoo.co.in

List of papers

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III. **Parihar VS**, Barbuddhe SB, Danielsson-Tham ML and Tham W. Isolation and characterization of *Listeria* species from tropical seafoods. *Food Control, 2008, 19, 566-569.*

IV. Peiris WIP, Lopez Valladares G, **Parihar VS**, Helmersson S, Barbuddhe SB, Tham W and Danielsson-Tham M-L. Gravad (Gravlax) and cold-smoked salmon, still a potential source of listeriosis. *Accepted in Journal of Foodservice.*

V. **Parihar VS**, Barbuddhe SB, Chakurkar EB, Danielsson-Tham M-L and Tham W. Isolation of *Listeria* species from farm bulk milk at the receiving dairy plant and cervico-vaginal swabs. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases 2007, 28, 1-5.*

VI. **Parihar VS**, Lopez-Valladares G, Danielsson-Tham M-L, Helmersson S, Barbuddhe SB Båverud V, Tham W. Molecular characterization of *Listeria monocytogenes* isolates from animals in Sweden and comparison with previously characterized human strains. *In Manuscript.*

Abbreviations

ActA - Protein required for actin polymerization
Bp – Base pairs
CAMP test - Christie, Atkins and Munch-Petersen test
CCL – Chemokine ligand
CFU – Colony-forming units
DNA - Deoxyribonucleic acid
DNTP – deoxynucleotide triphosphate
GC – guanine-cytosine
hly A - gene encoding the listeriolysin O protein
Hpt – Hexose phosphate transporter
Iap – Invasion associated protein
INF – Interferon
InlA – Internalin A protein
InlB –Internalin B protein
Kbp - Kilobase pairs
LLO – Listeriolysin O protein
L. monocytogenes – Listeria monocytogenes
LRR – Leucine-rich repeat
Mb - Megabase
NF – Nuclear factor
ORF – Open reading frame
PCR – Polymerase chain reaction
PFGE – Pulsed-field gel electrophoresis
PI-PLC – Phosphatidylinostol-phospholipase C
plcA - gene encoding PI-PLC
PLC – Phospholipase C
REA – Restriction enzymes analysis
RTE food - Ready-to-eat food
Spp. - species
Taq – *Thermus aquaticus*
TLR – Toll-like receptor
TNF – Tumor necrosis factor
TR – Tandem repeat
VNTR – Variable number of tandem repeat

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Introduction

Listeria monocytogenes is a rod-shaped facultative intracellular bacterium that causes severe food-borne infection in humans. Humans are mainly infected by *L. monocytogenes*, although, *L. ivanovii* has caused human infections, and at least one report of human listeriosis has been associated with *L. seeligeri* (Rocourt, 1986; Cummins *et al.*, 1994; Lessing *et al.*, 1994; Ramage *et al.*, 1999). In humans, the main clinical manifestations of listeriosis are meningoencephalitis, septicaemia and abortion. *L. monocytogenes* does not usually pose a high risk for healthy individuals, *i.e*. morbidity is low; however, mortality rates range from 20 to 30% (Farber and Peterkin, 1991; Rocourt, 1996) and can reach 80% to 99% in immunocompromised individuals including neonates, the elderly and pregnant women (Gray and Killinger, 1966; Farber and Peterkin, 1991; Rocourt, 1996).

Contaminated silage is the major cause of animal, particularly ruminant listeriosis. The common clinical manifestations in animals are meningoencephalitis, abortion, and mastitis (Osebold *et al.*, 1960; Bojsen-Moller, 1972). Animals may be diseased or asymptomatic carriers of *L. monocytogenes* and shed the organism in their faeces. Thus, it was earlier supposed that *L. monocytogenes* casued disease only by direct transmission from animals to humans. Veterinarians, technicians and farmers may periodically acquire a mild, self-limiting form of listeriosis when assisting cows during delivery (Kalkoff and Schiff, 1960; Cain and McCann, 1986). Today, the general theory is that ingestion is the main mode of infection with food the main vehicle of infection. A listeriosis outbreak in the Maritime Provinces of Canada (Schlech *et al.*, 1983) was related to food (coleslaw) but it was not until the outbreak in California from January to August 1985 (James *et al.*, 1985; Linnan *et al.*, 1988) that food (raw milk cheese) was seriously considered as an important vehicle for *L. monocytogenes*. Mastitis or contaminated milk play important roles in the dissemination of bacteria in dairy industry, where various raw milk products are made. In an outbreak, 2001, of gastrointestinal listeriosis involving more than 100 persons, Danielsson-Tham *et al.* (2004) found the same clonal type of *L. monocytogenes* among dairy animals, environment, equipment and cheeses manufactured on a Swedish summer farm and in consumers faecal samples. Similarly, the seafood industry may be contaminated and thus *L. monocytogenes* can be present in the final product. The organism has frequently been isolated from fish and fish products from different parts of the world. Cold-smoked or gravad fish (*Salmon salar*) caused a small outbreak of listeriosis in Sweden in 1994-1995 (Ericksson *et al.*, 1997) and once introduced into the food industry environment *Listeria* can persist for years (Unnerstad *et al.*, 1996). Food is a vehicle of *Listeria* transmission in 99% of human listeriosis cases (Mead *et al.* 1999). Risk assessment by the World Health Organisation (WHO) resulted in the guideline that 99% of all cases of listeriosis could be eliminated, if the level of *L. monocytogenes* never exceeds 1000 cfu/g food at the point of consumption

(WHO 2004). Nosocomial infection has also been described, placing medical physicians and other medical staff at risk.

L. monocytogenes is one of six species described under genus *Listeria*. Some of the biochemical and physical properties of genus *Listeria* are described in Table 1. Other characteristics of species *L. monocytogenes* are:

- Gram-positive.
- Short rods (0.4 - 0.5 µm × 0.5-2 µm).
- Motile by the presence of peritrichous flagella. Pronounced motility (+++) is seen in nutrient broth at 20-22°C. At 30°C, there is less motility (++) and motility is greatly reduced (+) at 37°C. At 20-22°C, *Listeria* spp are rotating (tumbling), interrupted by wiggling movements in any direction.
- Colony characteristics differ on different media. On nutrient agar, translucent, dew drop like opalescent colonies are formed. Grey-white colonies with a little odour of sour milk are exhibited on dextrose agar. On blood agar, colonies exhibit a surrounding clear haemolysis zone that can be appreciated distinctly after removal of the colony. Sheep have antilisterial antibodies in greater concentration than any other animals, thus sheep blood should be avoided in blood agar plates for listeria diagnosis (Seeliger, 1961). The identification of *L. monocytogenes* colonies on blood-free media such as tryptose agar is greatly facilitated by the use of a binocular microscope with oblique illumination (Girard, 1959). The white light should pass through the medium from below at an angle of 45°; colonies will appear small, round, finely textured and with a blue-green colour. If *L. monocytogenes* is cultured on selective agar, colonies should be transfered to freshly made blood agar before further tests are conducted.
- Capsules are not formed.
- Edges of *L. monocytogenes* often appear slightly pointed.
- Occurs singly, short chains or in diploforms producing V-shaped arrangements.
- Non acid-fast.
- Do not form spores.

Table 1. Physical and biochemical characteristics of genus *Listeria*

Characteristics	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. grayi*	*L. ivanovii subsp. ivanovii*	*L. ivanovii subsp. londoniensis*
Tumbling-motility	+	+	+	+	+	+	+
Catalase-production	+	+	+	+	+	+	+
Hemolysis	+	-	+	-	-	+	+
CAMP-test (*Staph. aureus*)	+	-	+	-	-	-	-
CAMP-test (*Rhod. equi*)	-	-	-	-	-	+	+
L-rhamnose	+	d	-	d	-	-	-
D-xylose	-	-	+	+	-	+	+
Hippurate	+	+				+	+
Ribose						+	-
N-acety-B-D-manosamine						-	+
Pathogenecity for mice	+	-	-	-	-	+	+

History and taxonomy

Phylum – *Firmicutes*

Class – *Bacilli*

Order – *Bacillales*

Family – *Listeriaceae*

Genus – *Listeria*

Species – *Listeria monocytogenes*

Listeria innocua

Listeria seeligeri

Listeria welshimeri

Listeria grayi

Listeria ivanovii

Subsp- ivanovii

Subsp - londoniensis

The history of the genus *Listeria* started when the causative organism of listeriosis was demonstrated by Murray, Webb and Swann in 1926. As typical monocytosis was observed in small laboratory animals, the organism was named

Bacterium monocytogenes. In 1927, a similar bacterium isolated by Pirie from gerbils (*Tatera lobengulae*) in South Africa had typical symptoms of listeriosis: the bacterium was named *Listerella hepatolytica*. When the two species were identified as identical, they were assigned the name *Listerella monocytogenes;* however, the name Listerella had been used by Jahn in 1906 for *mycetozoon* and by Cushman in 1933 for *foraminifera*. Thus, in 1940, Pirie suggested the name *Listeria monocytogenes* (Pirie, 1940).
In 1961, *L. dentrificans* (because of its ability to reduce nitrate) was added to genus *Listeria*. In 1966 and 1971, species *L. grayi* (in honour of M. L. Gray, an American microbiologist) and *L. murrayi* (in honour of E. G. D. Murray, a Canadian microbiologist) were added to genus Listeria. In 1977, Seeliger introduced *L. innocua* (because of its innocuousness). Wilkinson and Jones (1977) considered *L. grayi* and *L. murrayi* distinct from other *Listeria* species, and they were excluded from the genus: both speices were reincluded in *Listeria* due to similar murein variation of amino acid in their cell wall (Fiedler and Seger, 1983; Fiedler *et al.*, 1984). Rocourt *et al.* (1992) finally combined the two species into one species, *L. grayi*.

L. monocytogenes was first isolated from humans in 1929 by Nyfeldt at Blegdam Hospital, Copenhagen. Initially, it was assumed a variant of *L. monocytogenes* and was named *Listerella hominus*. Another variant was designated *Corynebacterium paravulum* by Schultz *et al.* (1934). Further studies revealed that the bacteria isolated by Nyfeldt and Schultz were different serotypes of *L. monocytogenes*. (Paterson, 1940a, b)
Complexity in the taxonomy of *Listeria* was further aggravated by a study by Wilson and Miles (1954), who suggested that *Listeria* bacteria resemble *Erysipelothrix* bacteria and should be included in a subgroup of *Erysipelothrix*. Both authors suggested the name *Erysipelothrix monocytogenes* instead of *L. monocytogenes*. The genus *Listeria* currently includes six species, of which one is divided into two subspecies (Boerlin *et al.*, 1992): *L. monocytogenes*; *L. innocua; L. welshimeri* (in honour of H. J. Welshimer, an American microbiologist)*; L. seeligeri* (in honour of H. P. R. Seeliger, a German microbiologist)*; L. grayi;* and *L. ivanovii, with* subspecies *ivanovii* (in honour of I. Ivanov, a Bulgarian microbiologist), and L. *ivanovii* subsp. *londoniensis* (in honour of E. G. D. Murray, who studied in London, Canada). The ninth edition of Bergey's Manual of Determinative bacteriology groups genus *Listeria* with genera *Brochothrix*, *Carnobacterium, Caryphanon, Erysipelothrix, Kurthia*, *Lactobacillus* and *Renibacterium* (Holt *et al.*, 1995). They are all been placed in Group 19, under the heading of "regular, non-sporing, gram-positive rods".

Serological cross-reactivity

There are 13 serovars of *L. monocytogenes* (Table 2). Serological cross-reactivity between the *Listeria* serotypes and other species of bacteria are reported (Seeliger and Jones, 1986) and some strains of *L. monocytogenes* could cross-react with strains of genus *Erysipelothrix* (Julianelle and Pons, 1939). Similar

overlapping serological reactions between serovars 4a, 4b of *L. monocytogenes* and *Streptococcus faecalis* are reported (Seeliger and Jones, 1986). Some cross-reactivity is observed between *L. monocytogenes* and *Escherichia coli* (Jaeger and Myers, 1954) and *Staphylococcus epidermidis* has serological similarities to *L. monocytogenes* (Welshimer, 1960).

Table 2. Distribution of various somatic (o) and flagellar (H) antigens of *L. monocytogenes* serovars.

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

Biodiversity of genus *Listeria*

Modern biological techniques and better communication has allowed the sequencing of 470 bacterial genomes. *L. monocytogenes*, *L. innocua* and *L. welshimeri* have been sequenced. Genome sequencing of *L. monocytogenes* comprises 2.8 million bp to 2.9 million bp, with G+C content of 38% and there are 2900 predicted protein-coding genes. The genome of *L. innocua* has 3,011,209 bp, a G+C content of 37%, and 2973 similar genes. The smallest genome is *L. welshimeri* consisting of 2,814,130 bp, an average G+C content of 36.4% and 2780 protein coding-genes (Table 3). Genomes from genus *Listeria* reveal high stability (Glaser *et al*., 2001) and a close phylogenetic relationship. One of the indicators of high stability is the lack of insertion sequences (IS), but transposons have been identified in two strains of serovar 4b (F2365 & H7858), two stains of 1/2a (EGDe & F6854) and from one strain of *L. innocua* (Buchrieser, 2007).

Table 3. Genomic features of genus *Listeria.*

Genomic features of three strains of *L. monocytogenes*

	4b (F2365)	1/2a (F6854)	4b (H7858)
Chromosome			
Length (bp) of chromosomes	2905310	2953211	2893921
G+C content of chromosomes	38%	37.8%	38%
No. of ORFs in chromosomes	2847	2973	3024
Phage regions of chromosomes	2	3	2
Plasmids	None	None	1
length of plasmids	-	-	82270
G+C of Plasmid	-	-	37.5%
No. of ORFs in Plasmids	-	-	94

Characteristic	*L. monocytogenes* (EGD-e)	*L. innocua*	*L. welshimeri*	*L. gayri*	*L.ivanov*
Size of chromosomes	2,944,528	3,011,208	2,814,130	NA	NA
G+C content	38	37.4	36.4	NA	NA
G+C of protein-coding genes	38.4	37.8	36.7	NA	NA
No. of protein-coding genes	2,855	2981	2,78	NA	NA
No. ofrRNA operons	6	6	6	NA	NA
No. oftRNA genes	67	66	66	NA	NA
No. ofProphages	1	5	1	NA	NA
No. of plasmids	0	1	0	NA	NA
No. of stain specific genes	218	121	311	NA	NA
No. of Transposons	1	0	0	NA	NA

NA=Information not available

L. monocytogenes is present in the environment and has been isolated from animals and humans. The ubiquitous and opportunistic ability of *L. monocytogenes* is due to extensive regulatory protein presence. More than 7% of *Listeria* genes predicted encode for regulatory proteins. Similarly, regulatory proteins are found in other bacteria such as *Staphylococcus aureus* (3%) and *Pseudomonas aeruginosa* (8%).

Inspite of high number of common genes and conserved genome, differences exist between the various *Listeria* genomes. All five stains characterized up to genome level exhibited 50 to 100 strain specific genes, some of which are related to ability of *L. monocytogenes* to infect animals and humans. In addition A118 family phages are present in *L. innocua* and in all serotypes of *L. monocytogenes* except serovar 4b. Such phages are absent in genome of *L. welshimeri* but *L. ivanovii* do have one phage similar to that of *L. innocua*. The small size of *L. welshimeri* is a result of deletions in all genes required for virulence (Figure 2). Absence of virulence genes suggest common evolutionary path for both *L. innocua* and *L. welshimeri* (Figure 1) (Buchrieser *et al*. 2007).

Figure 1. Phylogenetic tree of genus *Listeria*. This tree is based on 16S and 23S rRNA, *iap, prs, vclB*, and *idh*. (Reproduced with permission from Dr. Carmen Buchrieser, Institut Pasteur, Paris, France)

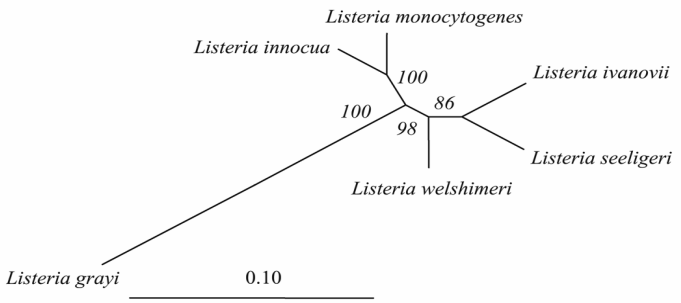
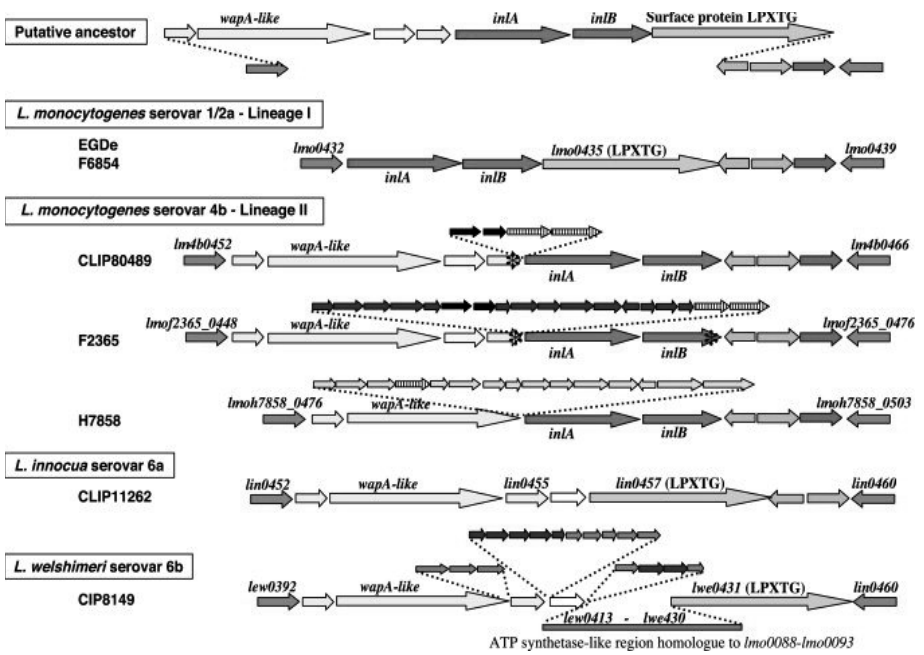


Figure 2. Comparison of the *inlAB* locus and the flanking regions in different species of genus *Listeria*. (Reproduced with permission from Dr. Carmen Buchrieser, Institut Pasteur, Paris, France).



PFGE

In conventional electrophoresis, negatively charged DNA molecules are pulled through an agarose gel by a field of electric current towards the anode. The pores in the agarose gel differ in size: small DNA molecules will pass through most pores and move rapidly in the gel, larger molecules move slower as they need to change configuration to pass through smaller pores. DNA molecules larger than 20kbp have restricted migration under uniform electric field and are hardly resolved by this type of electrophoresis: the molecules become trapped in the agarose matrix.

Larger molecules can be resolved by cyclically varying the orientation of the electric field in the gel during a run (Schwartz and Contour, 1984) (Figure 4). The method was later named pulsed-field gel electrophoresis (PFGE). Several theories have been proposed to explain the mechanism of enzyme-restricted DNA separation during PFGE and computer simulation and direct fluorescent visualization have shed light on the basic mechanisms or phenomena. As large molecules 'squeeze' through a pore, it is forced into an extended conformation; to squeeze through the first pore can take milliseconds to minutes, depending on the molecular size. If a larger molecule moving through a gel is suddenly forced to change direction because of a change in the orientation of the electric field, the molecule has to first reshape into a new configuration that will allow movement in a new direction: the larger the molecules, the longer it takes to reshape (Figure 3). Thus, the time difference for reshaping can be utilized for separate molecules. For example, although a 200 kbp and a 400kbp molecules move through the gel matrix at the same crest of wave, each time the direction of the field changes, the 200kbp molecule will move faster than the 400kbp molecule, as it is smaller.

