Zoonotic Aspects of *Listeria monocytogenes*

with Special Reference to Bacteriology

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Master of Science Programme in Veterinary Medicine
for International Students
Faculty of Veterinary Medicine and Animal Science
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The present thesis is a partial fulfilment of the requirements for an Masters of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of **Veterinary Public Health**

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“It seems that for success in science or art a dash of autism is essential”

To my Parents (Shri. Prabhu Lal Parihar and Smt. Shakuntla Devi)

Abstract

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Listeria monocytogenes is a non acid-fast, Gram-positive facultative anaerobic pathogen, which is considered as food- and feed-borne. Whereas poor quality silage is the main cause of animal listeriosis, contaminated food of animal origin is the main cause of human listeriosis. That the raw material for food is of animal origin does not necessarily mean that the *L. monocytogenes* bacteria also spring from animals. The bacteria may have contaminated the food product while processed. Knowledge of the direct or indirect transmission of *L. monocytogenes* between animals and humans, via e.g. foods, is limited. To highlight the zoonotic aspects of *L. monocytogenes* we need more comparative data concerning isolates of animal and human origin. The aim of the present study was to characterize clinical *L. monocytogenes* isolates from different animal's species and to compare the patterns with those obtained from previously characterized clinical human strains. Animal isolates were characterized by use of restriction enzymes *Asc* I and *Apa* I followed by PFGE. Out of 104 animal strains 47 belonged to clonal types identical or closely related to clonal types seen among clinical human strains. The clonal types shared by animals and humans may indicate that there is an exchange of *L. monocytogenes* strains between these two groups or there may be a common environmental pool of strains. On the other hand, 42 animal strains belonged to clonal types that were unfamiliar to our collection of human strains. Finally, 15 animal isolates distributed into eight clonal types yielded *Asc* I profiles familiar to our human clonal types yet unfamiliar *Apa* I profiles. Human and animal isolates of *L. monocytogenes* have rarely been compared by use of PFGE. Further studies is needed to highlight routes of transmissions between animals and humans, e.g., via food.

Keywords: *L. monocytogenes*, Zoonoses, Animals

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Introduction

Veterinarians, medical doctors and people involved in food science know listeriosis by various names (circling disease, silage sickness, leukocytosis, cheese sickness, tiger river disease) but few know who Gustav Hülphers was because he did not preserve his bacterial strains, which he named bacillus hepatis, later recognized as *Listeria monocytogenes* (Hülphers, 1911; McLauchlin, 2004; Hülphers, 2004). Fifteen years later Murray *et al.* (1926) also identified bacteria identical to *L. monocytogenes*, which caused monocytosis in rabbit and guinea pig. Isolates of these bacteria are still preserved (ATCC no. 15313; ATCC no. 4428) so the credit goes to Murray *et al.* for isolation of *L. monocytogenes* for the first time. Pirie finally named the species *L. monocytogenes* in 1940 and thereafter it is included in the 6th edition of Bergey`s Manual of Determinative Bacteriology (1948).

There are more then 350 zoonotic diseases known today, but listeriosis is given special attention due to the unique and changing concept of zoonoses. In the early 1980s listeriosis was classified under anthropozoonoses, which was changed to amphixenoses in the late 1990s. It lacks its true definition of zoonotic disease because of involvement of an inanimate reservoir (food) as the major cause of listeriosis. Up to 1961 *L. monocytogenes* was regarded as the one and only species of genus *Listeria* but later other species have been identified. Listeriosis is of great public health concern because of its high mortality (20 to 30%) and its common source epidemic potential. The most important aspect in food hygiene is the ability of the bacteria to survive in a wide range of temperatures and to make biofilms on various environmental surfaces, which serve as natural habitats or reservoirs (Duggan and Phillips, 1998).

