Zoonotic Aspects of *Listeria monocytogenes*

with Special Reference to Bacteriology

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Report - Masters of Science Programme in Veterinary Medicine
for International Students
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences
Report no. 45
ISSN 1403-2201

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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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Print: SLU Service/Repro, Uppsala 2004

“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

Parihar, V. S. 2004. Zoonotic Aspects of *Listeria monocytogenes* – with Special Reference to Bacteriology. Master's thesis. Department of Food Hygiene, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences.
ISSN 1403-2201.

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

Contents

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.

L. monocytogenes in animals

L. monocytogenes has been isolated from 42 animal species, both domestic and wild. The main reason of animals being at high risk for listeriosis is due to the ubiquitous nature of *Listeria* spp. and thus feed easily gets contaminated.

Listeriosis in cattle

There are three main forms of listeriosis in cattle:

- Meningoencephalitis (Scott, 1994; Gibbons, 1970)
- Reproductive form (abortion) (Osebold *et al.*, 1960)
- Mastitis (Gitter *et al.*, 1980)
- Other syndromes

Meningoencephalitis

Meningoencephalitis occurs in 8 to 10% of clinical cases of listeriosis. Adult ruminants are most commonly manifesting this type of clinical symptom (Rebhun, 1987; Gray and Killinger, 1966). The course of this form is from 1 to 2 weeks (Blood *et al*., 1994), the basic clinical signs closely relate to those of dummy syndrome and they depend upon the area in the pons and medulla affected. Morbidity is very low but mortality (fatality rate) is high. Meningoencephalitis is not observed in calves or lambs until the rumen is functional (Gray and Killinger, 1966). The main signs are: a) unilateral facial paralysis, b) head pressing due to destruction of basal ganglie, c) paralysis of tongue, d) propulsive circling towards affected part due to involvement of basal ganglie, e) palpebral reflex absent, f) dropped ear, g) loss of nasoliabialis muscle function due to involvement of 7th cranial nerve, h) Nystagmus, i) decreased consciousness and convulsions.

Reproductive form

The route of infection is mostly hematogenous but vaginal transmission also occurs. Abortion occurs mostly during the last trimester (Dennis, 1968). Both sporadic and clustered abortion has been reported (Blood and Radostits, 1989). Main clinical signs are: a) fever, b) retention of fetal membranes, c) pinpoint yellowish necrotic foci on cotyledonary villi, d) stillborn foetus.

Mastitis

L. monocytogenes is one of several causes of bovine mastitis but not as frequent as *Brucella* spp, *Mycobacterium bovis*, *Escherichia coli*, *Staphylococcus* spp, *Streptococcus* spp. Subclinical *L.*

monocytogenes mastitis is also seen. In a Danish report of 31 cases of mastitis due to *L. monocytogenes*, 18 were subclinical. In Yugoslavia *L. monocytogenes* was recovered from the milk of 32% of altogether 845 healthy cows distributed on seven different farms (Kovincic *et al.*, 1990). In listeriosis-aborted cows *L. monocytogenes* was isolated from milk for up to 13 days (Osebold *et al.*, 1960).

Other syndromes

a) keratoconjunctivitis (Morgan, 1977), b) enteritis, c) spinal myelitis.

Listeriosis in sheep

There are three main forms of listeriosis in sheep:

- Encephalitis
- Placentitis
- Gastrointestinal septicemia.

Encephalitis

This is called circling disease; clinical signs are similar to those of cattle but the disease is more peracute, death may occur in 4 to 48 hours. Main clinical signs are:
a) grazing is erratic, b) facial paralysis, c) drooping ear, d) lowered eyelid on the affected side, e) head and neck are laterally flexed away from the paralyzed side, f) prostration followed by coma and death.

Placentitis

Morbidity ranges from 1 to 20%, on average 10%. Abortion in sheep is one of the major problems in some areas such as Australia. Abortion occurs in the last trimester of gestation. Microscopic changes include thrombosis, vasculitis and neutrophil accumulation in the allantochorion. Micropathological signs in aborted foetus are focal hepatic necrosis, focal lung necrosis and bronchopneumonia. *L. monocytogenes* causes placentitis via the hematological route; foetus becomes infected secondary to uterine dysfunction.

Gastrointestinal septicemia

It is not as common as other forms of listeriosis in sheep but does occur in lambs.
The main clinical signs are:
a) dullness b) inappetance c) pyrexia d) diarrhoea e) death within 24 hours of clinical signs.