Figure 3. Movement of DNA in PFGE gel.

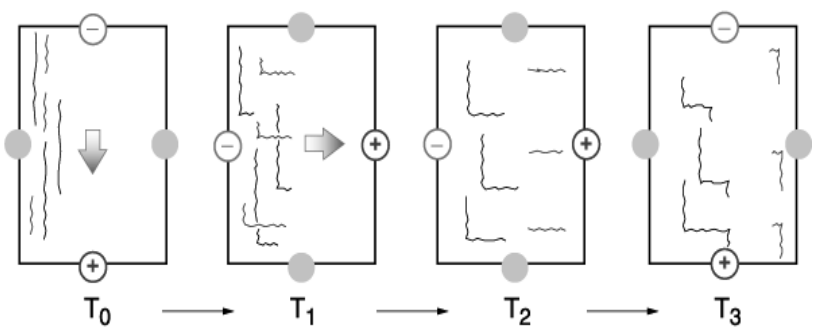
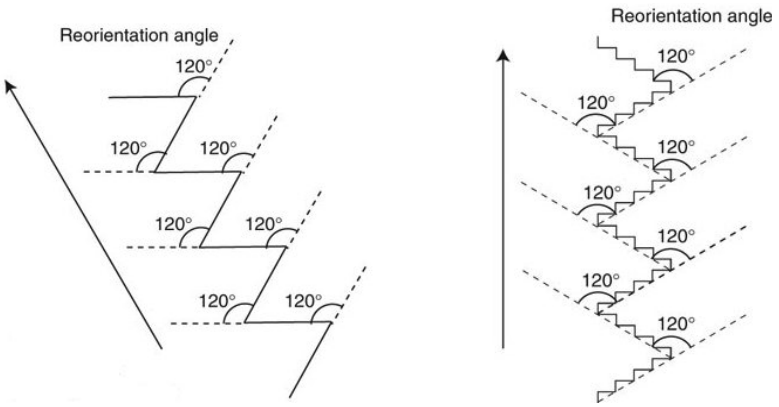
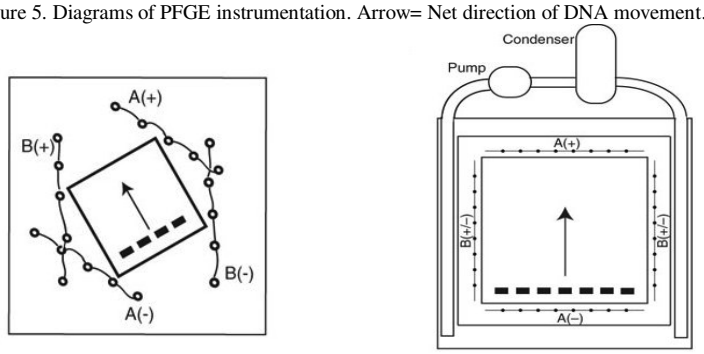


Figure 4. Arrow = Net direction of DNA movement. Solid line = Actual path of DNA molecule. Dashed line = Trajectory created by electric fields.



Depending upon field geometrics of electrodes (Figure 5), various models of PFGE have been tried to improve method, including: pulsed-field gradient gel electrophoresis (PFGGE); contour-clamped homogeneous electric field (CHEF); transverse alternating field electrophoresis (TAFE); rotating gel electrophoresis (RGE); rotating field gel electrophoresis (RFE); field inversion gel electrophoresis (FIGE); and orthogonal field alternating gel electrophoresis (OFAGE).

Figure 5. Diagrams of PFGE instrumentation. Arrow= Net direction of DNA movement.

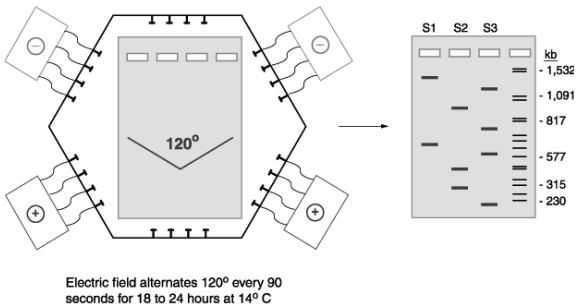


PFGGE (Pulsed-field gradient gel electrophoresis)

PFGGE produces inhomogeneous fields and uses earlier versions of Pharmacia Pulsophor apparatus (Schwartz and Contour, 1984).

CHEF (Contour-clamped homogeneous electric field)

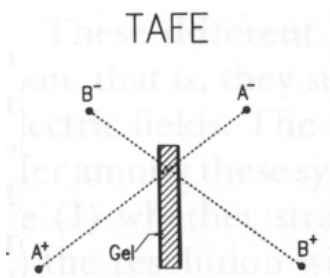
Figure 6. CHEF model of PFGE



A hexagonal electrode array allows the electric field to fan through an angle of 120°C between pulses. The individual electrode is clamped (fixed) to a specific voltage in such a way that a uniform electric field is set up within the hexagon (Figure 6). DNA molecules up to 2 mb can be separated with a CHEF alternating between two orientations 120° apart (Chu *et al.*, 1986). CHEF DR III from BioRAD was used in the present thesis.

TAFE (Transverse alternating field electrophoresis)

Figure 7. TAFE model of PFGE



The gel stands vertically (Figure 7) in a tank filled with buffer. This limits the size of the gel that can be used and low-percentage agarose gels are not strong enough to be run in a TAFE apparatus (Gardiner *et al.* 1986).

RGE (Rotating gel electrophoresis)

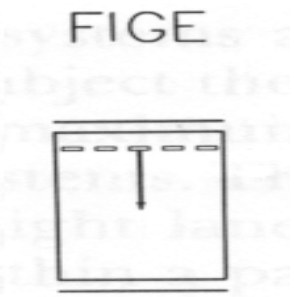
The electric field is stationary and the gel is rotated between fitted points controlled by micro switches. The apparatus turns the gel between two present orientations in a uniform electric field at present time intervals. The mechanical movement of the gel means that very short pulses and short pulse time cannot be attained (Southern *et al.*, 1987).

RFE (Rotating field electrophoresis)

The gel remains stationary and the electrode rotates around the gel at predetermined intervals. The mechanical movement of the electrodes means that very short pulses and short pulse time cannot be achieved (Ziegler *et al.*, 1987)

FIGE (Field inversion gel electrophoresis)

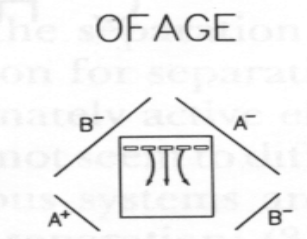
Figure 8. FIGE model of PFGE



FIGE is a conventional gel box in which the polarity of the electrodes changes periodically. The DNA molecules move in straight lines as they migrate through the gel. FIGE employs the limiting value of the 180° angle between two uniform fields (Figure 8). The system of field inversion electrophoresis is more complicated than that of alternating angle electrophoresis because different pulse times are used in the forward and reverse direction (Carle *et al.*, 1986).

OFAGE (Orthogonal field alternating gel electrophoresis)

Figure 9. OFAGE model of PFGE

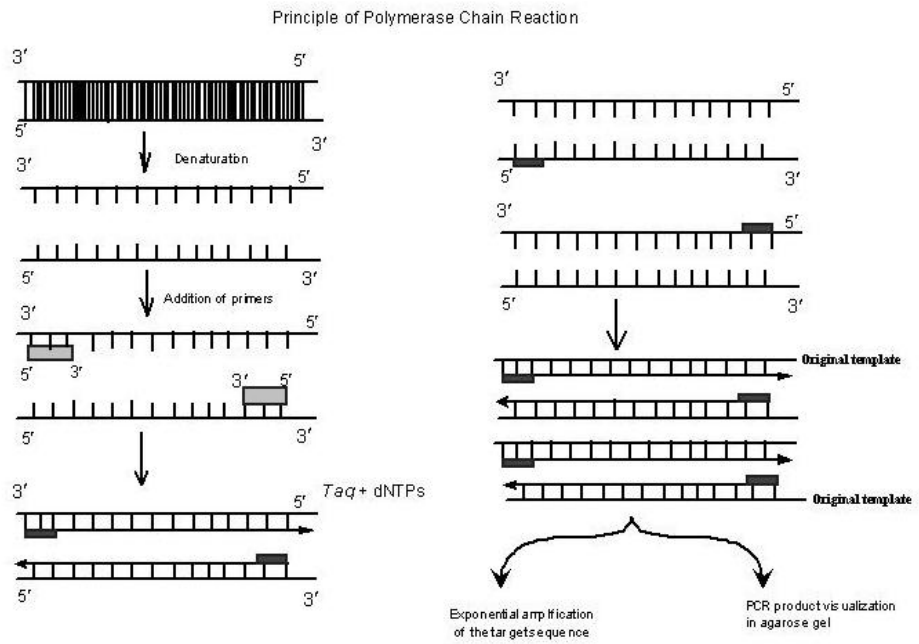


OFAGE (Figure 9) produces inhomogeneous fields and a commercial form of this apparatus is not available (Carle *et al.*, 1984).

Polymerase chain reaction

The polymerase chain reaction (PCR) was invented by Mullis in 1983, while working for a biotechnology company, Cetus Corporation, California, USA: he was awarded the Nobel Prize for Chemistry in 1993 for his remarkable invention. PCR is primarily a technique for amplifying DNA with the help of an enzyme in vitro. In PCR, a specific nucleotide sequence is copied several millions of times during thermal cycling (Figure 10); some new PCR techniques allow amplification of sequences up to 40 kb (Mullis and Faloona, 1987).

Figure 10. Principle of PCR instrument.



PCR requires several components:

- The sequence to be copied, the so-called template.
- The enzyme *Taq* DNA polymerase.
- Suitable environment for reactions including optimal temperature, pH, salt, buffer solution, divalent cation (magnesium Mg^{2+} ion or manganese Mn^{2+} ion).
- Building blocks of DNA (nucleotides) in the form of dNTPs (deoxynucleotide triphosphate). Conditions before thermal cycling are critical for the success of PCR. As *Taq* DNA polymerase exhibits some

activity at room temperature, products can be generated from annealing of primers to target DNA at locations with just a few complementary nucleotides at the 3 ends. Subsequent cycles generate non-specific products. Therefore, it is important to avoid polymerisation before the first temperature-controlled steps. Three methods can be applied to overcome this problem:

- Physical separation of an essential reaction component before first temperature step,
- Cooling of reagents at $0^{o}C$,
- Reversibly blocking enzymatic activity with an antibody.

There are several critical parameters for PCR techniques e.g.:

$MgCl_2$ concentration
It is important to determine the optimum $MgCl_2$ concentration as this varies depending upon the primer even for the same region of a given template.

Reagent purity
Purity of reagents is the most important parameter for amplification of templates and avoiding contamination.

Primer selection
An optimal primer maximizes the probability of hybridisation of the sequence of interest. Primers should be selected with care, including complementarities to the template, primer length, primer sequence, and primer-dimers.

Template
The amount and purity are important for template functioning.

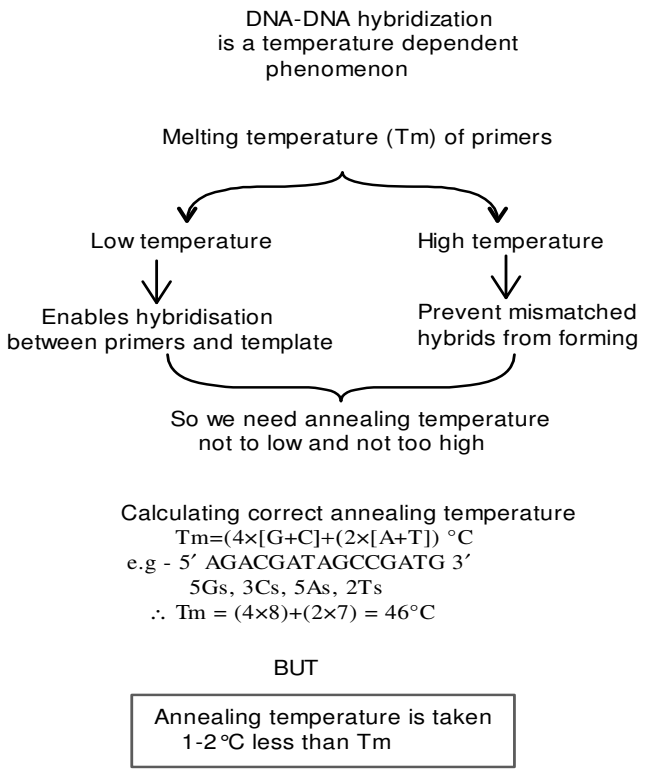
Taq
Taq is a thermostable DNA polymerase that should not be put through unnecessary denaturation steps. DNA polymerase is present in all living organisms and aids in the duplication of DNA in dividing cells during mitosis and meiosis inside the body; it is also used in vitro for the same function under controlled conditions. Natural DNA polymerase is not thermal stable. The bacterium *Thermus aquaticus* produces a thermal stable DNA polymerase, so called "*Taq*". However, *Taq* polymerase can make mistakes when copying DNA, called mutations, as it lacks 3---5 proofreading exonuclease activities: it has been replaced by DNA polymerase *Pwo* or *Pfu*, produced by *Archaea.*

dNTPs
Concentration of dNTPs should be used critically. Concentration of dNTPs should not be increased in an effort to increase efficiency of PCR.

Enhancers
Enhancers increase yield and specificity and overcome difficulties encountered with high GC content or long templates.

Thermal cycling parameters
a) Denaturation: a very fast and critical unimolecular reaction in which two strands of DNA are completely separated. If the amplification sequence is too long (>3 kb), the time should be minimized to protect the target DNA undergoing depurination by Tris-buffer.
b) *Annealing*: for improved PCR products, the primers need to anneal stably to the template. Temperature of this step depends upon the G+C content of the primers. Temperatures lower than 55^{o}C are used if the G+C content of primer is low, and temperatures greater than 55^{o}C are used if G+C content is high (Figure 11).
c) *Extensio*n: One minute per kilo base product is sufficient for this step
d) *Ramp time*: Time taken to change from one temperature to another.

Figure. 11. Ideal annealing temperature for primers to be used in PCR.



Several PCR models are available, including: Multiplex-PCR; Reverse Transcription PCR (RT-PCR); Nested PCR; Inverse PCR; Allele-specific PCR; Colony PCR; Asymmetric PCR; Assembly PCR; Helicase-dependent amplification PCR; Intersequence specific PCR; Hot-start PCR; Ligation-mediated PCR; Methylation specific PCR; Multi ligation-dependent probe amplification (MLPA) PCR; Quantitative PCR; Thermal asymmetric interlaced PCR; and Touchdown PCR.

Amplified fragment length polymorphism (AFLP)

AFLP is a PCR-based genetic fingerprinting technique developed in 1990s, also called AFLP-PCR.

AFLP is carried out in three steps:

- Digestion of cellular DNA with a combination of *Mse*I (four base cutters) and *Eco*RI (six base cutters). Ligation of complementary double strand adaptors to the ends of restriction fragments.
- Preselective PCR with primers complementary to the adaptors and restriction site fragments.
- Electrophoretic separation and visualization of fragments on denaturing polyacrylamide gels.

AFLP is a sensitive and reproducible method that simultaneously detects various polymorphisms in different genomic regions. The technology is used to identify the genetic variation in animals, plants and bacteria and is applicable to population genetics in humans.

The advantages of AFLP include: randomly generated polymorphism; differentiation between species at gene level; genetic diversity is easily identified; more bands per reaction and more polymorphic loci per primer are produced than with RAPD (Randomly amplified polymorphic DNA analysis); the technique is fast and no prior knowledge of sequence analysis is required; the method is suitable for large population screening, as there is a large multiplex ratio, *i.e*. each band is considered to be from different areas of bacterial genome; and, it is insensitive to template DNA concentration.

Multiple-Locus Variable Number of Tandem Repeat Analysis (MLVA)

The development of primers is accelerated by the availability of whole genome sequences for many bacteria. Tandem repeat loci (also called minisatellites) provide models for the construction of markers for bacteria and humans genome. Tandem repeats (having 2 to 5 bases short patterns) are easy to identify in genome sequence data. The density of tandem repeats in bacterial genomes varies from species to species and not all tandem repeats are polymorphic. Some tandem repeats are unstable with no long-term epidemiological value. Thus, for

each species of bacteria, tandem repeats must be carefully selected by representative collections of strains.
Nucleic acid such as DNA is subject to mutation and tandem duplication is one such mutation. A sequence of DNA is converted into two or more copies and these copies are connected in an unbroken sequence called tandem repeat.
For example:
AGCT is converted to AGCT*GCT*GCT**GCT**, in which a tandem repeat of triplet GCT appears. With time, these tandem repeats can undergo further mutation; leading to an increase or decrease in repeats number. Such tandem repeats occur in two forms: tandem repeats having 2 to 5 bases (TRs) and variable number of tandem repeats with more than 5 bases (VNTRs). In modern biology, tandem repeats are known for gene regulation and development of immune system cells and these repeats are used in DNA fingerprinting of bacterial strain. Although MLVA is a simple technique, there are limitations for analysis of long repeats, as they are difficult to detect by eye in printed sequences. To solve this problem, there are various mathematic theorems in algorithm such as computing alignment matrix, data compression techniques, simple sequences detection algorithm and Rival's algorithms.

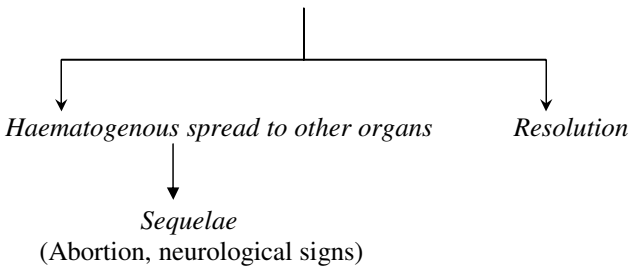
Most MLVA research focuses on short patterns in tandem repeats and uses PCR-analysis to discriminate a pathogen at strain level. The main principle is to detect a number of tandem repeats in the genome of an organism. MLVA has the potential to characterize bacteria such as *Escherichia coli* (Lindstedt *et al.*, 2003, 2004), *Staphylococcus aureus* (Sabat *et al.*, 2003) and *L. monocytogenes* (Murphy *et al.*, 2007). MLVA successfully discriminates among various clonal groups of *L. monocytogenes* but serotype specific discrimination is not possible (Murphy *et al.* 2007). MLVA is considered less expensive, faster, and less cumbersome than PFGE. Other typing methods are also compared with MLVA such as MLST (multi locus sequence typing) and AFLP, but no difference in discrimination is observed (Top *et al.*, 2004).

Randomly amplified polymorphic DNA analysis (RAPD)

RAPD is sometimes called arbitrary primed PCR (AP-PCR) analysis and can rapidly characterise various strains of a bacterial species. This method distinguishes species with identical 16S rDNA sequences. Usually, multiple arbitrary primers, each having 10 bases, is designed with no known target sequence. Initiation of amplification results in a single DNA strand with palindromic termini. The arbitrary primers target unknown sites in the genomic DNA. During subsequent PCR cycles, a number of other sequences is amplified in the genome. Electrophoresis of the PCR products yields a specific band pattern of different strains. The disadvantages of this method are that standardisation of concentrations of primes and templates are needed to make reproducible amplification products, and most of the RAPD markers are dominant, i.e. it is difficult to distinguish between similar DNA sequences amplified.