Direct transmission is possible, especially among veterinarians, performing gynecological interventions with aborted animals. Animals may be diseased or asymptomatic carriers of *L. monocytogenes* shedding the organism in their faeces. Thus, earlier it was believed that *L. monocytogenes* was causing disease by direct transmission from animals to humans. Today it is generally considered that ingestion is the main mode of infection and food being the main vehicle of infection. A listeriosis outbreak in the Maritime Provinces of Canada (1981) was indeed related to food but it was not until the outbreak of California from January to August 1985 (James *et al.*, 1985; Linnan *et al.*, 1988) that food was recognized as an important vehicle of *Listeria* transmission. According to Mead *et al.* (1999) food is an important vehicle of *Listeria* transmission in 99% of listeriosis cases. Risk assessment made by WHO has given the guideline that 99% of all listeriosis

could be eliminated if the *L. monocytogenes* level never exceed 1000 cfu/g food at the point of consumption. Nosocomial infection has also been described, placing medical physicians and other medical staff at risk.

The serovar 4b was the leading serovar responsible for human listeriosis cases in Sweden (Danielsson-Tham and Tham, 2004), Finland (Lukinmaa *et al.*, 2004), Canada (Pagotto *et al.*, 2004) and United kingdom (McLauchlin *et al.*, 1991; Newton *et al.*, 1992) during the 80s and 90s but during 2000 to 2003 most (70 to 80%) of the human listeriosis were due to serovar 1/2a and 1/2c. The reason for this change in seroprevalence is not clear but is attributed to change in food habits and more attention given to control and eradication of serovar 4b. The incidence of listeriosis appears to be rising, especially in developed countries, which is believed to be due to more consumption of ready-to-eat food with extended shelf life. Cases in both humans and animals have been reported to occur during specific seasons (the peak season for humans being autumn and for animals it is spring). The correlation between the two variables (peak value and season) is not yet understood. To prevent the transmission of *L. monocytogenes* we have to understand its ecology, including the zoonotic aspects.

History and taxonomy

On March 30, 1910 G. Hülphers described bacteria (bacillus hepatis) isolated from a colony of rabbits (Hülphers, 1911; McLauchlin, 2004; Hülphers, 2004). The description given by Hülphers corresponded well with the bacterial findings in rodents later presented by Murray *et al.* (1926). Soon, also Pirie isolated similar bacteria from gerbils in South Africa (1927). As gerbils were found near the Tiger river station he called the disease 'Tiger river diseases' and named the bacteria *Listerella hepatolytica* after the name of a British surgeon, Lord Joseph Lister. In 1940, the bacterium was finally named *Listeria monocytogenes* which was the only recognized species of genus *Listeria*, but in 1961 *L. dentrificans* was added. In 1966 and 1971 species *L. grayi* and *L. murrayi*, respectively, were also added to genus *Listeria* and in 1977 Seeliger introduced *L. innocua*. Wilkinson and Jones (1977) indicated that *L. grayi* and *L. murrayi* are distinct from other *Listeria* species so those species were excluded from the genus. Later *L. grayi* and *L. murrayi* were included again 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 brought the two species in one species *L. grayi*.

Taxonomy of the genus *Listeria* has been problematic. *L. monocytogenes* was previously in the family *Corynebacteriaceae*

(Stuart and Pease, 1972) but in the 8th edition of Bergey`s Manual of Determinative Bacteriology, *Listeria* along with *Erysipelothrix* and *Caryophanon* were grouped as uncertain affiliation. On the basis of DNA-DNA hybridization, Stuart and Welshimer (1974) suggested a new family *Listeriaceae* to accommodate genera *Listeria* and *Morraya*. Today, the genus *Listeria* belongs to the *Clostridium* subbranch together with *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Brochothrix*. *Listeria* includes six species, of which one is divided into two subspecies: *L. monocytogenes*, *L. innocua, L. welshimeri, L. seeligeri, L. grayi* and *L. ivanovii* subsp. *ivanovii* and L. *ivanovii* subsp. *londoniensis* (Boerline *et al.*, 1992). Only *L. monocytogenes* causes disease in both animals and humans. However, occasional human infection with *L. ivanovii* and *L. seeligeri* has been reported (Gilot and Content, 2002). *L. ivanovii* is known to cause spontaneous abortions in sheep.

Cultural characteristics

Listeria is aerobic and facultatively anaerobic. After 24 hours, incubation colonies on nutrient agar are round, 0.5-1,5 mm in diameter, translucent, smooth, with glistening surface (S forms). Prolonged incubation makes colonies rough (R forms). Colonies show hemolytic activity on blood agar, which distinguishes *L. monocytogenes* from some other species of genus *Listeria*. Stabbed in semisolid medium, inverted “pine tree” like growth appears below 3 to 5 mm of the surface. *L. monocytogenes* exhibit positive CAMP reaction on sheep blood agar (5% v/v) with *Staphylococcus aureus* but not with *Rhodococcus equi*.

Morphology

Microscopically *Listeria* appears as regular, short rods with rounded ends, 0.4-0.5 micrometer in diameter and 0.5-2 micrometer in length. Sometimes it is arranged in Y or V forms but usually it occurs singly or in short chains. *Listeria* is motile with peritrichous flagella when cultured at room temperature (20-22ºC). *Listeria* rotates around its long axis with the help of actin-based motility; average time per rotation is 507±106 micrometer per sec and average distance per rotation being 29.4±11.8 micrometer (Robbins and Theriot, 2003). *Listeria* does not form spores or capsules and is nonacid-fast, Gram-positive but older cultures may appear Gram-negative. If smears are not stained properly they may resemble *Haemophilius influenza* (Gray *et al.*, 1966).

Identification of *L. monocytogenes*

L. monocytogenes colonies exhibit blue green iridescence on agar when seen with oblique light and narrow beta-hemolytic zone on blood agar.

Microscopic examination

a) Staining: Gram-positive rods
b) Morphology: Singly, arranged in Y or V forms or short chains. In broth culture longer bacilli with palisade formation are seen.
c) Motility: Tumbling motility when cultured at 20 to 22 ºC.

Biochemical reactions

Table 1. Characteristics of Listeria species (Boerlin *et al.*, 1992; Seeliger and Jones, 1986).

Characteristics	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. grayi*	*L. ivanovii subsp. ivanovii*	*L. ivanovii subsp. ivanovii*
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	+	-	-	-	-	+	+

Other methods for identification are ELISA (Enzyme Linked Immunosorbant Assay), DNA/RNA hybridization and PCR-based techniques.

Animal inoculation

In the Anton test, ophthalmic pathogenicity in rabbit is studied and keratoconjuctivitis is taken as a positive test. Pathogenicity is also confirmed by intra-peritoneal inoculation of mice and guinea pigs or inoculation of the chorioallantoic membrane in egg.

Differentiation of L. monocytogenes from related species

Table. 2. Characters most useful in differentiating the genus *Listeria, Brochothrix, Erysipelothrix, Lactobacillus* and *Kurthia.*

Taxon	Motility	Oxygen requirement	Growth at 37˚C	Catalase	H_2S production	Fatty acid type	Mol % G+C
Brochothrix	-	Facultative	-	+[b]	-	S, A, I	35.6-36.1
Erysipelothrix	-	Facultative	+	-	+	S, A, I, U	36-40
Lactobacillus	-[a]	Facultative	+	+	-	S, U	34-53
Kurthia	+[d]	Aerobic	+	-[c]	-		36.7-37.9
Listeria	+	Facultative	+	+	-	S, A, I	36-39

[a] Most strains are non-motile but some exhibits motility.
[b] Catalase production depends on medium and temperature of incubation.
[c] Weak production of catalase. [d] Non-motile strains do occur.
S, straight-line saturated; U, monounsaturated; A, anteiso-methy-branched; I, iso-methy branched

Growth requirements of *Listeria monocytogenes*

Glucose is an essential carbohydrate for growth of *L. monocytogenes* and is synthesised by the Embden-Meyerhof pathway both aerobically and anaerobically. Although glucose-6-phosphate and 6-phoshogluconate-dehydrogenase have been extracted from *L. monocytogenes*, the pathways for these metabolites have not been not reported yet (Bergey's Manual, 8th edition). Out of 331 transporter genes, 88 (26%) genes in the genome of *L. monocytogenes* are responsible for carbohydrate metabolism (Glaser *et al.*, 2001).