Pathogenesis

The pathogenesis of a disease is the mechanism by which an etiological factor causes the disease. Features of pathogenesis of listeriosis can be outlined as: oral entry of *L. monocytogenes;* colonization of the intestine; intestinal translocation; and replication in organs such as liver and spleen (Figures 12 & 13).



Pathogenesis of listeriosis is influenced by host factors and bacterial factors. Host factors include intrinsic and extrinsic factors such as genetic, behaviour, age, and interaction of microorganism to host. Bacterial factors include proteins released by *L. monocytogenes* in the host being further classified according to their role in various steps of pathogenesis:

Step 1 - Bacterial contact and adherence to the host cell.
Step 2 - Formation of the first vacuole or phagocytic cup formation.
Step 3 - Escape from the first vacuole.
Step 4 - Cytosolic replication and movement.
Step 5 - Spread of Listeria from cell-to-cell in the host.
Step 6 - Lysis of two-membrane vacuole (second vacuole).

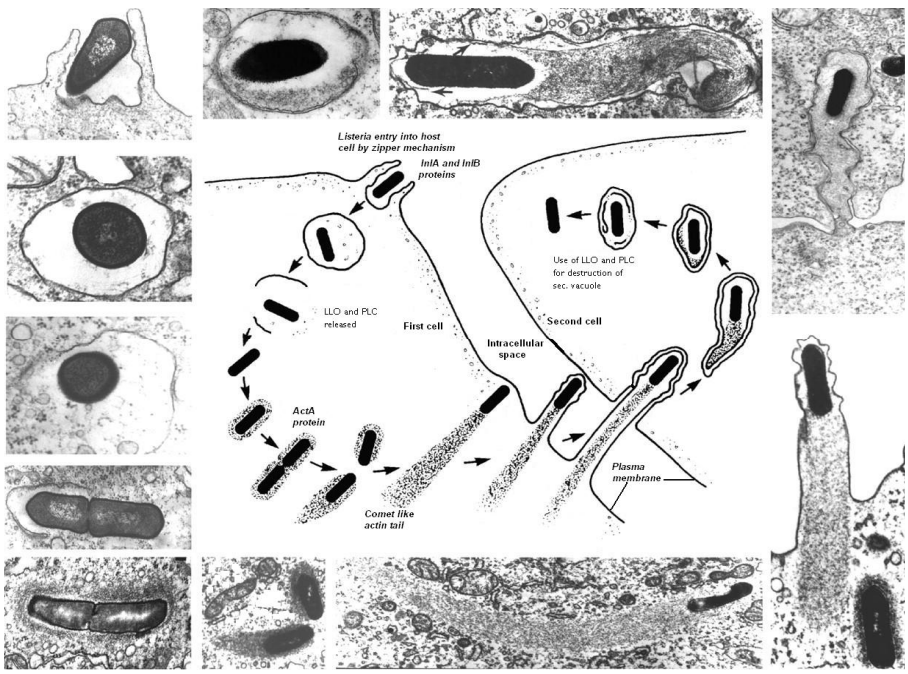
Step 1
L. monocytogenes provokes its internalisation into mammalian cells that are nonphagocytic such as epithelial cells and hepatocytes. Two major internalisation factors (proteins) are the products of *inlA* and *inlB* genes. Internalisation is initiated by bacterial surface protein InlA or InlB binding to their respective host receptors. The InlA receptor is E-cadherin (Mengaud *et al.*, 1996). InlA-mediated uptakes utilize components of adherens junctions hinged to F-actin and myosin. InlB-dependent entry involves cytosolic adaptors joining Met to F-actin regulators, including phosphoinositide 3-kinase and activators of the Arp2/3 complex.

Step 2
This is initiated by the transient signals occurring after the formation of the first ligand-receptor complexes and that propagate around the invading microbe. These signals induce actin polymerisation and membrane extension in host cells. Finally, actin depolymerisation occurs after the closure and retraction of the phagocytic cup.

Step 3

L. monocytogenes gene such as *hly* (see **Paper III**) encodes a pore forming cytolysin called listeriolysin O (LLO) that is responsible for *Listeria* escape from the phagocytic vacuole. *Listeria* secretes two types of phospholipase C (PLC) that assists lysis of the vacuole. Version one of PLC is active for PI-PLC (phosphatidylinositol-PLC) and is unique for two proteins such as PI (phosphatidylinositol) and glycosyl-PI-anchored protein. The second version, PC-PLC (phosphatidylcholine-PLC), has a wide substrate range. Both versions act synergistically with LLO in lysis of first and second vacuoles (Camilli *et al.*, 1993; Gedde *et al.*, 2000). In the absence of LLO, PC-PLC assists lysis of the primary vacuole.

Figure 12. Entry, cytosolic life and intercells movement cycle of *L. monocytogenes.*



Step 4

Hexose phosphate transporter (Hpt) assists in intracellular replication. *Listeria*-induced polymerisation of actin filaments in the cytosol is responsible for bacterial movements with the help of a transmembrane motif in the carboxyl-terminal domain. As protein ActA is distributed asymmetrically on bacterial surfaces, this leads to propulsive effects of actin polymerisation.

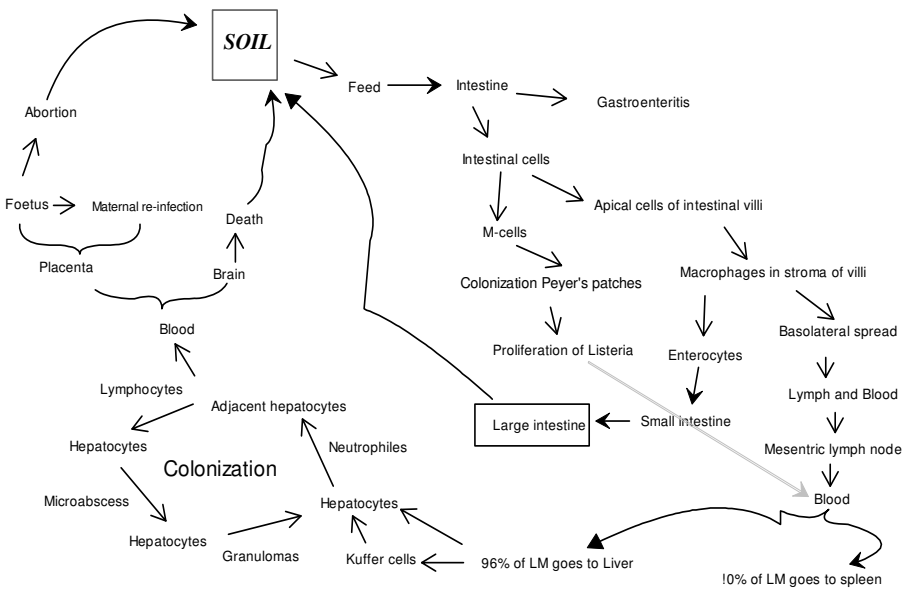
Step 5

L. monocytogenes needs to spread to new host cells as a means of obtaining fresh supplies of nutrients and avoiding humoral and cellular immune responses (Auerbuch *et al.*, 2001). Bacterial spread from cell to cell is through exploitation of host actin polymerisation. Cytosolic *L. monocytogenes* is soon surrounded by a dense cloud of host-derived F-actin, which forms a comet- tail structure at one bacterial pole (Tilney and Portnoy, 1989). After 2h of internalisation, *L. monocytogenes* begins to move in the host cytosol at a velocity of 0.1-0.4 μm/sec. The *L. monocytogenes* surface-based protein ActA is needed for actin-based motility within the host cytosol and appears the only *L. monocytogenes* protein required to nucleate actin polymerisation. The length of the actin tail reflects the rate of bacterial movement and efficiency of cell-to-cell spread.

Step 6

This step needs the action of two phospholipases to facilitate the escape of *Listeria* from the double membrane vacuole created when polar actin polymerisation propels a bacterium from one cell into another (Tilney and Portnoy, 1989; Mounier *et al.*, 1990).

Fig.13. Life cycle of *L. monocytogenes*.



Pathogenicity islands of genus *Listeria*

Only two species of genus *Listeria* (*L. monocytogenes* and *L. ivanovii)* are considered pathogenic, causing listeriosis. Both species have pathogenicity island designated LIPI-1, but *L. ivanovii* has an additional pathogenicity island, LIPI-2. Other loci are present on chromosomal DNA responsible for virulence in *L. monocytogenes* and *L. ivanovii*.

LIPI-1 contains genes such as prfA, plcA, plcB, hly, mpl, actA, orf XYZ, orf BA

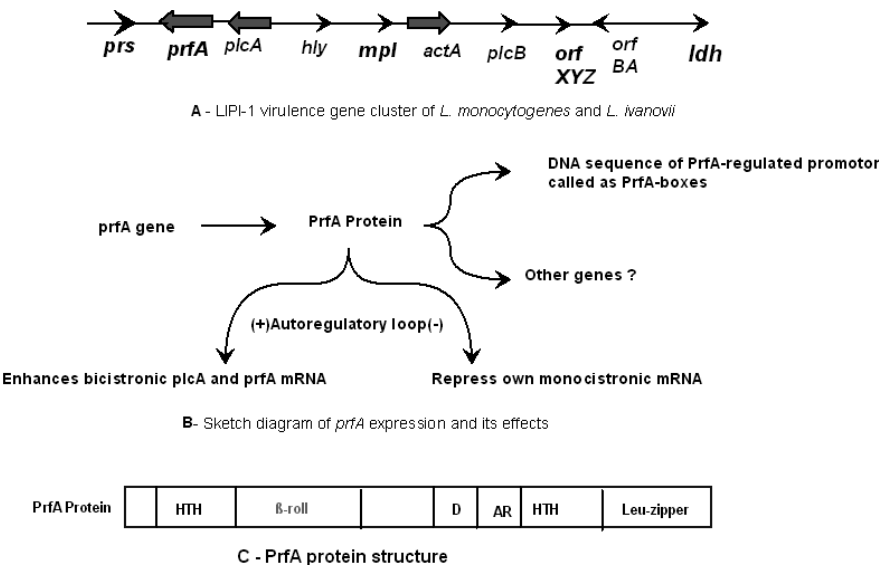
The LIPI-1 cluster or island (Figures 14 & 15) of virulence genes is identical in both *L. monocytogenes* and *L. ivanovii*. The island consists of virulence genes and the expression products of these genes are important for the intracellular life of bacteria. This cluster is present between the housekeeping gene *prs* (phosphoribosyl synthetase) and *idh* (lactate dehydrogenate). The central position is taken by the *hly* gene that is responsible for production of listeriolysin O (LLO) (Gedde *et al.*, 2000). The gene *plcA* produces phosphatidylinsitol-specific phospholipase C (Pl-PLC) (Gandhi *et al.*, 1993) (see **Paper I**).

The gene *plcB* produces a zinc-dependent phospholipase C protein (PlC-B), which has similarities to clostridal phospholipase C. This protein (enzyme) is produced in inactive form and is then modified by Mpl protease. PlC-B along with LLO helps *Listeria* disrupt the primary vacuole. The main role of PlC-B is to dissolve double-membrane phagosome (second vacuole), thus helping the pathogen to bypass the humoral immune system in the host.

The gene *actA* utilize host cell action for making a propulsive tail. ActA proteins in *L. monocytogenes* and *L. ivanovii* have same function but differ in amino-acid composition (Kreft *et al*, 1995). Amino acids in the ActA protein also differ among *L. monocytogenes* strains (Sokolovic *et al.*, 1996).

PrfA is the transcriptional activator of all genes (Figure 14)

Figure 14. PrfA activity



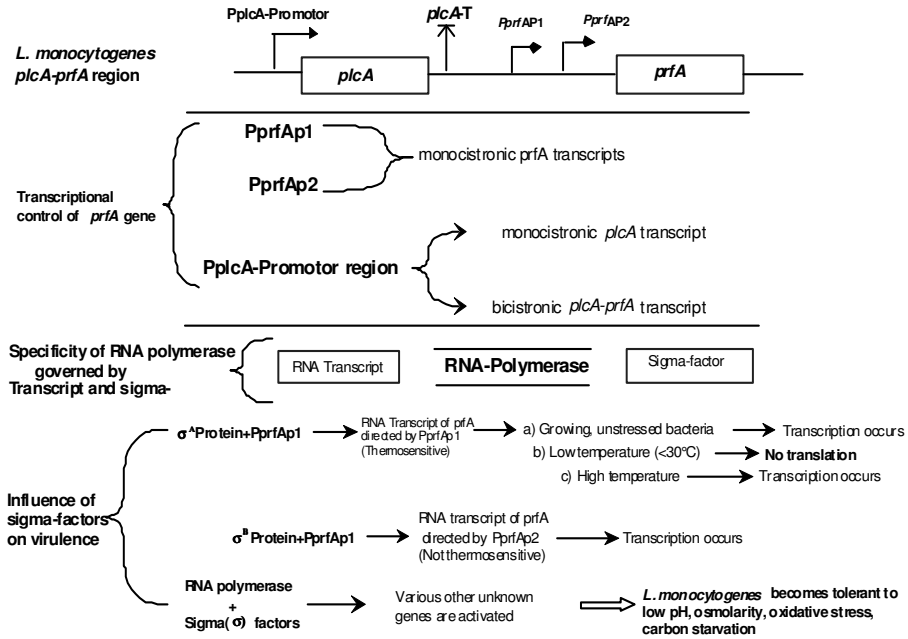
Other virulence-associated genes identified responsible for tolerance to bile salt *(bsh)*, acid (*gad)*, and osmotic stress (*htq)* have been identified.

LIPI-2 contains internalin genes and *smcL* gene (sphingomyeline C)

LIPI-2 is a large (22 kb) gene cluster consisting of several internalin genes and the *smcL* gene. The products of *smcL* gene selectively lyse sheep erythrocyte membrane (Gonzalez-Zorn *et al.*, 1999): this may be the reason *L. ivanovii* mainly causes disease in sheep. The majority of LIPI-2 *inl* genes are PrfA dependent, but *smcL* is independent of PrfA regulation. Enzyme encoding by *smcL* exhibits similar functions to β-toxin in *S. aureus* and SMase of *B. cereus*. The DNA composition of LIPI-2 differs from other *L. ivanovii* genomes and is unstable: this indicates LIPI-2 has a recent origin of in the evolution of genus *Listeria.*

The main products of other loci include (Ripio *et al.*, 1997; Milohanic *et al.*, 2000): stress tolerant mediators; Clp (intraphagosomal survival); ClpP (Stress protease); ATPase (member of HSP-100 stress protein family); member of HSP-100 stress protein family; Ami protein (attachment to host); and PrfA-dependent HPT (hexose phosphate transporters)..

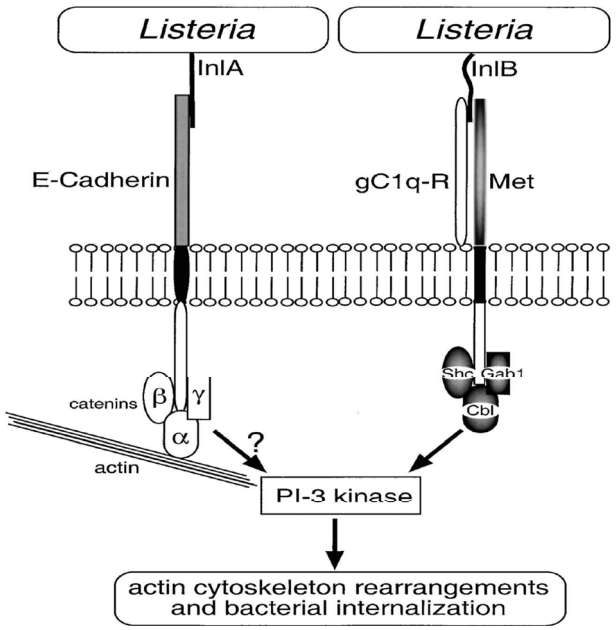
Figure 15. Activity of various virulence genes and protins in the LIPI-1 cluster.



Internalins

In addition, important internalin islets assist internalisation of *L. monocytogenes* into non-phagocytic host cells. *L. monocytogenes* and *L. ivanovii* produce internalins that facilitate internalisation of pathogens in non-phagocytic cells (Gaillard *et al.*, 1991). Internalin proteins are coded from genes present in islets at different locations. Internalin genes undergo recombination and transferral during evolution. All internalin proteins consist of novel domains containing varying numbers of leucine-rich repeats (LRR), making this protein family unique among pathogens of different genera. LRR were first identified in *Erwinia chrysanthemi* and were later successfully purified from *Yersina pestis* (YopM-prtotein), *Bordetella pertussis* (filamentous haemagglutinin) and *Shigella flexneri* (IpaH-protein). Leucine-rich repeats of internalin proteins have a right-handed helix that turns after each leucine repeat (Figure 16).

Figure 16. Mechanism used by *L. monocytogenes* to enter cells (reproduced by permission from Dr. Jorge Galan of Yale University, USA).

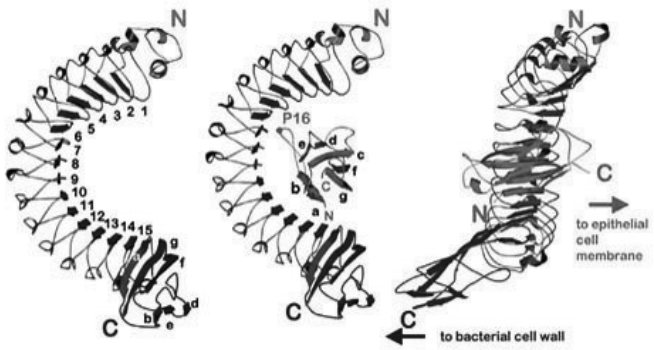


Two internalin protein families are known:

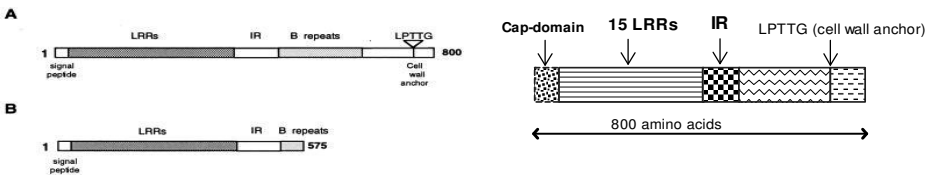
- Internalin family made up of large proteins of 70-80 kDA, with the C-terminal attached to the cell wall: they include InlA, InlB, InlC2, InlD, InlE, InlF, InlG, and InlH (Figure 17).
- This family consists of small internalin proteins of 25-30 kDa and no C-terminal. These types of proteins are mostly released in the extracellular medium, one example is InlC.

In *L. monocytogenes* internalin genes have three locations: position 1, *inlG* (C2D/H) internalin islet, this locus is between two housekeeping genes *ascB* and *dapE*; position 2, *inlA, inlB;*and position 3, *inlC*, this gene is alone and its products (proteins) are secreted in small quantity. *L. ivanovii* consists of sets or loci of genes responsible for internalins: position 1, *i-inlC* and *i-inlD; and* position 2, LIPI-2, a large internalin islet inserted between two housekeeping genes. *L. monocytogenes* genes encode large surface-anchored internalins and few genes encode small-secreted internalins. In *L. ivanovii,* many genes encode small-secreted internalins but no gene encodes large surface-associated internalin (Engelbrecht *et al.*, 1998a, 1998b, Gonzalez-Zorn *et al.*, 2000).