Requirement of major and minor elements

Not much information is available about the requirement of *L. monocytogenes* for major and minor elements, but iron has been shown to be an important factor for growth and regulation of virulence genes (Trivett and Meyar, 1971; Litwin and Calderwood,

1993). It has been reported that *L. monocytogenes* is unable to produce iron chelating agent siderophores. Iron acquisition from the environment is operative by different known mechanisms:

- The ferric citrate induced uptake (Adams and Roper, 1990); which includes surface bound reductase (Deneer *et al.*, 1995) and an extra cellular reductase which needs Mg^{2+}, FMN (flavin mononucleotide) and NADH (nicotinamide adenine dinucleotide) for its action (Barchini and Cowart, 1996; Cowart and Foster, 1985).
- Transferrin-binding protein at cell surface (Hartford *et al.*, 1993).
- Siderophores or siderophore-like substance (Simon *et al.*, 1995).
- Iron-catecholamine complexes.

L. monocytogenes requires six amino acids and four vitamins in the medium for growth. The six amino acids are isoleucine, leucine, cystein, argenine, methonine and valine. At least four vitamins are needed, such as biotin (required for the carbon monoxide fixation), riboflavin (used in oxidoreduction reaction), thiamine (help in the decarboxylation of keto acids and transaminase reactions), and thioctic acid (transfer of acyl group in oxidation of ketoacids). The complete pathway for the biosynthesis of amino acids is identified but not for vitamins (Glaser *et al.*, 2001). *L. monocytogenes* possesses proteins for synthesis of vitamin B_{12} and this synthesis is carried out by an oxygen-independent pathway.

Environmental requirements

Oxygen

L. monocytogenes is carbon dioxidophilic (microaerophilic) and contains the enzyme catalase to decompose H_2O_2. Friedman and Alm (1962) observed that catalase activity is low in medium having glucose concentration of 10% (v/v). Genes that code for the anaerobic pathway of *L. monocytogenes* are: *cbiD*, *cbiG* and *cbiK*.

pH

Three cardinal points of pH for *L. monocytogenes* are minimum (4.3), optimum (6.8) and maximum (9.6). Acid tolerance of *L. monocytogenes* is an important factor for its survival in the human and animal gut. Pre-exposure of *L. monocytogenes* to mild acidic stress enables the bacteria to adapt further to acid and heat tolerance because of cross protection (Farber and Pagotto, 1992). *L. monocytogenes* uses multiple mechanisms to adapt to acidic stress depending upon its growth phase; one of these mechanisms is growth phase dependent acid resistance (AR), which becomes stimulated when bacteria approach the stationary phase. Once the bacteria adapt to environmental stress by AR, subsequent lethal

doses of acid are tolerated by the adaptive acid tolerance response (ATR) (Davis *et al.*, 1996) mechanism. Acidic pH mediates rapid escape of *L. monocytogenes* from vesicles (Glomski *et al.*, 2002).

Temperature

Three cardinal points of temperature are minimum (0.5ºC), optimum (30 to 37ºC) and maximum (45ºC). It has been estimated that *L. monocytogenes* needs 35 hours at 4º and 41 minutes at 35º as generation time in milk products (Marth *et al.*, 1986).

Methods of characterization of *L. monocytogenes*

Serotyping

Serotyping is a phenotypic method for serological analysis of flagellar and somatic antigens. Seeliger and Höhne (1979) described the method of obtaining antisera against *L. monocytogenes* somatic (o) and flagellar (H) antigen from immunized rabbits. On the basis of serotyping, the *L. monocytogenes* species is divided into 12 serovars.