Fig.17. Structure of internalin-A and its domains of *L. monocytogenes*. (with permission from Prof. Partho Ghosh, Dept. of Chemistry and Biochemistry, University of California, San Diego, USA)



Structure of InlA'(A) Uncomplexed InlA': cap domain-pink, LRR-domain-violet, Ig-like interrepeat domain-blue. ß strands of the LRR are numbered; those of the Ig-like domain are indicated by letters.



Structure of Internalin A protein (InlA) of *L. monocytogenes*

This protein has 800 amino acids and two main parts, or terminals, known as N-terminal and C-terminal (Lecuit *et al.*, 1999). N-terminal consists of signal peptide and 15 LRR units and C-terminal consists of three longer LRR and cell wall anchor (LPXTG, hydrophobic 20 amino acids, charged amino acids) (Figure 17). LRR makes covalent bonds with the peptidoglycan of the cell envelope with the help of sortase.

Structure of Internalin B protein (InlB) of *L. monocytogenes*

InlB, which is partially buried in the cell wall, interacts with cytoplasmic membrane and consists of N-terminal, which starts with a signal peptide followed by 15 units of LRR, and C-terminal regions, which lacks LPXTG and hydrophobic tail (Bohne *et al.*, 1996). A three-dimensional structure of InlB reveals a LRR region with a long, slightly curved β-strand-loop-310-helix-loop (Marino *et al.*, 2004) (Figure 18). An inter-repeat (IR) region, which resembles immunoglobulin, projects at a 90° angle from LRR base, and after the IR is a highly extended L-shape molecule called B-repeat (Figures 19-21). The units after this have 80 amino acid sequences starting with GW (Gly-Trp) dipeptide,

which is known as the cell surface anchor (Csa); this unit attaches to lipoteichoic acid of the cell wall of *L. monocytogenes*.

Figure 18. Various domains of internalin-B of *L. monocytogenes.* (with permission from Prof. Partho Ghosh, Dept. of Chemistry and Biochemistry, University of California, San Diego, USA)

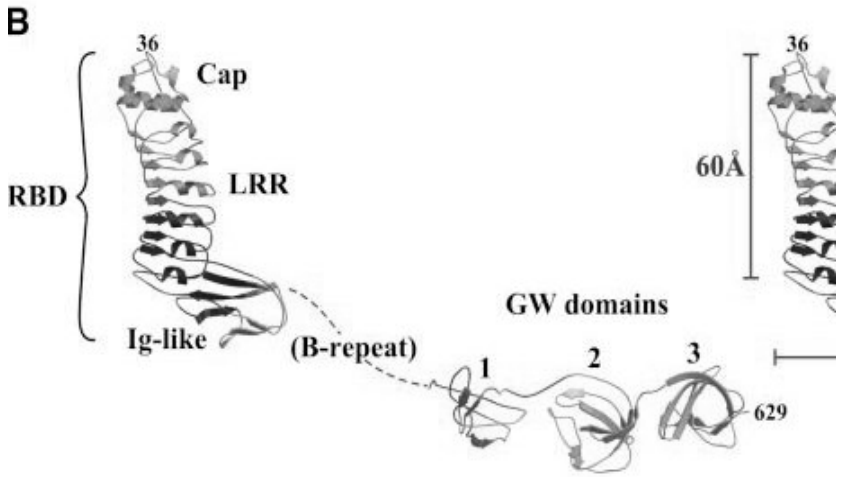


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Figure 19 Model of InlB mediated Met activation by receptor clustering (reproduced by permission from Prof. Dr. Drik Heiz of German Research Center for Biotechnology, Braunschweig, Germany).

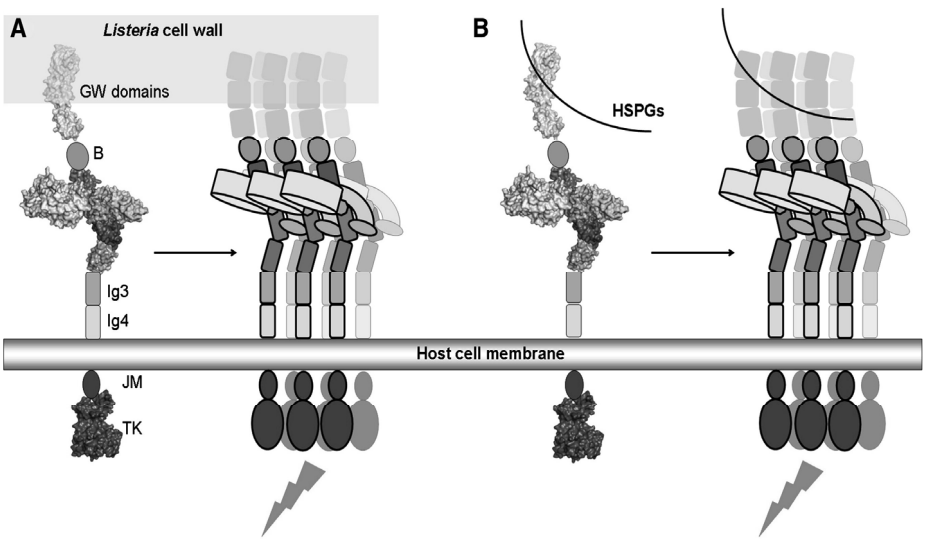


Figure 20. Primary interface between InlB, LRR and Met Ig1. (reproduced by permission from Prof. Dr. Drik Heiz of German Research Center for Biotechnology, Braunschweig, Germany).

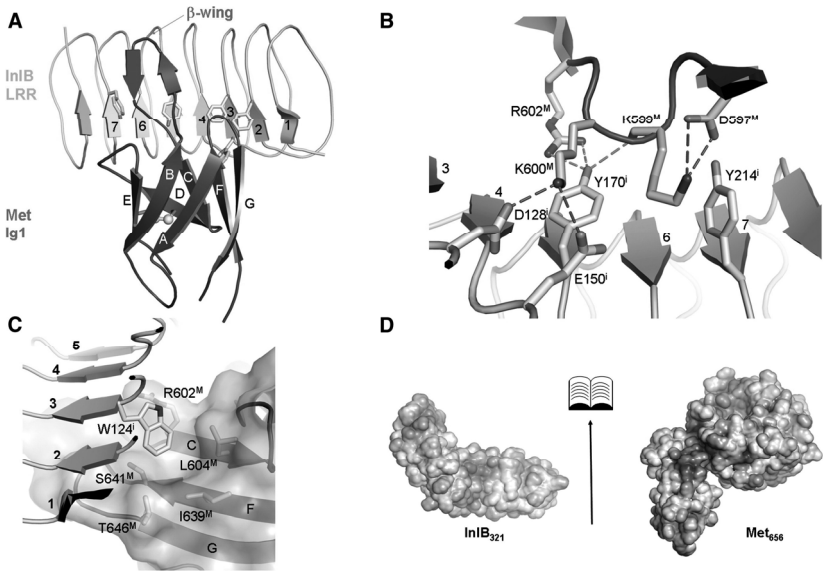
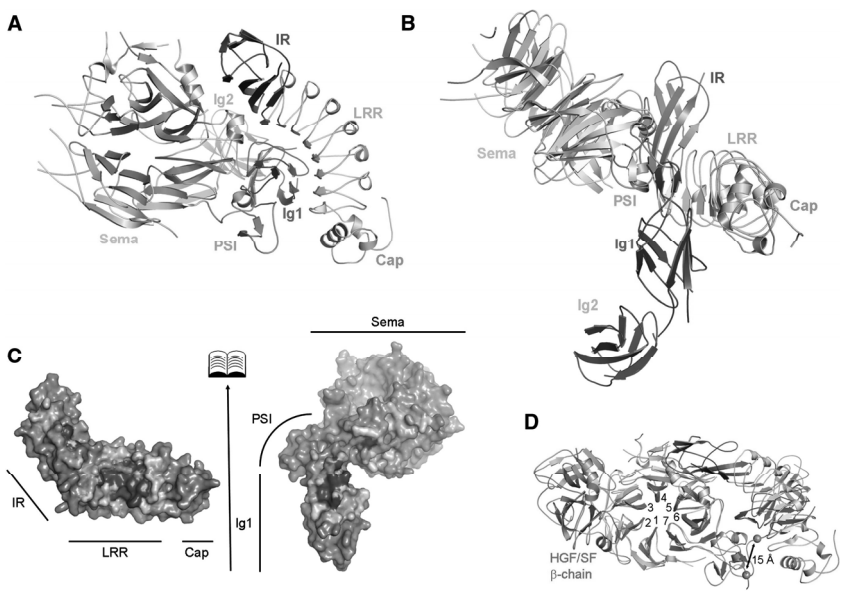


Figure 21. Structure of the Met-InlB complex reveals two interfaces that are distinct from the HGF/SF beta chain. (reproduced by permission from Prof. Dr. Drik Heiz of German Research Center for Biotechnology, Braunschweig, Germany)



***Listeria* science after genome sequencing** (Buchrieser, 2007; Vazquez-Boland, 2002)

The main striking features about genome of *L. monocytogenes* are: a large number of *surface proteins*; an abundance of *transport proteins*; and an *extensive regulatory repertoire*.

Surface proteins

Lipoproteins
These types of bacterial proteins have important interaction with host cell. This large family of surface proteins are encoded by 2.5% of the genes in *L. monocytogenes*. *Listeria* lipoproteins include 28 putative substrate-binding proteins (SBPs) of an ABC transporter system, 19 proteins are involved in different enzymatic activities or other functions and 21 proteins have unknown function. Lipoproteins are anchored to the cell membrane of the bacteria by covalent N-terminal lipidation. These proteins are directed by a specific signal peptide sequence having conserved cysteine residues called lipobox. These proteins maturate through two-steps.

LPXTG
About 41 proteins (1.4%) of *L. monocytogenes* belong to this family. The characteristic feature of this LPXTG family is the LPXTG motif at C-terminal sorting signal that makes covalent bridges with peptidoglycan in the cell wall followed by a hydrophobic domain of about 20 amino acids and positively charged residues at the end. The sorting signal is a substrate for sortase enzyme that cleaves the LPXTG motif between theonine and glycine residues and helps to form an amide bond between the carboxy group of theonine and cell wall precursors. The LPXTG protein in *L. monocytogenes* is InlA.

Surface proteins with **non-covalent attachment to cell wall** are further divided into subfamilies (Figures 25 & 26):

- *GW proteins* - first characterized from InlB of *L. monocytogenes.* They are required for entry into many eukaryotic cell types. The C-terminal of InlB consists of three conserved tandem repeats of 80 amino acids called GW, as they start with dipeptide Gly-Try. GW binds InlB to lipoteichoic acid and this motif interacts with eukaryotic cell molecules such as glycosaminoglycans (GAG) and gc1q-R (Figure 16). Serovar 1/2a (EGDe) genome contains eight additional proteins with variable numbers of GW modules (seven putative autolysin-like Ami or Auto proteins).
- *WxL domain* – the cell wall associated domain of 160 to 190 amino acids is called WxL because of two conserved sequence motifs with Trp-x-Leu signatures. These proteins are present in low G+C Gram-positive bacteria and belong to the CscA family of surface proteins (Figure 24).

- *LysM domain* – P60 and MurA proteins are of this type. They have 1 to 4 copies of 40 amino acid domains called lysine motif (LysM). LysM domain is found in enzymes involved in bacterial wall degradation.
- *Hydrophobic tails* – proteins that have signal peptide may be retained in the membrane bilayer by hydrophobic segments present at the N-terminal or C-terminal of a protein. Proteins associated with membranes at C-terminal contain a carboxy-terminal stretch of hydrophobic residues and a few charged residues serving as stop-transfer signals. These types of proteins include ActA. Proteins may be tethered to the cell membrane by an amino-terminal hydrophobic stretch, which may be the signal peptide if it remains uncleaved. SrtA, SrtB and penicillin binding proteins (PBPs) belong to this class of proteins.
- *Peptidase peptidoglycan binding domain* – this domain is of 70 amino acids long with three alpha helices. Protein Lmo1851 of *L. monocytogenes* has this property. The three helices are N-terminal signal domain, a transmembrane domain and C-terminal domain.

Non-conventional secreted surface proteins
These proteins have no signal peptides and are coined with cytoplasmic functions, such as glycolytic enzymes, chaperons, heat shock proteins, proteins involved in detoxification and adoption to atypical conditions, nucleic acid metabolism, transcription, and translation. Usually these proteins are obtained after bacterial cell lysis but new evidence indicates that some of these proteins may be specifically transported to the surface by alternative pathways. Auxiliary secretory protein SecA2 is involved in the export of a subset of listerial proteins. Superoxide dismutase is a protein dependent on SecA2 for its secretion and surface association.

Transport proteins
The predominant class corresponds to ABC transporters. Of 331 transport proteins detected in *L. monocytogenes*, 86 are for transport of carbohydrate that is mediated by phosphoenolpyruvate-dependent phosphotransferase system (PTS).

Regulatory repertoire proteins are further classified into three subfamilies:
- GntR subfamily with 24 proteins
- Two-component system with 15 histidine kinases and 17 response regulators.
- BglG –are associated with PTS and have 18 proteins.

Adhesion of *L. monocytogenes* to host cells (Bierne and Cossart, 2007; Bierne *et al*, 2005; Bierne *et al*., 2007)

Adhesion is an important step in the life cycle for many pathogenic bacteria invading cells. The purpose of internalisation of bacteria is to gain access to an intracellular niche that can provide an environment for growth without selective pressure and to subvert host cell signalling pathways allowing them to avoid host defence mechanisms.
Internalisation of pathogens does occur in some immune cells such as macrophages, but this leads to destruction of the pathogen. However, some pathogens have evolved sophisticated mechanism and various adaptations to get inside of non-phagocytic cells without being destroyed.
Adhesins are specialised surface proteins that help the process of adhesion between the host and bacteria. Adhesin proteins bind to complementary receptors, or epitops, present on the target location of host cells. Host receptors (proteins) are present in extra cellular matrix (ECM), which is present in intracellular compartment of tissue. Three main proteins are recognised in ECM: structural proteins such as collagen and elastin; specialized proteins, such as fibrillin, fibronectin and elastin; and proteoglycans (Table 4).

Comparative genomics and sequence analysis (Figure 25) have revealed the domains in various proteins that exhibit adhesion properties to host cells. On this basis, *L. monocytogenes* proteins also have specialized domains, including (Figures 22 & 23):

- *Collagen binding domain* – Proteins Lmo0159, Lmo0160, Lmo0880, and Lmo2576 are predicted to have this type of domain and are similar to C_{an} protein of *S. aureus*. C_{an} is having an N-terminal A region that carries the collagen binding activity and a C-terminal region of B repeats. The C_{an} region is made up of three subdomains N1, N2 and N3. The collagen binding protein present in *L. monocytogenes* is similar to N2 subdomain of this family.
- *Fibronectin binding domain (FbpA)* – Lmo1829 is a *L. monocytogenes* fibronectin binding protein that helps *Listeria* to colonize the liver and intestine and is homologous to PavA protein of *S. pneumoniae*. Lmo0721 is another listerial fibronectin binding protein but lacks an anchoring signal.
- *Proteoglycan binding domain* - Binding of GW with eukaryotic cell molecules such as glycosaminoglycans (GAG) enhance the interaction of InlB to its cellular receptor Met, this suggests a similar interaction may explain the role of Ami in *L. monocytogenes* adhesion to host cells.
- *Arginine-glycine-aspartate (RGD) motif* – Is present in various ECM proteins and interacts with integrins. Three *L. monocytogenes* proteins contain this type of domain - ActA, LPXTG protein (Lmo1666), and the lipoprotein (Lmo0460).
- *Mucin-binding domain* - One protein of *L. monocytogenes* (Lmo0576) contains a mucin-binding protein (MucBP). *L. monocytogenes* also contains

12 LPXTG proteins that exhibit this type of domain. MucBP are only present in *Lactobacillus spp* and *Listeria spp*.

- *Ig-like domain* – Further divided into five sequence families: C_{an} collagen binding domain; C_{an} protein B-type domain; LRR-adjacent domain; PKD repeat domain; and Big 3 domain.

Figure 22 Different types of surface proteins found in *L. monocytogenes*. (Reproduced by permission from Dr. Hélène Bierne)

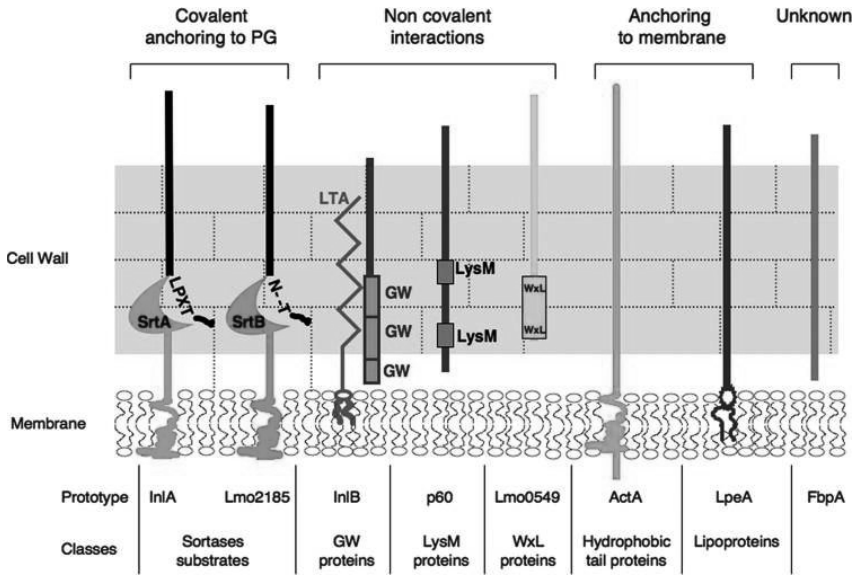


Figure 23 Surface proteins predicted to be involved in protein processing, folding, and anchoring at the cell surface. AA= amino acid. (Reproduced by permission from Dr. Hélène Bierne).

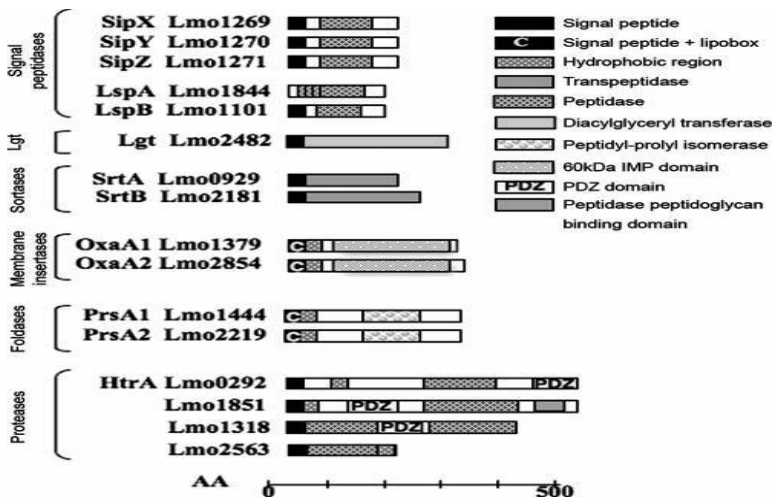


Figure 24 Two Csc clusters in *L. monocytogenes*. The first exhibits four-component Csc cluster, with predicted surface-anchoring domain of Csc protein. AA - amino acid (reproduced by the permission of Dr. Hélène Bierne).