Table 3. Classification of *L. monocytogenes* serovars

Serogroup	Serovar					
1/2	1/2a	1/2b	1/2c			
3	3a	3b	3c			
4	4a	4b	4c	4d	4e	4ab
7						

Phage-typing

Phage-typing for bacteria was described for the first time in 1945. It is a valuable tool for rapid screening of bacterial strains in epidemiological surveys. Bacteriophages are viruses that infect bacteria, causing lysis, *i.e*. absence of bacterial growth, on nutritious media. Different phages have different target bacteria. By phage-typing, one bacterial strain is tested against a battery of phages. Each bacterial strain is characterized by its sensitiveness to specific phages. *Listeria* phages are double stranded DNA classified as

- *Siphoviridae* (non contractile tails)
- *Myoviridae* (contractile tails).

Ribotyping

Ribotyping is a genotypic method for characterization of various bacterial strains by using a single probe because of similarity of ribosomal genes (Graves *et al.*, 1991; Graves *et al.*, 1994). In this method, *Eco R*I is used to digest bacterial DNA followed by southern hybridization probing with the rRNA operon of *Escherichia*

coli. In a modified version, automated ribotyping is made possible by using different enzymes to improve the characterization of different strains of *L. monocytogenes* (De Cesare *et al.*, 2001). On the basis of ribotyping, Nadon *et al.* (2001) described three lineages of *L. monocytogenes*. Lineage I consists of the serovars 1/2b, 3b, 3c and 4b whereas lineage II included 1/2a, 1/2c and 3a. Lineage III contained serovars 4a and 4c. The typeability and reproducibility of this method are good for *L. monocytogenes* but have limited discriminatory power for serovar 4b (Swaminathan *et al.*, 1996; Bille and Rocourt, 1996).

Multilocus enzyme electrophoresis (MEE)

By the use of MEE genomic relationship of various strains is studied by estimating the relative electrophoretic mobility of water-soluble cellular enzymes. The main reasons for the variation in electrophoretic mobility are

- Allelic variation
- Genetic variation

Cells are enriched in nutritious medium and lysed by ultrasound treatment and after removing debris by centrifugation the supernatant with enzymes is electrophoresed in starch gels. The migration length (electromorph) depends on the amino acid sequence of the enzyme. Thus, the migration pattern can be correlated to the genome. The combination of different electromorphs for one strain is called electrophoretic type (ET)

Random amplification of polymorphic DNA (RAPD)

In bacterial genomes some DNA stretches tend to vary moderately or greatly among different strains. These stretches can be informative for specific species. Multiple arbitrary primers, each of about 10bp are designed. They will anneal to matching sequences on the target bacterial genome. Sequences will be amplified using PCR and electrophoresed followed by staining. Each strain will show a characteristic band pattern.

Advantages of using RAPD

a) it is a cheap method, b) multiple bands appear on the gel, c) easy to read.

Disadvantages of RAPD

a) highly purified DNA required, b) difficult to interpret band profile in terms of alleles and loci, c) low reproducibility, d) needs standardization due to sensitivity of reaction conditions, e) high risk of contamination

Pulsed-field gel electrophoresis

In 1984, Schwartz and Cantor introduced a new concept of electrophoresis named pulsed-field gel electrophoresis (PFGE). The

first commercial PFGE was introduced by Pharmacia-LKB (Uppsala, Sweden). The main advantage of PFGE is its ability to separate double stranded DNA in the range of a few kilo base pair (kbp) to 10000 kbp by orientation of an electric field periodically across the gel. The enzymes used for restriction of DNA are called infrequent cutters because instead of the normal 4 bases they recognize 6 to 8 base sequences; this makes PFGE a macro-restriction analyzer rather than micro-restriction analyzer as in traditional electrophoresis.