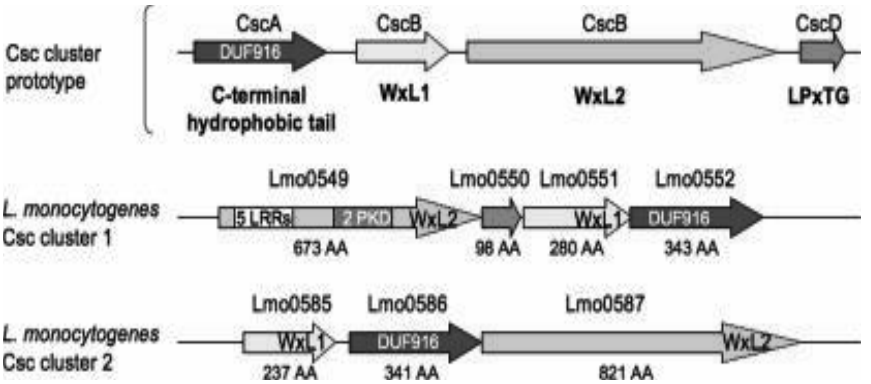


Figure 25. In Silico analysis of surface proteins of the genus *Listeria.*

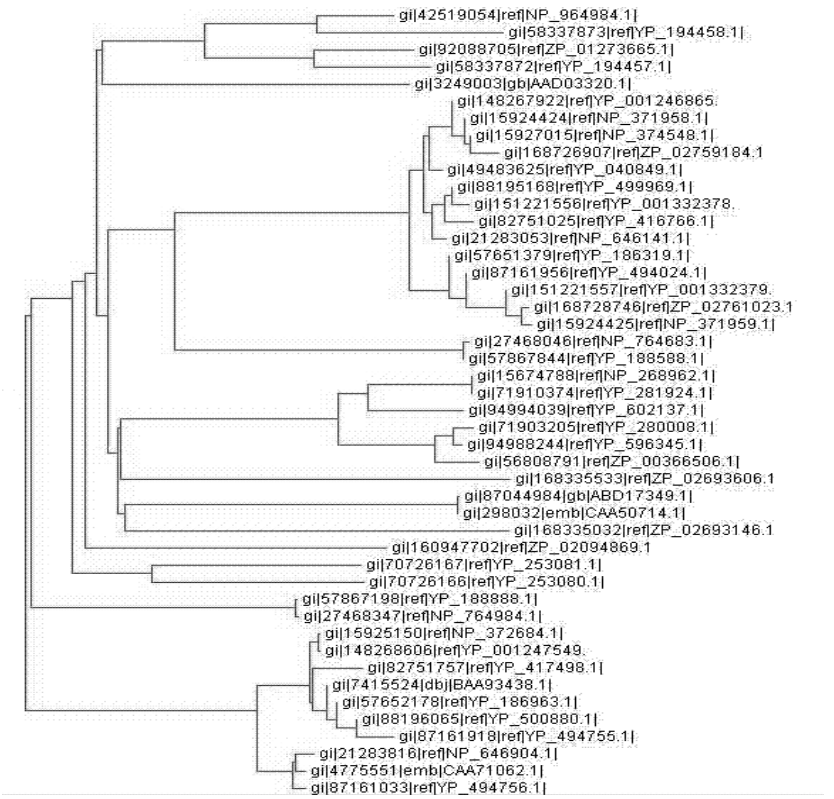
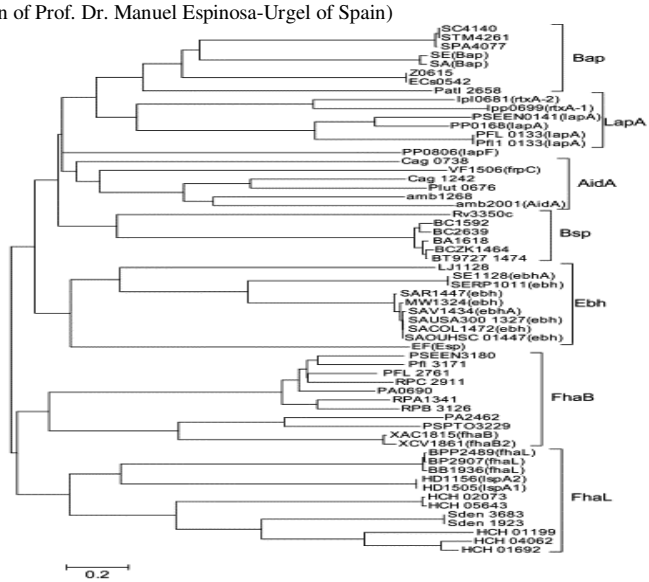


Table 4. Representative motif in large microbial adhesions.

Family of Protein	Protein name	Organism	VWF-A	aa-Rich domain	Cadherin	RGD motif	Other motifs
LapA family							
	PSEEN0141	*Pseudomonas entomophila L48*	+	T, G	+	-	
	Ipl0681(rtxA-2)	*Ligionella pneumophola str. Lens*	+	-	-	-	
	Ipp0699(rtxA-1)	*Ligionella pneumophola str. Paris*	+	-	-	-	
AidA family							VCBS
	VF1506(frpC)	*Vibro fischeri ES114*	-	T	+	+	VCBS
	Ava4160	*Anabaena variabilis*	-	T	-	+	VCBS
	VC1451	*Vibrio cholerae 01 biovar eltor*	-	T	-	+	VCBS
Bap family							
	AAK38834 (Bap)	*Staphylococcus aureus*	-	T, D, N	+	-	Gram+anchor
	AAY28519 (Bap)	*Staphylococcus epidermidis*	-	T, D, N, S	+	-	Gram+anchor
	Z0615	*Escherichia coli 0157:H7 EDL933*	-	T	-	-	
	ECs0542	*Escherichia coli 0157:H7 Str. Sakai*	-	T	-	-	
	SC4140	*Salmonella enterica sv. Choleraesuis*	-	T	+	-	
	SPA4077	*Salmonella enterica sv. Paratyphi A*	-	T	+	-	
	STM4261	*Salmonella typhimurium LT2*	-	T	+	-	
Ebh family							
	SACOL1472	*Staphylococcus aureus COL*	-	T, N, Q, K	-	+	Leu zipper
	SAR1447(ebh)	*Staphylococcus aureus MRSA252*	-	T, N, Q, K	-	+	Leu zipper
	SAV1434(ebhA)	*Staphylococcus aureus Mu50*	-	N, Q, K	-	-	
	SERP1011(ebh)	*Lactobacillus johnsonii NCC 533*	-	A	-	-	Gram+anchor
FhaL family							
	BB1936(fhaL)	*Bordetella bronchiseptica RB50*	-	A	-	-	Leu zipper
	BP2907(fhaL)	*Bordetella pertussis Tohama 1*	-	A	-	-	Leu zipper
FhaB family							
	PA0690	*Pseudomonas aeruginosa PAO1*	-	A, G	-	+	Leu zipper
	RPC2911	*Rhodopseudomonas palustris BisB18*	-	A, G	-	+	Hemagglutinin
	PSEEN3946	*Pseudomonas entomophila L48*	-	-		-	Hem, autotras
Unclassified							
	YPN3592	*Yersinia pestis Nepal516*	-	T	-	+	Big-1, intimin
	LJ0484	*Lactobacillus johnsonii NCC 533*	-	T	-	-	Gram+anchor
	LBA1392	*Lactobacillus acidophilus NCFM*	-	T, P	-	-	Gram+anchor
	nfa8110	*Nocardia farcinica IFM 10152*	-	A, G, P, D	-	+	Leu zipper

Figure 26. Phylogenetic relationship between various surface proteins of prokaryotes (reproduced with permission of Prof. Dr. Manuel Espinosa-Urgel of Spain)



Immune system response to *L. monocytogenes* infection

Adaptive immunity

Mackaness (1962) highlights the role of T-cell mediated immune responses for *L. monocytogenes*. Long-term immunity is mainly due to $\alpha\beta$ T-cells and $CD8^+$ T-cells (among the family of $\alpha\beta$ T-cells). $CD8^+$ T-cell mediated adaptive immunity is independent of IFN-γ (Harty and Bevin, 1995). $CD4^+$ T-cells have fewer roles in immunity against *L. monocytogenes*. $CD4^+$ T-cells may assist in IFN-γ production for the activation of macrophages, thus, there will be less chance of *L. monocytogenes* escaping the phagosome (Portnoy *et al.*, 1989). *L. monocytogenes* secretes fewer proteins into the cytosol than other cytosolic intracellular bacteria. The protein LLO in cytosol elicits the production of $CD8^+$ T-cells and is antigenic, despite lower quantities in cytosol. The reason for lower quantities of LLO in cytosol could be: LLO production is down regulated after *L. monocytogenes* enters a cell, or LLO is rapidly degraded by the proteasomes of the infected host cells.

$CD8^+$ T-cells provides immunity to *L. monocytogenes* and can be classified into two sub-populations: MHC-class-1a restricted/classical MHC-class-I $CD8^+$ T-cells and MHC-class-1b-H2-M3 restricted/non-classical MHC-class-1 $CD8^+$ T-cells (Figure 27).

MHC class-1a restricted/classical MHC-class-I $CD8^+$ T-cells

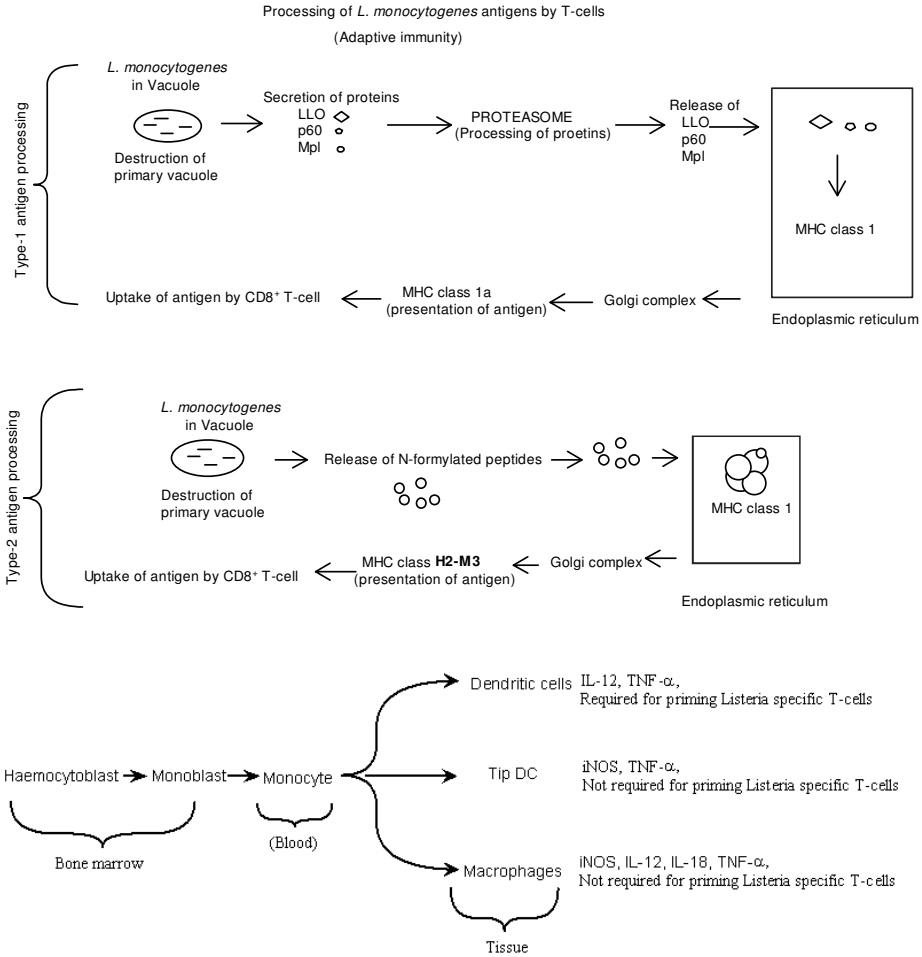
LLO is quickly degraded as it contains a sequence know as pest-sequence that enhances proteasome mediated destruction of protein (Villanueva *et al.*, 1995). Aside from LLO, antigen $_{P}60$ also induces production of $CD8^+$ T-cells. $_{P}60$ contains at its amino-terminal a specific amino acid that is recognized by proteases. $CD8^+$ T-cell proliferation (clonal expansion) is independent of the quantity and duration of antigen presentation. The clonal expansion of $CD8^+$ T-cells is not fully understood, but in vitro studies indicate the $CD8^+$ T-cells antigen presentation stage is transient and followed by prolonged proliferation of $CD8^+$ T-cells, and may be influenced by initial inflammatory response.

MHC-class-1b-H2-M3 restricted/non-classical MHC-class-1 $CD8^+$ T-cells

Some $CD8^+$ T-cells are produced in the cell and are MHC unrestricted. MHC unrestricted cells are mainly under the control of MHC class 1b molecule H2-M3. This molecule of T-cells recognises bacterial peptides with N-formyl methionine at their N-terminal (Lenz *et al.*, 1996; Princiotta *et al.*, 1998; Gulden *et al.*, 1996). Such peptides are directly secreted into cytosol without being processed by proteasomes. H2-M3 bound T-cells have characteristic features and these types of T-cells are not restricted to one class of peptide recognition. H2-M3 T-cells acquire peak level at 5-6 days after primary infection, and their immune response is quicker than MHC-class-1a immune response. H2-M3 T-cells produce IFN-γ, a feature required by the host for early clearance of

bacterial load, but such responses have poor memory for clearing bacterial reinfection; one reason for less memory is attributed to competition from MHC-class-1a restricted T-cells. MHC-class-1a T-cells interfere with clonal expression of memory cells, but not with primary H2-M3 restricted T-cells.

Figure 27. Sketch diagram of adaptive immunity of *L. monocytogenes*.



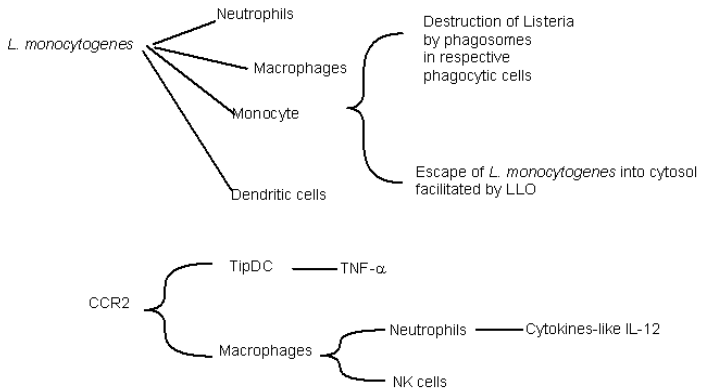
Innate immune responses to *L. monocytogenes*

Innate immune response is a stepwise response with sequential involvements of activation and down-regulation of signalling pathways. Production of interferon-γ (INF-γ) by natural killer cells results in activation of macrophages and tumour-necrosis factors (TNF-α) (Tripp *et al.*, 1993). Signalling through TNFRp55 and lymphotoxin-β receptor is important for defence against infection with *L. monocytogenes*. Neutrophils and macrophages are the principle cells for killing *L. monocytogenes*. The recruitment and proliferation of monocytes indicate an inflammatory action by the host tissue, in response to infection with *L. monocytogenes,* and is essential for primary defence of the host body.

The mechanisms of bacterial clearance by granulocytes or monocytes are not fully understood, although, the production of nitric oxide and oxidative burst are the two possible bactericidal properties of granulocytes. The recruitment and migration of granulocytes to the infection site are limited to the understanding of studies made on lymphocyte function-associated antigen 1 (LFA1)-deficient mice (Miyamoto *et al.*, 2003). Infected macrophages are the source of TNF-α, iNOS and CC-Chemokine ligand-2 (CCL2) (Miyamoto *et al.*, 2003). The circulating monocytes express a chemokine receptor CCR2 that binds to CCL2, but macrophages are not solely responsible for the production of TNF-α and iNOS. A new family of dendritic-cells producing TNF-α and iNOS have been called TipDC (Serbina *et al.*, 2003). The major production of TNF-α and iNOS is in uninfected cells rather than heavily infected macrophages in white-pulp of spleen (Figure 28).

On the host cell surface, Toll-like receptors (TLR) transmit signals in response to microbial molecules. Many TLRs such as TLR2 and TLR5 play a role in defence. TLR2 transmits signals to the host cell when they have contact with any of peptidoglycan, lipoteichoic acid and lipoprotein of bacteria. TLR5 transmits signals to the host cell in response to bacterial flagellin proteins. However, the role of both TLRs needs to be further elucidated. TLR5 interacts with flagellar antigen of *L. monocytogenes* and triggers the intracellular adaptor protein MyD88. MyD88-independent pathways are important in innate immune response against *L. monocytogenes* (Seki *et al.*, 2002; Serbina *et al.*, 2003). As with MyD88, another transcriptional nuclear factor- κв (NF-κв) is involved in the expression of different genes involved in immune response. NF-κв is triggered by *L. monocytogenes* invasion of cytosol and simultaneously LLO (listeriolysin O) production increases.

Figure 28. the body's innate immune response to *L. monocytogenes* and various cells produced in response to infection.



L. monocytogenes lineages

Lineages are primarily used to understand the epidemiology including transmission of *L. monocytogenes* from all animate and inanimate reservoirs, and to cluster serovars occurring frequently within a host and to explain genetic relatedness among different serovars based on prevalence or incidence of listeriosis. Lineages are branches of a phylogenetic tree classified on DNA, RNA or protein sequences. Defining lineages based on incidence or prevalence of a serovar in a host can be problematic and thus it is suggested that clusters of isolates with same serovar be called groups/divisions rather than lineages.

The University of California, Berkley, CA, USA, (www.universityofcalifornia.edu) defines lineages as "a continuous line of descent; a series of organism, population, cells, or genes connected by ancestor relationships". Various cluster analyses of *Listeria* strains highlight the difficulty in defining particular groups and several methods are used to differentiate *L. monocytogenes* beyond species level, as a result various groups or lineages are constructed. Isolates of *L. monocytogenes* can be divided into two divisions based on allelic profiling known as electrophoretic types (ETs) (Piffaretti *et al.* 1989) and 45 ETs of *L. monocytogenes* have been detected: division I represents strains of serotypes 4b, 1/2b and 4a, and division II serotypes 1/2a and 1/2c. There is no evidence of gene differences among strains of *L. monocytogenes* causing infection among human, sheep, rabbits and horses. Piffaretti *et al.* (1989) put forward an explanation for the high prevalence of ET 1 in various epidemic episodes as: ET 1 adapts to particular food, for example, cheese; ET 1 is abundant in the environment.

The reason for creating groups is to identify isolates more prone to cause disease and attempts to highlight genomic divisions of *L. monocytogenes* are based on PFGE (Brosh *et al.*, 1994), ribotyping (Graves *et al*, 1994) and serotyping

(Nadon *et al*, 2001). Attempts to clarify the biology of lineages based on gene expression and polymorphism among isolates of *L. monocytogenes* from humans, animals and food have been unsuccessful (Jacquet *et al.*, 2002). A microarray method was applied to test the virulence of strains capable of causing listeriosis (Doumith *et al.*, 2004) and the parameters were based on the presence or absence of virulence genes: various subgroups were identified. Virulence genes were present in all isolates of *L. monocytogenes* recovered from clinical cases or environment; however, the distribution of 55 genes coding for putative surface proteins of internalin/LPXTG/GW motif differed. The heterogeneity in virulence was a temporarily phenomenon encountered in various strains of *L. monocytogenes* that are influenced by the environment of proliferation (Doumith *et al.*, 2004). Thus, strains of all serovars of *L. monocytogenes* can act as potential pathogens infecting both human and animals.

Figure 29 Lineages proposed by different authors.

Unnestrad and Tham et al.

Serovar 1/2a strains division byPCR-REA

PCR-REA group	Strain origin	No. of strains in group
1/2a:I	Human	26
	Animal	20
	Food	18
	Environment	6
		Total 70
1/2a:II	Human	10
	Animal	13
	Food	3
	Environment	4
		Total 30

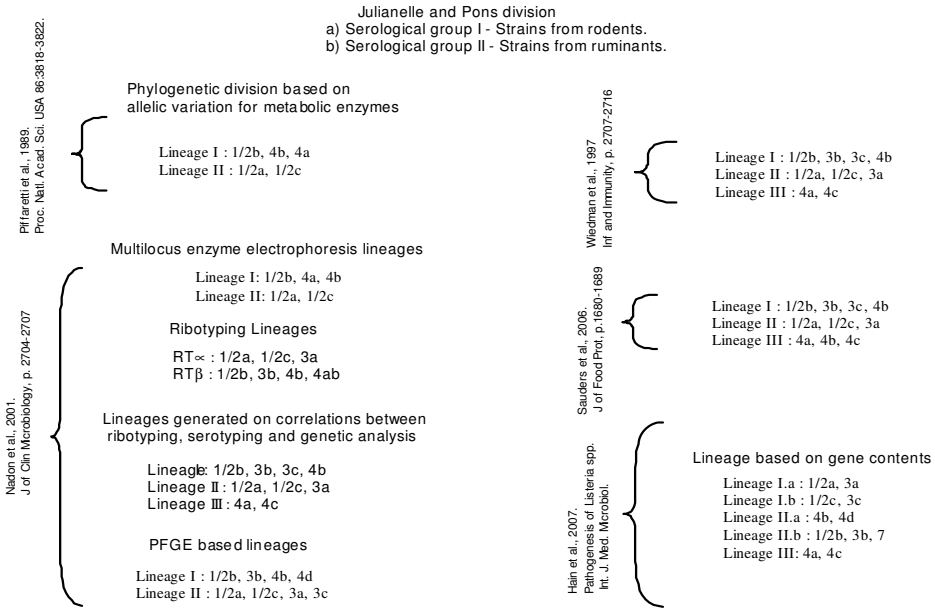
Ericsson and Tham et al.

Division of *L. monocytogenes* serovars by PCR-REA method

Group one
4b.I
Group Two
4b.II
Group Three
1/2b
3b
Group Four
1/2c
1/2a

conti…

Lineages of *L. monocytogenes* suggested by different authors



Listeriosis status in India

Animal listeriosis in India

Listeriosis in animals from the Indian subcontinent has been reported mainly in the form of sporadic cases and occasionally as outbreaks. The first report of animal listeriosis in India was from sheep in the state of Hyderabad in 1936 (Mahajan, 1936) but isolation was unsuccessful until 1950 (Vishwanathan and Iyyar, 1950). Subsequently, *L. monocytogenes* has been isolated from a number of animal species including goat, chicken, cattle, buffalo, cow and pig. Outbreaks in animals are mainly confined to small ruminants (sheep and goat) but there are reports of sporadic cases in large ruminants such as buffalo and cow (Uppal *et al.*, 1981; Rahman *et al.*, 1985).
The type of animal husbandry practised might influence the occurrence of the disease. The average livestock holding per farmer is smaller than in Western countries. Debeaking poultry is considered a predisposition in the occurrence of listeric infections (Vijayakrishna *et al.*, 2000).

Most Indian cases reported are from last trimester of pregnancy in ruminants (Kapur and Sadana, 1974). In 1977-78, large numbers of abortions were reported among sheep in the state of Jammu and Kashmir (Vishwanathan and Uppal, 1981) but *L. monocytogenes* was only isolated from one sample. Similar studies in northern India revealed prevalence of *L. monocytogenes* in small ruminants (sheep and goat). An epidemiological survey in the state of Himachal Pradesh indicated the presence of *L. monocytogenes* in migratory flocks of sheep and goat (Sharma *et al.*, 1996; Nigam *et al.*, 1999) and sporadic cases and outbreaks of listeriosis are reported among swine (Dutta and Malik, 1981; Rahman *et al.*, 1985; Dash *et al.*, 1998). *L. monocytogenes* has been isolated from cow and buffalo in north India (Dutta and Malik, 1981). Outbreaks in poultry are rare but isolation of *L. monocytogenes* is common from faeces. Studies on the prevalence of *L. monocytogenes* are mainly limited to serological analysis (Jain *et al.*, 1996, 1997), and currently only 20 animal (cow) isolates of *L. monocytogenes* from India have been serotyped and all belong to serovars 4b (Parihar *et al.*, 2007).

Human listeriosis in India

Little attention is focussed on isolating *L. monocytogenes* among the human population in India and there are few reports of *L. monocytogenes* in clinical samples in India (Bhujwala & Hingorani, 1975; Gupta *et al.*, 1997; Dhanashree *et al.*, 2003), although antibodies against listeriolysin O have been detected in occupational groups (Barbuddhe *et al.*, 1999). The epidemiological data available in India is inadequate either because of failure to identify the isolate, low incidence rate or lack of awareness (Malik *et al.*, 2002). *L. monocytogenes* is one of the etiological factors causing abortion and premature birth (Bhujwala, *et al.,* 1973). Two cases of human listeriosis were recorded in India by Rocourt (1991).

In Chandigarh (Northern India), Dhawan and Dhall (1963) failed to isolate *Listeria* from the cervix of 100 cases of unsuccessful pregnancy and 60 cases of unhealthy cervical and vaginal discharges. Whereas, Krishna *et al.*, (1966), working in Mumbai (Maharashtra), isolated *L. monocytogenes* from the cervix of 14% of 150 patients with bad obstetric history *i.e*., past history of abortions, miscarriages, stillbirths or neonatal deaths. In addition, *L. monocytogenes* has been isolated in 3/100 (Bhujwala, *et al.,* 1973) and 9/670 women with a bad obstetric history (Bhujwala and Hingorani, 1975) and 4/40 women with a history of abortions (Stephen *et al.,* 1978) (**Paper I**).
Bhujwala, *et al.* (1973) isolated *L. monocytogenes* from an aborted foetus and blood of the mother; however, no isolations were made from 150 healthy controls with either primigravidas or history of obstetric mishaps (Bhujwala, *et al.,* 1973; Bhujwala and Hingorani, 1975). *L. monocytogenes* has been isolated from blood taken from a 4-hr-old baby, a 6-week-old child with congenital heart disease and digestive failure, and an 18-month-old severely malnourished child (Gupta *et al.,* 1997) and a perinephric abscess, presented as abdominal pain, in a 5-yr-old malnourished child (Gomber *et al.,* 1998).

Although *L. monocytogenes* is isolated from ruminants in India, isolation from milk and milk products is rare (Bhilegoankar et al, 1997) and suggests a low rate of contamination (Arora *et al.*, 2007). One reason could be that milk is boiled before consumption and before making most milk products.
The prevalence of *L. monocytogenes* in tropical fish is low (Karunasagar and Karunasagar, 2000) and few reports reflect the presence of *L. monocytogenes* in tropical environment (Jayasekaran *et al.*, 1996) and tropical fish (**Paper VI**).

Listeriosis in Sweden

The next International Symposium on Problems of Listeriosis (ISOPOL XVII) will be held in Porto, Portugal, May 7-10, 2010. That year, *i.e*. 2010, it will be 100 years since Gustav Hülphers, later professor at the Royal Veterinary College in Stockholm, Sweden, discovered *Listeria monocytogenes*. This discovery was published in Swedish in 1911 (Hülphers 1911). and the title of the paper was, in translation,''Liver Necrosis in Rabbit Caused by a Hitherto Unknown Bacterium''. The paper was for the first time translated to English by Nigel Rollison and published by permission of Johan Beck-Friis, Editor of Svensk Veterinärtidning, in the proceedings of ''ISOPOL XV, International Symposium on Problems of Listeriosis'' (Sept. 12-15, 2004, Uppsala, Sweden, Abstract 147a). In the same proceedings, Dr. Jim McLauchlin, a Listeria expert from the United Kingdom, published an abstract (Abstract 147b) in the same proceedings titled ''Was Hülphers the First to Describe Listeria monocytogenes?''

Citation from the abstract of Dr. McLauchlin:
''Firstly this (Hülphers paper) provides a picture of the meticulous work of a veterinary microbiologist in the early Twentieth Century which is instantly recognisable to anyone with microbiological experience. Secondly this provides strong suggestive evidence that the gram-positive bacterium so carefully described by Hülphers was indeed *Listeria monocytogenes*.''

Unfortunately, the isolates of Hülphers were probably not saved.

Animal listeriosis in Sweden
The first reports of animal listeriosis in Sweden were written by Lilleengen (1942) and Wramby (1944). Lilleengen provides a thorough literature review of listeriosis in different animal species; rodents, sheep, cattle, hens, pig, silver fox and human. After this, there is an account of a case of listerellosis in a hen-capercailzie, which had died a natural death and sent to the Game-Investigation Section at the Royal Veterinary College in Stockholm. Wramby reported that infection with *Listerella monocytogenes* had been diagnosed on several occasions in animals such as dog, rabbits and poultry at the National Veterinary Institute *e.g*., a case of listerellosis in a dog with septicaemia in 1929 (Investigator: Prof. H. Hedström) was, according to Wramby, the first identified case of listeriosis in dog in the world. From 1940 onwards, a more thorough search for listerella infection started.

Nilsson and Karlsson (1959) present results from routine diagnostic examination at the National Veterinary Institute in Sweden. *L. monocytogenes was isolated* from 141 animals of various between during 1948 and 1957: the most common were poultry, chinchilla, cattle, hare, sheep, rabbit and fox. These results differ from Wramby (1944) in the large number of chinchillas included. The authors concluded: ''Since *L. monocytogenes* can cause serious disease among human

beings and dissemination of the organism through foods is conceivable, it is important that veterinarians concerned with food hygiene should be thoroughly acquainted with this species.'' This was written over 20 years before the food-borne route was generally accepted.

During 1958-1972, *L. monocytogenes* was isolated from 195 animals, most commonly poultry, chinchilla, sheep, hare, cattle, grouse and roe deer (Nilsson and Söderlind 1974). The observations on anamnesis, pathological-anatomical findings and latent infections are in agreement with previous observations and the authors suggest that animals with latent infections constitute a hazardous infectious reservoir for human, partly due to direct contact, partly through contaminated foods.

The two publications cover the period 1948-1972 and report the procedures for identifying *L. monocytogenes*, pathogenicity test of isolates, clinical picture and pathological lesions in animals. Twenty isolates from 1948-1957 were serotyped and they were to serotype 1. **Paper VI** includes isolates of *L. monocytogenes* from cases of listeriosis in 177 animals during 1986-2006. The animals were of 22 different species and the study is focused on the characterization of *L. monocytogenes* isolates compared with human isolates from the same time period. The serovar distribution among animal isolates was 1/2a (72%), 1/2b (2%), 4b (24%) and other serovars (2%).

Human listeriosis in Sweden

The first human case reported in Sweden was presented at a meeting of the Pathology Association in Malmö by Linell in May, 1958. The case was a 7-day-old child with *granulomatosis infantiseptica* and meningitis. The findings werer later published by Linell *et al*. (1959). In the same journal and issue, Brandt and Berg (1959) presented a fatal case of meningitis connected with findings of *L. monocytogenes*. The disease was made notifiable in Sweden in 1960.

Clusters of human listeriosis without known source have been described in Sweden. The human disease, previously unknown in Sweden, was diagnosed at the same time in four cities; Malmö, Gothenburg, Stockholm and Uppsala (Bergman *et al.* 1960). Larsson *et al*. (1978) report two outbreaks of hospital-acquired infection. In the first outbreak, case 2 was infected by case 1, with congenital listeriosis. In the second outbreak, an infant with congenital listeriosis probably infected two infants who both fell ill with listerial enterocolitis at the age of 3 days. The authors consider the thermometer used for diagnosing *anal atresia* was a possible transmitter and it was later found that the same thermometer had been used for several children without being disinfected between consecutive cases. Bernander *et al*. (1981) describe a cluster of six cases of listeriosis in Västerås during July-September 1981. Five of the cases lived in the same housing area and the sixth had his parents living in the same area. The possible sources in common were food and animals kept in a recreation centre in the housing area, including sheep, goats and rabbits. During 1959-1961, Listeria

infections were diagnosed in 16 mother and infant cases in Uppsala. The Listeria isolates belonged to at least three different serotypes. Bacteriological investigations were on *e.g*., eggs and poultry but no Listeria bacteria were identified (Ekelund *et al*. 1962): this investigation was done 19 years before the food-borne route for *L. monocytogenes* was actually confirmed. Larsson (1979) reported that during 1958-1974, serotypes 1 and 2 account for 50 (45%) cases and serotype 4b account for 40 (36%) cases. However, during the period 1976-1985, 69% of strains were serovar 4b, the majority of which were of two different molecular subtypes (Ericsson *et al*. 1996). In **Paper II**, between 1986 and 1999, 43.9% of *L. monocytogenes* isolates were serovar 4b, and 50.2% were serovar 1/2a: although 2000 to 2007, only 12.8% were serovar 4b and 71% were 1/2a.

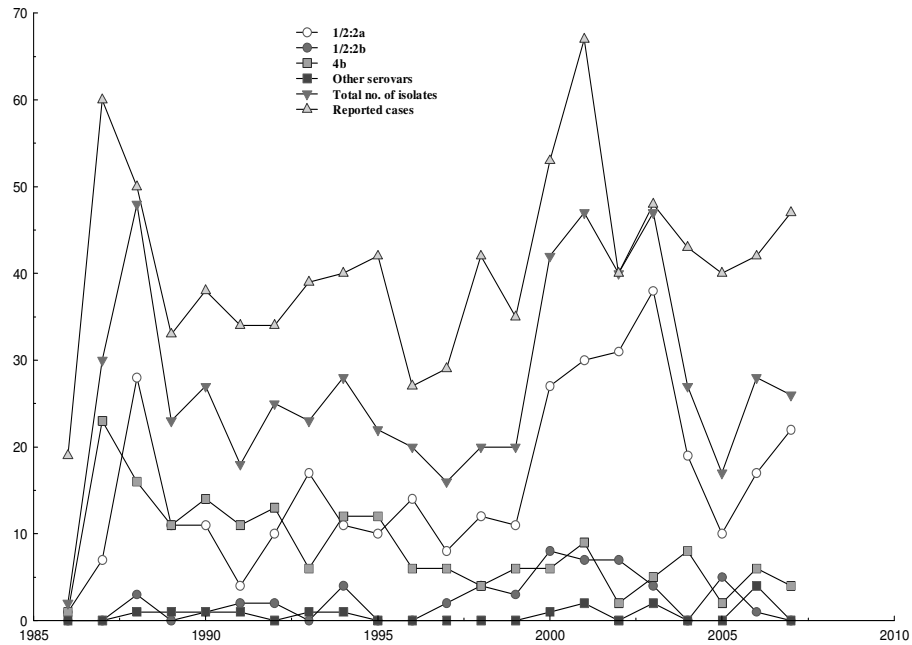


Figure. 30. Human listeriosis in Sweden. No. of cases/strains with serovars.

In 1987, the largest number of listeriosis cases hitherto was reported from the National Swedish Bacteriological Laboratory. Soft cheeses (due to cheese-borne outbreaks abroad) and animals were discussed as possible infectious sources. However, only 3 out of 30 patients confirmed that they had eaten soft cheese during the four weeks preceding the illness and 14 out of 30 patients had had contact with animals (Niklasson and Arneborn 1988).

The first case of food-borne listeriosis reported in Sweden was a 70-year-old woman. The same type of *L. monocytogenes* was isolated from samples of medwurst from the patient's refrigerator and the local retail store, and in

cerebrospinal fluid from the patient (Loncarevic *et al.* 1997). The first outbreak of rainbow trout-borne listeriosis ever reported included eight patients (Ericsson *et al.* 1997). The first outbreak of gastrointestinal listeriosis reported in Sweden occurred during the summer 2001 when at least 120 people fell ill in due to consumption of fresh cheese made from raw milk and manufactured on a summer farm (Danielsson-Tham *et al.* 2004). In these three incidents PFGE was used for characterization of *L. monocytogenes.*

In Sweden today *L. monocytogenes* serovar 1/2a isolates are increasing and serovar 4b isolates are decreasing (**Paper II**). The increase of 1/2a may be due to the lack of awareness of this serovar, and conversely, the decrease of 4b is due to more awareness of the serovar especially in the food industry. No similar serovar shift in animal listeriosis is apparent **(Paper VI)**; serovar 1/2a isolates were the majority during the whole period (1986-2006).

L. monocytogenes and its sources

Environment as source

The environment is regarded as the natural habitat of *L. monocytogenes* which can be isolated from herbage, sewage and soil. Aerobically spoiled silage favours the growth of *L. monocytogenes* and is the direct source of infection in animals, which can act as origin for contamination of food. *L. monocytogenes* can survive under adverse environmental conditions (Figure 32), thus, permitting colonization and multiplication. Agricultural soil is often a good source of *L. monocytogenes* as it receives manure and treated sewage as biofertilizer. In 1975, the incidence of *L. monocytogenes* in Germany was estimated to be 9.7% in cornfield, 13.3% in grain fields, 12.5% of cultivated fields and 44% of uncultivated fields (Weiss and Seeliger, 1975). Examination of grass leaves and stems from crops before harvesting revealed no *L. monocytogenes* but after harvesting the same crops gave 9/10 positive samples (Fenlon, 1985).

Faecal material as source

L. monocytogenes has been isolated in faecal samples from a wide variety of animal species and humans. Isolation is documented from pigs (Sichert *et al.*, 1958; Hale, 1959; Iida *et al.*, 1991b), chicken (Zeitoun and Debevere, 1991; Lawrence and Gilmour, 1994) and fish (Parihar *et al.*, 2008). Humans excrete *L. monocytogenes* symptomatically or asymptomatically, with a prevalence of 1.8 to 9% in a healthy human population Ralovich (1984).

Sewage as source

L. monocytogenes is present at levels greater than 1800 cfu/L in effluents associated with animals and sewage sludge in England (Watkins and Sleath, 1981) and is present during sewage treatment and at storage (Al-Ghazali and Al-Azawi, 1986). The final effluent containing less than 3-7 cfu/ml is subject to land disposal in many countries, this can lead to contamination of crops and vegetables grown on this land, as demonstrated in one food borne outbreak of human listeriosis (Schlech *et al.*, 1983).

Water as source

Sewage contaminates water and the presence of *L. monocytogenes* in fresh water is reported from various countries (Watkins and Sleath, 1981; Gugnani, 1999; Schaffter and Parriaux, 2002): in the Netherlands 21% of surface water was contaminated with *L. monocytogenes* (Dijkestra, 1982). However, no water-borne cases of human listeriosis appear to have been documented.