The basic need for PFGE is unsheared DNA, thus DNA is prepared by embedding intact microorganisms in agarose plugs. The plugs with microorganisms are treated with suitable lysozyme to degrade the cell wall and then all proteins and RNA are digested with proteinase K. Before adding restriction enzymes, proteinase K is inactivated by phenylmethysulphonyl-fluoride (PMSF) or 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride. The most appropriate restriction enzymes for *L. monocytogenes* are *Asc* I, *Apa* I and *Sma* I. The basic theory of pulsed-field gel electrophoresis is still a matter of debate. By changing orientation of the electric field small-sized DNA will begin to move in the new direction more quickly than the larger DNA. Three models are used to describe the migration and behavior of DNA during PFGE: repetition model, the chain model and bag model (Chu *et al*., 1986). Various types of PFGE are: a) single inhomogeneous field, b) double inhomogeneous field, c) field inversion gel electrophoresis (FIGE), d) homogeneous crossed field electrophoresis. There are two limitations of PFGE. First, DNA preparation involves several incubation steps that will make this procedure time-consuming. Second, PFGE requires expensive, specialized equipment. In the current study we used both double inhomogeneous field (Pharmacia) and FIGE (CHEF-Bio Rad) procedures.

Virulence factors of *L. monocytogenes* (see review by Dramsi *et al.*, 1996; Dussurget *et al.*, 2004; Wehland and Carl, 1998)

Ability of *L. monocytogenes* to cause disease depends, *i.a*., upon the expression of virulence factors and immune status of individuals. Usually individuals having weakened cell-mediated immunity are more susceptible to *L. monocytogenes*. Genetic susceptibility to listeriosis is uncertain but intrinsic susceptibility to *L. monocytogenes* exists in certain inbred mice. *L. monocytogenes* is one of the most invasive bacteria known and is capable of crossing intestinal (Marco *et al*., 1997), transplacental (Gray and Killinger, 1996; Lecuit *et al*., 2004) and blood brain barriers (Uldry *et al*., 1993; Berche, 1995) of the host, but the normal route of infection is by crossing intestinal barriers particular through the M cell of Payer’s patches.

InlA

InlA is an 800-amino acid surface protein required for internalization of *L. monocytogenes* into host epithelial cells, such as macrophages, fibroblasts and epithelial cells. The main receptor for this protein is E-cadherin present on the host cell membrane. E-cadherin is a calcium dependent cell adhesion glycoprotein and is species-specific due to its amino acid (proline) at location 16. It has been reported that 96% of clinical strains of *L. monocytogenes* express full-length InlA as compared to 65% food-associated strains.

InlB

InlB is an 630-amino acid protein located on the same operon as InlA and is required for *L. monocytogenes* to be able to internalize fibroblasts, hepatocytes, epithelial and endothelial host cells. Tyrosine kinase Met or hepatocyte growth factor receptor (HGF) has been identified as the main receptor of InlB on host cells. InlB triggers bacterial entry by interacting with Met, through the concave surface of the LRR (leucine-rich repeats) region.

Clp proteases and Clp ATPase

Clp proteases are caseinolytic proteins that act both as chaperones and proteolytic enzymes. Chaperones are the proteins important for the adaptation of the bacteria in adverse environmental conditions. ClpP serine protease is critical for the growth of *L. monocytogenes* under stress conditions and mediates the escape from vacuoles. Clp ATPase are named as ClpC and ClpE. ClpC is a stress protein and helps in intracellular survival of *L. monocytogenes* and it also modulates ActA protein expression. ClpE plays an important role in the pathogenesis.

Ami

Ami is an 917-amino acid amidase. The main function of this protein is lytic against the *L. monocytogenes* cell wall but also helps in adhesion to host cells.

Protein p60

p60 is a 60-kDa protein that catalysis the final stages of cell division in *L. monocytogenes*. This is encoded by invasion associated protein gene (*iap*). p60 is secreted on the cell surface and into the surrounding medium. The central part of p60 is threonine-asparagine repeats. Studies conducted on mutation of the p60 coding gene indicate that this protein is important in phagocytosis of *L. monocytogenes*. The name *iap* is suggested to be changed to *cwhA* and the protein p60 to ‘‘Cell wall hydrolase A’’.

FbpA

FbpA is a 570-amino acid protein and a substrate for the SecA2 pathway. FbpA is an important factor for the efficient colonization of *L. monocytogenes* into the liver and spleen of the mouse. It also helps in preventing degradation of the virulence proteins by modulating levels of listeriolysin O and InlB.

Listeriolysin O (LLO)

Listeriolysin O (LLO) is a 60-kDa protein. As *Listeria* are engulfed by the host cell, they are enclosed within an intracellular vacuole that is surrounded by a membrane. LLO is a pore-forming toxin, essential for lysing the vacuolar membrane in the host cell, thus facilitating the escape of *L. monocytogenes* from the vacuole. Activation of LLO stimulates various host cellular responses such as interleukin-1 secretion in macrophages, apoptosis, cell adhesion protein expression, cytokines in spleen cells and mitogen-activated kinase in HeLa cells lines. Most of these responses are Ca^{2+} dependent.

ActA

ActA is a 639 amino acid protein encoded by *actA* gene. Once *L. monocytogenes* has escaped from the primary phagolysosomes into the host cytoplasm, it starts to multiply by using nutrients from the host cell cytoplasm. In order for these bacteria to move directly to another host cell, a single bacterial surface protein, ActA, assembles and activates (polymerization) host cell actin cytoskeletal molecules (filaments) at the bacterial surface. Within 3 hours of initiation of infection, polarized actin tail filaments (up to 40 micrometer - nearly the full length of the host cell) rapidly propel *L. monocytogenes* as a comet-shaped apparition in the cytoplasm towards neighboring cells at a speed of up to 1.5 μm/sec. The host cell generates the force required for intracellular bacterial movements. Portions of the membrane of host cells bulge outwards and neighboring cell bulge inwards. The so-formed double-membrane structures are engulfed by neighboring cells and thus a intracellular vacuole is formed (secondary phagolysosome). This phagolysosomes will be lysed by LLO and PLC. The procedure described above allows the bacterium to spread from cell to cell without leaving the intracellular environment and thereby avoiding the host immune response.

Hexose phosphate transporter (Hpt)

L. monocytogenes uses Hpt to get sugar from the cytosol of the host cells. The main sugar utilised is glucose-1-phosphate. The PrfA dependent Hpt is similar to eukaryotic glucose-6-phosphate (G6P) transporter.

Phospholipase C (PLC)

Two phospholipases C with overlapping activities are also involved in the invasion and spread of *L. monocytogenes*. Those are the phosphatidylinositol-specific PLC (PI-PLC or PLC-A; encoded by plcA) and a broad-range phosphatidylcholine-specific PLC (PC-PLC or PLC-B; encoded by plcB). Together with LLO, PI-PCL aids in the escape from the primary phagolysosome, whereas PC-PLC is active during the cell-to-cell activity, including formation of the secondary phagolysosome.

Metalloprotease

Metalloprotease is zinc-dependent and, together with host cell cysteine protease, it activates phospholipase. The chief action of this protease is to cleave off the precursor part from the active part of PI-PLC and PC-PLC system.

Sortases

Sortases are transpeptidases responsible for anchoring surface protein and virulence factors to the cell wall. The genes encoding these proteins are *srtA* and *srtB*: *srtA* is responsible for anchoring of InlA to the peptidoglycan, and *srtB* encodes anchoring of proteins containing C-terminal NXXTN motif such as SvpA.

Auto

Auto is a surface protein with autolysin activity and is encoded by *aut* gene in *L. monocytogenes* but *L. innocua* lacks this gene. Inactivation of auto decreases the invasiveness of *L. monocytogenes* into epithelial and fibroblastic cell lines. Recent studies have shown that *aut* is not present in *L. monocytogenes* 4b strains.

Bile salt hydrolase (BSH)

BSH is believed to protect *L. monocytogenes* from bile salt toxicity and is encoded by *bsh* gene. It is sigma B dependent and is regulated by PrfA. Activity of BSH increases at low oxygen tension. BSHs is also produced by other enteric bacteria such as *Clostridium* spp, *Bacteroides* spp and *Enterococcus* spp. Deletion of *bsh* leads to decrease in fecal carriage and colonization of *L. monocytogenes* in liver, thus BSH is a unique protein involved in both hepatic and intestinal listeriosis phases.