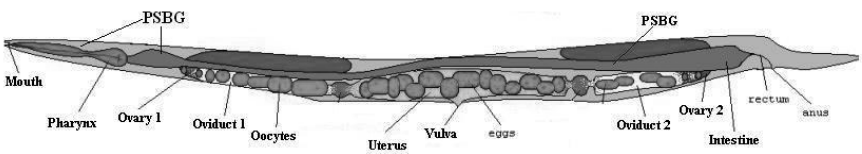
Caenorhabdits elegans as source

Role of various vertebrates and invertebrates have been documented in transmission of zoonotic pathogens but role of *C. elegans* (Figure 31) is rarely

highlighted. Gary *et al*. (2006) exhibited that the nematode *C. elegans* is capable of shedding various important food-borne pathogens such as *Escherichia coli O157:47*, *Salmonella enteric* and *L. monocytogenes*. Ingested pathogens get protected by various bactericidal products present in soil or water. It has been demonstrated that both live and dead *C. elegans* can harbor pathogens for several days after exposure. Various studies have demonstrated that *Salmonella* and *E coli* come into soil mainly from wild and domestic animals and can be ingested by *C elegans* that usually flourish in manure rich environment due to easy availabity of *E coli* in such places.

Thus, manure (treated or untreated) spread to farm soil as fertilizer can act potential source of various humans and animal pathogens and can be transmitted to population via feed and food originating from that soil. Recent studies have also exhibited the ability of different pathogens to form biofilms inside the gut of *C. elegans*. Study conducted by Raoult, 2008 demonstrated that plague causing etiological agent *Yesinia pestis* (transmitted by *Xenopsylla cheopis*, *Nosophyllus fascicticus* and *Pulex irritans*) is able to form biofilms in *C. elegans* and thus acting as possible reservoir of the pathogen (Houhamdi *et al*., 2006).

Figure. 31. Hermaphrodite *C. elegans* anatomy, PSBG = Possible site for bacterial growth. From Wikipedia.



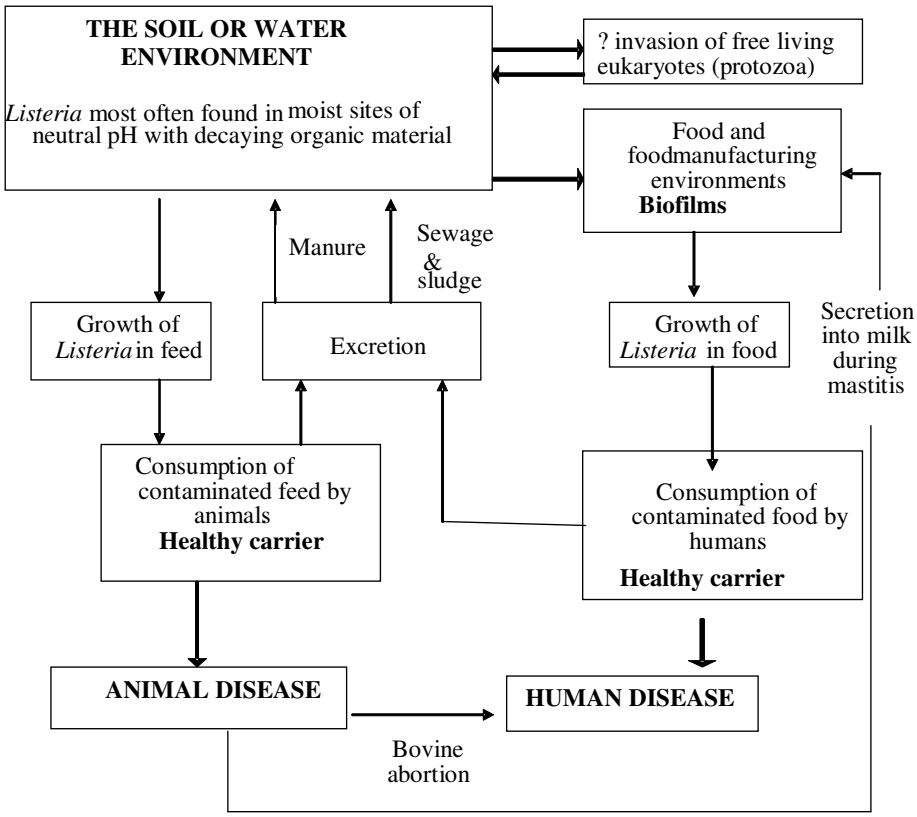


Figure 32. Interactions of *Listeria* with environment

Aims

The overall aim with this thesis was to acquire further knowledge about the sources and routes for human listeriosis.

The specific aims were to:
- characterise *L. monocytogenes* isolates from different animal species isolated 1986 – 2006 in Sweden.

- characterise human isolates of *L. monocytogenes* from cases of invasive listeriosis in Sweden 1986 – 2007.

- analyse changes in *L. monocytogenes* serovars and pulsovars during the study period.

- compare the patterns of *L. monocytogenes* animal strains with patterns of clinical human strains.

- study the present occurrence of *L. monocytogenes* in gravad and cold-smoked salmon bought in Sweden and compare the findings with an earlier investigation.

- compare serovars and pulsovars of *L. monocytogenes* isolated from fish with serovars and pulsovars of human strains isolated in Sweden.

- generate information on the evidence of *Listeria* species in seafood from West coast region of India.

- analyse clinical samples from human patients, in India, with decreased cell mediated immunity for presence of *L. monocytogenes* and to investigate the presence of virulence-genes in the isolated strains.

- investigate the presence of *L. monocytogenes* in farm bulk milk samples and in cervico-vaginal samples collected from dairy cows, in India, with reproductive disorders and to look for the virulence gene *hlyA* in the isolated strains.

Human Listeriosis, Sources and Routes
Summary of **Papers I-VI** (For references, Tables and Figures, see **Papers I-VI**)

MATERIALS AND METHODS

The material in this thesis **(Papers I – VI)** consist of *L. monocytogenes* isolates from human and animal cases of listeriosis in India and Sweden, from seafood in India and Sweden, and from farm bulk milk at a receiving dairy plant and cervico-vaginal swabs from India. In **papers II and VI** the isolates have been received, thus species L. *monocytogenes* has already been defined. In **Papers I, III, IV** and **V** bacteria have been isolated from samples.

Samples

Clinical samples (*n*=114) (**Paper I**), collected from various hospitals in Nagpur and Bhopal in Central India during the period May 2006 to July 2006, included placental tissue obtained from pregnant women with poor obstetric history like repeated abortions, intra-uterine deaths and still births, and blood samples from kidney dialysis patients, HIV infected patients and immunocompromised patients having various clinical conditions (tuberculosis, typhoid, eosinophilia and prolonged illness). In **Paper III,** raw/fresh seafood samples (n=115) were collected from retail outlets in Goa, India, during the period April 2006 to July 2006. It consists of two main categories of samples, i.e., finfishes (n=104) and shellfishes (n=11). The samples were collected in UV sterilized polyethylene sachets from their respective places. They were then brought to the laboratory and immediately processed for microbiological analysis. In **Paper IV**, fifty-six salmon products were purchased from retail stores In Sweden between June and September 2005. Fifty-three products were bought in Sweden and three in Germany. The purchases were from 16 manufacturers and included 30 vacuum-packed gravad, one modified-atmosphere gravad, 19 vacuum-packed cold-smoked and six modified-atmosphere cold-smoked products. All products purchased in Sweden were kept under refrigeration (4ºC) and analysed on the recommended best before day. The products bought in Germany were frozen for 16 hours and kept at room temperature for 10 hours during transportation to Sweden, and refrigerated until analysis. **In Paper V**, farm bulk milk samples (*n*=123) were collected at a receiving dairy plant in India during April 2006 to July 2006. The samples were collected aseptically in sterile glass containers. Cervico-vaginal swabs (n=20) from cows suffering from reproductive disorders were also collected from two dairy farms. Samples were transported and maintained in cold chain till processing.

Isolation of Listeria spp. *from samples*

In Paper I, placental tissue was processed by United States Department of Agriculture (USDA) method. Briefly, 25 g of the sample was homogenized in 225 ml of Pre Enrichment Broth (PEB) (Tryptic Soy broth with 0.6% yeast extract). After incubating for 24 h at 30°C, 10 ml of PEB was transferred to 90 ml of University of Vermont Medium (UVM) I and incubated for 24 h at

30°C. 0.1 ml of UVM I was inoculated to UVM II and also streaked on Dominguez-Rodriguez isolation agar (DRIA) and Polymixin Acryflavin Lithium chloride Ceftazidime Asculin Mannitol (PALCAM) agar plates. UVM II was incubated at 37°C for 24 h and then streaked on to DRIA and PALCAM agar plates. Agar plates were incubated at 37°C for 48 h. Blood samples (5 ml) were collected in BHI broth or in sterile plain bottles and cultured directly on 5% sheep blood agar. In **Paper III**, the procedure adopted for the isolation of *Listeria* spp. from seafood samples was a modification of USDA and FDA methods. Briefly, 25 g of the sample was homogenized in 225 ml of Pre Enrichment Broth (PEB) (Tryptic Soy broth with 0.6% yeast extract)) using a Stomacher 400-laboratory blender (Seward Medical, London, UK) and incubated at 30°C for 24 h. PEB sample (10 ml) was transferred to 90 ml of University of Vermont Medium I (UVM I) and incubated at 30°C for 24 h. UVM I (0.1 ml) was transferred to UVM II and incubated at 30°C for 24 h. The enriched inoculum from UVM II was streaked onto Dominguez-Rodriguez isolation agar (DRIA) and Polymixin Acriflavin Lithium chloride Ceftazidime Asculin Mannitol (PALCAM) agar plates and incubated at 30°C for 48 h.

Enrichment and cultural procedures for isolation and detection of *L. monocytogenes* in salmons (**Paper IV)** were according to NMKL, with some modifications. From each product, 25g was transferred to a sterile stomacher bag and macerated with the help of a rolling pin. First, enrichment broth 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. Products determined as *L. monocytogenes* positive by the enrichment procedure were stored in the freezer (-20ºC) to await the quantification procedure. From each product a 10g of fish sample was macerated in a stomacher bag with the help of a rolling pin and 90ml of peptone water was then added. Ten-fold serial dilutions of 1ml portions of the suspension were prepared in pepton water. The original suspension and each dilution were then 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. Milk samples (**Paper V**) were analysed according to McClain and Lee. Briefly, 25 ml of milk was inoculated in 225 ml of Pre Enrichment Broth (PEB) (Tryptic Soy broth with 0.6% yeast extract). After incubating for 24 h at 30°C, 10 ml of the enrichment was transferred to 90 ml of University of Vermont Medium I (UVM I) and incubated for 24 h at 30°C. Subsequently, 0.1 ml of UVM I was transferred to UVM II and also streaked onto Dominguez-Rodriguez Isolation agar (DRIA) and Polymixin Acryflavin Lithium chloride Ceftazidime Asculin Mannitol (PALCAM) agar. UVM II was incubated at 30°C for 24 h and then streaked onto DRIA and PALCAM agar plates. Plates were incubated at 37°C for 48 h. Cervico-vaginal swabs (**Paper V**) were transferred to 10 ml UVM I and incubated at 30°C for 24 h. The enriched inoculum from UVM I was transferred

to UVM II and incubated at 30°C for 24 h. The enriched inoculum from UVM II was streaked onto DRIA and PALCAM agar plates.

Identification of Listeria spp. **(Papers I, III, V)**
Grey green colonies with black sunken centers from PALCAM agar plates and greenish yellow, glistening, iridescent colonies of about 0.5 mm diameter surrounded by diffuse zone of aesculin hydrolysis from DRIA, suspected to be *Listeria* spp., were picked up (at least 3-7 per plate) and cultured on brain heart infusion agar. All the isolates were subjected to standard biochemical tests such as Gram's stain, catalase test, tumbling motility at 20-25°C and 37°C, acid production from glucose, mannitol, rhamnose, xylose and , α-methyl D-mannopyranoside α-methyl D-mannoside, nitrate reduction, methyl red test and Voges-Proskauer test. *L. monocytogenes* isolates identified biochemically were tested for hemolytic activity by blood agar plate method and CAMP test (Christie, Atkins, Munch Petersen test).

Identification of isolates of L. monocytogenes (**Papers IV**)
From each sample enrichment, five presumptive *L. monocytogenes* colonies, or all when fewer were present, were picked from the platings. In addition, 1-7 presumptive *L. monocytogenes* colonies were picked from the platings of each quantification procedure. Colonies were streaked onto blood agar for purity and presence of haemolytic activity. Isolates were tested for Gram reaction and motility (20ºC for 10 hours in Brain Heart Infusion). *L. monocytogenes* were identified with rhamnose and xylose fermentation tests.

Detection of virulence-associated genes **(Paper I)**
The PCR was standardized for the detection of virulence associated genes namely, *plcA, prfA, hlyA, actA and iap* of *L. monocytogenes* by following the methodologies described with suitable modifications. In brief, the standard strain of pathogenic *L. monocytogenes* (MTCC 1143) was grown overnight in brain heart infusion broth at 37°C. The culture (approximately 0.5 ml) was then centrifuged in a microcentrifuge (Sigma, USA) at 6000 g for 10 min. The recovered pellet was resuspended in 100µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 min. and then snap chilled in crushed ice. The obtained lysate (5µl) was used as a DNA template in PCR reaction mixture. The primers used in this study for detection of the genes *L. monocytogenes* used in this study were synthesized by Sigma Aldrich, USA. The DNA amplification reaction was performed in Px2 Gradient Thermal cycler (Thermohabaid, UK) with a pre-heated lid. Adequate positive and negative controls were included in each PCR run. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L); stained with ethidium bromide (0.5µg/ml) and visualized by a UV transilluminator (UVP Gel Seq Software, England).

Polymerase Chain Reaction (**Paper III**)
The primers for detection of presence of the *hlyA* gene of *L. monocytogenes* used in this study were ordered from Sigma Aldrich, St. Louis, Mo., USA. The sequences of oligonucleotide primers were 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3' and 5'- GCA ACG TAT CCT CCA GAG TGA TCG -3' for *hlyA* (Paziak-Domanska et al., 1999). The PCR protocol (See reference in **Paper III**) for detection of the hlyA gene in *L. monocytogenes* was used with some modification. *L. monocytogenes* MTCC 1143 was used as a standard and grown overnight in BHI broth at 37°C. Subsequently, the culture (1 ml) was centrifuged in a microcentrifuge (Heraeus, Germany) at 6000 rpm for 10 min. The pellet was resuspended in 100µl of sterilized DNAse and RNAse-free milliQ water (Millipore, Bedford, MA, USA), heated in a boiling water bath for 10 min. and then snap chilled in crushed ice. The obtained lysate (5µl) was used as a DNA template in PCR reaction mixture. Bacterial DNA was also extracted employing DNA extraction kit (Genei, Bangalore, India). The standardized PCR protocol for 50µl reaction mixture included 10X PCR buffer (100mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 0.2 mM dNTP mix, 2 mM $MgCl_2$ and 0.1 µM of forward and reverse primers, 1 unit of Taq DNA polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume. The DNA amplification reaction was performed in Gradient Thermocycler (Thermohybaid, UK) with a pre-heated lid in PCR tube (0.2 ml). The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 min followed by 35 cycles each of 15 s denaturation at 95°C, 30 s annealing at 60°C and 1 min 30 seconds extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L); stained with ethidium bromide (0.5µg/ml) and visualized by a UV transilluminator (UVP Gel Seq Software, Cambridge, England).

Bacterial strains received **(Papers I, II, III, V, VI)**
Isolates of *L. monocytogenes* from human cases of invasive listeriosis (n=601) occurring between 1986 and 2007 in Sweden were received from clinical microbiological laboratories throughout the country (**Paper II**). In **Paper VI,** 177 isolates of *L. monocytogenes* from 22 animal species (1986-2006) were received from the National Veterinary Institute, in Uppsala, Sweden. Both human and animal were frozen in 80% brain heart infusion broth (Merck, Darmstadt, Germany) and 20% vol/vol glycerol at -70°C until analysis. The reference strains of *L. monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135), were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India (**Papers I, III, V**).

Serotyping of L. monocytogenes *isolates* (**Papers II, IV and VI**)
All *L. monocytogenes* isolates were 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, Liverpool, UK) according to manufacturer's instructions, with the following 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 3000 rpm 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 isolates were passed three times through nutrient medium with 10.5% (w/v) agar and a supplement of 5% (v/v) bovine blood before serotyping. Plates were incubated at 30ºC for 24 hours after each passage. These cultures were inoculated into 3ml Brain Heart Infusion Broth and kept at 30ºC overnight, after which 3ml of 1% formal saline were added. The tube agglutination test was conducted with H antiserum and the prepared cell suspension.

PFGE (Pulsed-field gel electrophoresis) of L. monocytogenes *isolates* (**Papers II, IV and VI**)

L. monocytogenes isolates 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 (4-8ºC), centrifuged (Wifug, 5500rpm) for 5 min and the supernatant discarded. The pellet was suspended in 5ml of TN buffer (10mM Tris HCl, pH 8.0, 5M NaCl), vortexed, cooled and centrifuged again, and then the supernatant was discarded. The pellet with the small amount of liquid was vortexed and suspended in 0.9ml of lysozyme solution (1mg lysozyme/ ml TN buffer), and then incubated in a water bath at 37ºC for 45 min. Fifteen ml SeaKem Gold agarose (1.2%) supplemented with 1.67ml ESP (1g N-lauroylsarcosin, Sigma-Aldrich, St Louis, MO, USA + 100ml 0.5 EDTA, pH 8 + 200mg pronase, Roche Diagnostics, Mannheim, Germany) was prepared and maintained in a water bath 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, GE Health Care, Uppsala, Sweden) 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 in water bath at 55ºC for 48 h.

The plugs were cut into half longitudinally and transferred to Eppendorf tubes containing 0.5ml PEFA (3.5mg Pefablock SC, Roche Diagnostics GmbH, Mannheim, Germany) in 10ml TE solution (10ml 1M Tris HCl, pH 8.0 and *aqua dest.* 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. The solution used for restriction with *Asc* I contained 870µl of *aqua dest.,* 108µl of NE 4 buffer (10x conc., New England Biolabs, Beverly, MA, USA), 10µl acetylated BSA (Bovine Serum Albumin 10mg/ ml, Promega Corporation, Madison, WI, USA)

and 12µl of *Asc* I (10 units/ml, New England BioLabs, Beverly, MA, USA). In **Paper IV** isolates were also cleaved with restriction enzyme *Apa* I. *Apa* I solution contained 870µl of acqua dest, 98µl of Buffer A (10x conc., Roche Diagnostics, Mannheim, Germany), 10µl acetylated BSA and 22µl of *Apa* I (10 units/ml, Roche Diagnostics, Mannheim, Germany). 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, 27.5g 0.45M Boric acid, 20ml 0.5M EDTA, pH 8.0 and *aqua dest.* up to 1000ml]). Samples were incubated at room temperature for more than 30 min. 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 (GE Health Care, Uppsala, 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, Hercules, CA, USA) run time being 20h with initial pulse time of 1.0s and final pulse time of 15.0s. After electrophoresis, the gel was 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. Gels with profiles were visualized with short-wave ultra violet. (312 nm) light and photographed with a Polaroid camera. 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). Through serogroups and *Asc* I profiles, different types of *L. monocytogenes* were obtained. For each type, a reference strain was selected, *e.g.*, the reference strain type 1/2:48 belonged to serogroup 1/2 and to *Asc* I profile no. 48. Thus, all isolates sharing serogroup 1/2 and *Asc* I profile no. 48, were regarded as identical with the reference strain. If serovar and profiles of two isolates were indistinguishable, they were considered as belonging to the same *L. monocytogenes* type, whereas, a two to three fragment difference with one or both enzymes was considered closely related, a four to six fragment difference was possibly related, and seven or more fragment differences were unrelated.

RESULTS AND DISCUSSION

Reports of listeriosis from humans in India are scanty, either because of failure to identify the isolate, its rarity, low incidence rate or lack of awareness. The epidemiological data available in the country to date is neither adequate nor reliable for assessing the extent of infection in human beings and animals, because it is based on conventional diagnostic tests employing conventional antigens which show considerable cross-reactivity with other bacteria. The disease largely remains undiagnosed and under reported, mainly because of the non-availability of a reliable, rapid and simple diagnostic test.

In **Paper I** (**Isolation of *Listeria monocytogenes* from human clinical cases in India)** *L. monocytogenes* was isolated from two of the six placental bits from women with poor obstetric history, one patient with renal failure (out of 81) and three of the 16 immunocompromised patients. Placental bits were collected from women aborted in second trimester of gestation. Earlier studies from India indicated the incidence rate of *L. monocytogenes* in pregnant women with bad obstetric history ranging between 1.34% and 4%. Pregnant women are at higher risk of getting *L. monocytogenes* infection due to tropism of internalin for E-cadherin molecules present on the syncitiotrophoblasts. Our study indicated that abortion could be a common form/manifestation of listeriosis in India.

L. monocytogenes was isolated from one blood sample from patient with renal failure and three blood samples of immunocompromised individuals. The patient with renal failure was undergoing kidney dialysis. Interestingly, all the isolates except one isolated from patient with renal failure were positive for the *hlyA, actA* and *iap* genes. The isolate recovered from patient with renal failure was positive for the *hlyA* and *actA* genes. None of the isolate was positive for the *plcA* gene. Reasons for this discrepancy are unclear. Earlier studies on clinical isolates from bovine mastitis and reproductive disorders in India also indicated the lack of one or more virulence-associated genes. The source of infection could not be determined here, however, transmission of *L. monocytogenes* can be possible from animals to humans and from food to humans.

In **Paper II** (**Characterization of Human Invasive Isolates of *Listeria monocytogenes* in Sweden 1986-2007**) the cases of listeriosis were distributed over all the country and no seasonal variation in the frequency of listeriosis was observed. The characterization of human *L. monocytogenes* isolates causing invasive listeriosis during the period 1986 to 2007 in Sweden revealed a decrease in serovar 4b strains. During the first part of the period studied, 1986 to 1999, 43.9% of *L. monocytogenes* isolates belonged to serovar 4b, and 50.2% belonged to serovar 1/2a. However, during the latter period, 2000 to 2007, only 12.8% belonged to serovar 4b and 71% to 1/2a. Serovar 4b has been and is still prevalent in human listeriosis globally: the few exceptions are Finland, Italy, Sweden and parts of USA, where serovar 1/2a cases are predominant. The predominance of serovar 1/2a in some countries may be linked to public health authorities increasing the control on serovar 4b due to warnings from scientists

that this serovar is a cause of concern, as it has been involved in numerous outbreaks. Other scientists have called attention to particular serovar 4b strains and the necessity to identify and avoid those in the food chain. Serovar 1/2a prevails in food in several countries. May be it is now time to focus on serovar 1/2a by increasing control measures in the same manner as it has successfully been done with serovar 4b in some countries.

The discrimination achieved by serotyping is often rather low, as most human isolates belong to the serovars 1/2a, 1/2b, and 4b: this was also the observed in the present study. Therefore, serotyping should always be accompanied by a highly discriminatory typing method, e.g. PFGE. In total, in the present study, 345 isolates of serovar 1/2a divided into 114 different pulsovars were found, that is approximately three isolates per pulsovar. In contrast, serovar 4b was represented by 186 isolates classified into 28 different pulsovars that is 6.6 isolates per pulsovar. Thus, serovar 1/2a appears more heterogeneous than serovar 4b. Similar observations have been reported elsewhere. In the present study 44 pulsovars (*Asc* I profiles) were included in a dendrogram. Type 4b:6 was identical according to serovar, phagovar and pulsovar to the epidemic strain in the Swiss outbreak due to a soft cheese 1983 to 1987. This strain has not been seen in Sweden since 1997, in contrast, type 1/2a:15 was introduced into Sweden during 2000. There were 12 cases due to type 1/2a:4A in 1988. Likewise 12 cases due to type 1/2a:9A in 2003. Those clusters indicate common source outbreaks. In 2002, 35 samples of vacuum-packed cold smoked and gravad salmon bought in Sweden were analysed for *L. monocytogenes*. Ten of the samples were positive for *L. monocytogenes*. The quantitative analysis showed that the number of *L. monocytogenes* in two of the samples were 2000 and 4000 CFU/gram, respectively. Twenty isolates were characterized from those products and they were all shown to be type 1/2a:9A. One of the samples harboured <10 CFU/gram of type 1/2a:4A. Type ½:4A is one of the most frequently encountered types of *L. monocytogenes* in humans as well as in vacuum-packed salmons in Sweden. It is reported from the literature that strains with indistinguishable PFGE profiles may display different serovar. Whether serovar 1/2b and 4b strains (lineage I) are more prone to infect humans than strains of serovar 1/2a (lineage II) is under discussion. Hybridization results have exhibited that all virulence factors are present in strains of all serovars, suggesting that all strains can cause disease. From the data in the present study, we are not convinced that any one particular serovar is more adapted to humans than others are. In the light of **Paper II** it could be speculated about common sources. All together, during 1987-1988, there were 110 cases reported, which was an unusual large figure. In **Paper II** there are 59 strains/cases representing 1987-1988, i.e. 53.6% of 110. Type 1/2a:4A are represented by 11 isolates (18.6%). This pulsovar has also frequently been isolated from vacuum-packed cold-smoked and gravad salmon or rainbow trout in Sweden. The consumption of fresh salmon (mainly vacuum-packed) per inhabitant in Sweden was nearly doubled in 1985 compared with 1980.

In tropical regions, *L. monocytogenes* and other *Listeria* species have been isolated from fishery products on a regular basis since the late 1990's. The prevalence of *L. monocytogenes* varied from 4% to 12% in surveys from temperate areas. Out of 115 samples (**Paper III, Isolation and characterization of *Listeria* species from tropical seafoods**) of tropical seafood, 28 were tested positive for *Listeria* spp. thereby indicating the prevalence of 24%. Out of the 28 isolates of *Listeria* spp., 10 (9%) isolates were haemolytic and CAMP positive, and were *L. monocytogenes*. The remaining 18 isolates were *L. innocua*. Higher occurrence of *L. monocytogenes,* 12.1 % in fresh shell fishes and 17.2% in fresh fin fishes from tropical seafood has been reported from India. Earlier reports suggested the absence of *L. monocytogenes* in tropical fish. Other studies have found that the prevalence of *L. monocytogenes* in raw fish is quite low, ranging from 0 to 1%. In Iceland 56% of fresh fish on sale were contaminated with *L. monocytogenes* and other *Listeria* species. An overall prevalence 3% of *L. monocytogenes* was observed in European fish. *L. monocytogenes* is considered a psychrotrophic pathogen and may thus be less common in tropical water.
The frequent presence of *L. innocua* in tropical fish, in the present study, is in agreement with other. As in other raw foods, fishery products more frequently contain *L. innocua* than *L. monocytogenes.* Since both species share ecological niches, the presence of *L. innocua* is considered as an indicator of possible contamination with *L. monocytogenes*. In general, *L. monocytogenes* is not usually found on fish captured from open waters. However, contamination may take place long before the fish raw material reaches retail trade or processing factories. Potential sources of *L. monocytogenes* on fishing vessels include contamination from water and ice, soiled surfaces and boxes, as well as contamination from human and avian sources. Since *L. monocytogenes* is commonly found in coastal waters and in surface waters of lakes, fish captured or cultivated in these waters may possibly carry this microorganism. Little is known of the potential *Listeria* contamination of fish and fish products at the retail level. Products that are purchased in bulk and re-packaged prior to sale may be vulnerable to *L. monocytogenes* contamination. Process contamination in particular has proved to be an important source of *Listeria* contamination in food production and numerous studies show that in-house *L. monocytogenes* flora contaminates seafood during processing.

The industry costs for contamination of a product with *L. monocytogenes* can be extremely prohibitive. The incidence of *L. monocytogenes* in raw/fresh seafoods may have serious impact on the its processing as it tends to contaminate the processing table as well as sanitizing the surfaces of seafood processing plants and also in the processing and production of frozen seafoods for exports. Contamination in seafood establishments may occur from any food contact surface as well as from secondary contamination sites and equipment support structures. It is indeed necessary to create awareness among the retailers of the risks that can arise from contamination of foods with *L. monocytogenes* so that appropriate measures can be instituted. Sanitary conditions of food contact

surfaces and handling areas, and personal hygiene practices should reduce the potential contamination with *L. monocytogenes* at the retail level.

Isolation of *L. monocytogenes* from seafood suggests that there is a risk of acquiring listeriosis through seafoods in India. *L. monocytogenes* will be killed by cooking and raw or semi-raw seafood (graved or cold-smoked) are not consumed in India. However, *L. monocytogenes* in raw seafoods may pose a health risk in kitchen if contaminating cooked food or other ready-to-eat food. Considering outbreaks of listeriosis associated with different foods, avoidance of consumption of insufficiently cooked seafoods by at-risk populations is recommended. Diligent enforcement of sanitary conditions of food contact surface and handling areas, and personal hygiene practices should reduce the potential contamination of fishery products by *L. monocytogenes* at the retail level.

Since seafood is popular and associated with human listeriosis, prevalence studies are important in the identification of health hazards. In **Paper IV**, **''Gravad and cold-smoked salmon, still a potential source of listeriosis''**, *L. monocytogenes* was isolated from gravad salmon and cold-smoked salmon. The occurrence of *L. monocytogenes* was 12.9% in gravad and 28.0% in cold-smoked salmons. Prevalence of *L. monocytogenes* in gravad fish has been reported to be as high as 26.9%, whereas, in cold-smoked fish, overall prevalence is approximately 10% with a contamination rate of between 0% and 75%. REA/PFGE typing of the 56 salmon isolates identified five *Asc* I profiles all identical to previously characterized human clinical strains. Restriction with *Apa* I confirmed that salmon *L. monocytogene*s strain type 1/2:48 was identical to human strain type 1/2:48, whereas, salmon *L. monocytogenes* strain types 1/2:114 and 4:2 were closely related to the corresponding human strain types This suggested an association between human cases of listeriosis and the consumption of gravad or cold-smoked salmon. The remaining salmon strain types (1/2:49 and type 1/2:93) were unrelated to human strain types according to the information obtained from *Apa*.

The highest level of *L. monocytogenes* contamination found in the present study was 1500 cfu/g in cold-smoked salmon, but the level was low (<100 cfu/g) in most products. In an outbreak of listeriosis, in Sweden 1994/1995, associated with gravad and cold-smoked rainbow trout, the levels ranged from <100 cfu/g to 25 000 cfu/g. The high contamination level found in earlier investigations might have been due to a more generous best before day for these products, *i.e.* up to 42 days. In a gastrointestinal listeriosis outbreak, in Finland 2001, associated with cold-smoked rainbow trout, 190 000 cfu/g of *L. monocytogenes* was found. 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. And, ready-to-eat foods are sometimes kept in consumer's refrigerator beyond the recommended best before day that will give *L. monocytogenes* possibility to multiply.

In the present study, isolates of *L. monocytogenes* were mainly serovar 1/2a (76.8%) and the remainder were serovar 4b. Also in other studies the most predominant serogroup identified in gravad and hot and cold-smoked fish is serogroup 1/2. Even though overall reported incidence of listeriosis associated with serotype 4b is high, pathogenicity of different serovars cannot be distinguished on practical grounds. Therefore, all strains of *L. monocytogenes* should be considered as pathogenic. *L. monocytogenes* isolated from cold-smoked fish of manufacturer A harboured two serovars (1/2a and 4b), each with unique *Asc* I profiles, implying that serotyping and genotyping of several isolates from each food sample is important. Furthermore, products from the same manufacturer (A) purchased in both Germany and Sweden contained *L. monocytogenes* strains with the same *Asc* I and *Apa* I profiles, 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, it can colonize, multiply and persist in the food-processing environment. Products from manufacturer A were processed in another European country and distributed to European countries, *e.g*. Sweden and Germany, which suggests that one processing plant can distribute and introduce pathogenic *L. monocytogenes* strains into several countries. Previous studies have confirmed the introduction of pathogenic strains of *L. monocytogenes* into the human population through the processing environment. Due to the findings in the present study, producer A was released from their production commissions in Europe

In **Paper V** (**Isolation of *Listeria* species from farm bulk milk at the receiving dairy plant and cervico-vaginal swabs**), *Listeria* spp. were isolated from 30 (24.4%) of 123 bulk milk samples and four (20%) of 20 cervico-vaginal swabs. *L. monocytogenes* was confirmed from 17.9%, *L. innocua* from 1.6%, *L. seeligeri* from 3.3% and *L. welshimeri* from 1.6% bulk milk samples. *L. monocytogenes* and *L. innocua* were isolated from 2 (10%) samples each of cervico-vaginal swabs. In other studies, *L. monocytogenes* has frequently been isolated from farm bulk tanks in various countries. The incidence of *L. monocytogenes* in milk samples from farm bulk tanks was 5.3% in Northern Ireland and 4.9% in Ireland. In Mexico 13% of bulk milk samples were positive for *L. monocytogenes*. In Spain, *L. monocytogenes* and *L. innocua* were detected in 3.6 and 2.7% of 774 milk samples, respectively. The reasons for such difference could be explained as varying environmental conditions between different locations as well as the methods of the detection. Presence of *L. monocytogenes* in raw milk may be due to contamination from the environment or from udder infection. This has practical significance in milk industry since *L. monocytogenes* can persist for longer period of time by making biofilms. One of the reasons for contamination of raw milk with *L. monocytogenes* has been attributed to faeces and environmental contamination during milking, transportation and storage. Poor silage quality was reported as a factor for raw milk contamination. However, silage was not widely used in the region of our study. In the present study the samples were obtained from tinned cans, so that

environmental contamination during transportation could not be excluded. Under unhygienic milking practices indirect contamination of bulk milk is likely to occur if *L. monocytogenes* is present in feeds, faeces, udder surface or bedding.

L. monocytogenes (10%) and *L. innocua* (10%) were isolated from cervico-vaginal swabs. Reports on the findings of *L. monocytogenes* from vaginal samples of cows are largely lacking. However, a prevalence of 4.4% in buffaloes with reproductive disorders has been reported. Also, *L. monocytogenes* (2.4%) and *L. ivanovii* (0.8%) has been isolated from vaginal samples of buffaloes. In the control preparations, the *hlyA* gene of *L. monocytogenes* was amplified to its respective base pair, 456 bp represented by a single band in the corresponding region of the DNA marker. The isolates were subjected for detection of the *hlyA* gene. All *L. monocytogenes* isolates were positive for the *hlyA* gene.

In India, raw milk is widely consumed and the incidence of milkborne disease is unknown. In rural areas, the consumption of raw milk and derived products is traditionally been a very common practice. *Listeria* is of particular concern to the food industry because it can grow at refrigerator temperatures and is ubiquitous in nature. Considering the level of incidence of *L. monocytogenes* in cattle related and bulk milk samples, it seems likely that *L. monocytogenes* may be transferred to humans by raw milk or milk that have not been correctly pasteurized or that have been contaminated post pasteurization with raw milk. This study highlights *L. monocytogenes* to be a potent health risk for consumers in India where most of the population still depends upon homemade unpasteurised milk and milk products. Further studies are needed to identify risk factors for the presence of *Listeria* in raw milk, and to assess the public health impact.

In **Paper VI**, **''Molecular characterization of *Listeria monocytogenes* isolates from animals in Sweden and comparison with previously characterized human strains''**, isolates of *L. monocytogenes* (n=177) from 22 animal species were characterized by serotyping and restriction enzyme analysis followed by PFGE. The animal strains (**Paper VI**) and human strains (**Paper II**) isolated between 1986-2006 in Sweden are compared.

The serovar distribution in animals during the study period were: 72% of 1/2a; 2% of 1/2b; 24% of 4b; and 2% other serovars. The corresponding figures for humans were: 57 % of 1/2a; 9 % of 1/2b; 31 % of 4b; and 3%. There was an increase in human serovar 1/2a strains in Sweden during the latter part of the period: 1986-1999 yielded only 50% *L. monocytogenes* serovar 1/2a strains among humans, whereas, 2000-2006 yielded 68% of the same serovar. The percentages of serovar 1/2a strains among animals during the same two periods were stable, 72% (1986-1999) and 71% (2000-2006). The most common serovar 1/2a pulsovars among animals were 1/2a:1 (12 strains), 1/2a:63 (9 strains) and 1/2a:4B (7 strains). These three pulsovars comprised 28 (15.8%) of the total number of animal strains (n=177) during 1986-2006. The corresponding numbers

of strains in humans were 1/2a:1 (9 strains), 1/2a:63 (9 strains) and 1/2a:4B (28 strains) and comprised only 8.0% of the total number of human strains (n=572). The most common serovar 1/2a pulsovar in humans were 1/2a:4A , represented by 7.9% (45 strains) of strains: this pulsovar accounted for only 2.3% (4 strains) in animals. The most common pulsovars among serovar 4b strains in animals were 4b:3 (11 strains) and 4b:6 (10 strains) and constituted 50% of the 42 serovar 4b strains. The corresponding figures in humans during the whole period studied were 40 strains of 4b:3 and 16 strains of 4b:6 strains constituting 31.5% of 178 serovar 4b strains.

Pulsovar 4b:6 caused a cheese-borne outbreak in Switzerland in 1983-1987. Although not rare in human listeriosis patients in Sweden before to 2000 (the Swiss cheese was also sold in Sweden), pulsovar4b:6 has not been identified in humans in Sweden since 2000. Conversely, pulsovar 4b:6 has been more common in animals since 2000, *i.e.,* 8/10 cases during the study period: the animals affected were cattle, sheep, roe deer and dog. In general, animal 4b strains, except 4b:6 were evenly spread throughout the period; whereas, human 4b strains were most common in the earlier period, *i.e.* 1986-1999. Although many animal and human in this investigation shared sharing pulsovars, they did not appear at the same time or with the same proportion of strains. During the period studied, no human case was reported as caused by animals.

The same *L. monocytogenes* pulsovars causing diseases in both humans and sheep have been reported and 29% of ruminant clinical isolates had indistinguishable DNA PFGE fingerprints to strains isolated from documented human listeriosis outbreaks. One explanation to common pulsovars could be that they are widely distributed in the environment and as such could be picked up by both humans and animals. The pulsovars shared by both animals and humans may indicate that there is an exchange of *L. monocytogenes* strains between these two groups due to either direct or indirect transmission. Direct transmission of *L. monocytogenes* from animals to humans is reported among professionals having close contact with diseased animals o healthy carriers such as animal handlers and veterinarians.

Indirect transmission may occur by consumption of food products from diseased animals, for example, an on-farm manufactured raw milk cheese partly made with milk from a goat with an udder infection caused an outbreak of febrile gastrointestinal listeriosis involving at least 120 people in 2001. Direct transmission and especially indirect transmission from animal to human via food is rarely reported in the literature and may be due to the difficulties in following the infectious route or that indirect transmission is rare.

From 76 animal pulsovars, 34 (represented by 46 strains including 39 1/2a strains) were unfamiliar to the human strain collection. These strains might not yet have been transmitted to the human population or certain clonal types of *L. monocytogenes* are adapted to specific hosts, *i.e*., some of the strains not sharing

pulsovars with human strains belong to clonal types with attenuated virulence for man.

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