L. monocytognes in humans

L. monocytogenes is mostly responsible of human listeriosis but occasionally infection with *L. seeligeri* and *L. ivanovii* has been

reported. The first human case of listeriosis was reported by Nyfeldt in 1929. Today, listeriosis is regarded as a food-borne disease of serious public health concern due to the great mortality rate (20-30%). Listeriosis is mainly sporadic but outbreaks do occur in humans (Gellin and Broome, 1989). Incidence of human listeriosis varies between countries ranging from 4.4 to 7.4 per million of the population annually (Lorber, 1997). All *L. monocytogenes* strains have similar pathogenicity, regardless of geographic origin (Corral *et al.*, 1990; Brosch *et al.*, 1992). However, Jones *et al*. 1994 reported some differences in the prevalence of listeriosis in that the disease is over-represented in social classes 1 and 2 (professionals) and under-represented in classs 4 and 5 (semiskilled and unemployed). Although debilitated immune status due to, *e.g*. diabetes mellitus, cardiovascular disease, neoplastic disease, pregnancy or hemodialysis failure (Nieman and Lorber., 1980) are important factors in the pathogenesis of listeriosis, other factors also play important role such as:

- Genetic
- Behavior
- Age

Genetic

Genetic polymorphism of host cell surface receptors like E-cadherin may lead to differences in susceptibility to *L. monocytogenes*.

Behavior

Use of antacids or H^+ pump inhibitors (*e.g*., Losec®) leads to decrease in stomach acidity and thus *L. monocytogenes* may avoid lethal effects from gastric acid. Use of alcohol can be related to the gastroenteritis form of listeriosis due to impaired cellular immunity (Farber and Peterkin, 1991).

Age

Newborn and young children and older persons are more susceptible to *L. monocytogenes* because of an immature or inefficient immune system. Rocourt and Brosch. (1992) found that 22% of the total cases of listeriosis occur at an age of below 1 month and 31% of total cases occur in people older than 60 years.

Table 4. Classification of human listeriosis.

Noninvasive form	invasive form	
(Gastrointestinal)	Septicemia	Neuropathic
I.P is 18 to 24 hours	I.P is 2-weeks	I.P is 1-week
Fever	Malaise	Malaise
Diarrhoea	Fatigue	Ataxia
Vomition	Abdomnal pain	Meningitis
Nausea	Fever and chills	
Arthralgia		
Headache		

I.P- incubation period

Pregnant women

Listeriosis is most common in the third trimester but listeriosis cases have been reported in all stages of pregnancy. Cell-mediated immunity decreases during pregnancy, so pregnant woman are at higher risk of getting *L. monocytogenes* infection. Pregnant women may also be more prone to listeriosis due to the tropism of internalin for E-cadherin molecules present on the syncitiotrophoblasts (Cossart, 2004).

The main clinical symptoms are:
a) mild influenza, b) meningitis; rare but is distinguished from other bacterial meningitis on the basis of its special predilection for brain parenchyma, c) bacteremia, d) bloody vaginal discharge, e) endocarditis; occurs in 10% of pregnant women with listeriosis and mostly affects the left side of the valve and mortality can reach up to 50% (Lorber, 1997), f) gastroenteritis, g) focal infections such as cellulitis, conjunctivitis.

Perinatal infection

Transmission of *L. monocytogenes* from mother to foetus or neonates has been frequently reported but cross-infection postpartus is also possible (Lecuit *et al.*, 2004). Perinatal infection is classified on the basis of debut of clinical symptoms:

- Early onset of listeriosis
- Late onset of listeriosis.

Early onset of listeriosis (*granulomatosis infantisepticum*)

Occurs in foetus or neonates within 1st week after delivery and is characterized by a serious septicaemia with respiratory distress, pneumonia and purulent conjunctivitis. The foetus may die *in utero* with or without accompanying spontaneous abortion. Prognosis is usually poor (Farber and Peterkin, 1991). The pregnant woman often has fever, headache and myalgia due to bacteremia.

Late onset of listeriosis

This form of neonatal listeriosis occurs within the 2nd-4th week of life and is manifested as meningitis (McLauchlin, 1992). The infant gets infected while passing mother's birth canal or as a result of post-partum cross-infection.

L. monocytogenes in food

The role of *L. monocytogenes* as a food-borne pathogen was recognized following outbreaks of human listeriosis caused by the consumption of contaminated foods in North America and Europe during the mid-1980s. *L. monocytogenes* can be considered as an environmental contaminant transmitted to humans mainly by food, thus the prime route of *L. monocytogenes* infection is oral. Presence of *L. monocytogenes* in food may be due to contaminated raw material or due to faulty handling. Although the presence of *L. monocytogenes* has been reported from a wide variety of foodstuffs, the incidence in tropical food is very low (Fuchs and Surendran, 1989; Jeyasekaran and Karunasagar, 1996) which may be due to differences in cooking habits and/or lack of facilities to isolate *L. monocytogenes*.

Davis *et al.* (1996) found that acid tolerance of *L. monocytogenes* increases during the exponential phase of bacterial growth if the bacteria had been exposed to sublethal acidic conditions previously and this phenomenon will be also expressed in *Listeria* cells that are in the stationary phase (King *et al.*, 2003). Increased tolerance to lethal acidic environmental conditions also leads to increase in virulence. Furthermore, *L. monocytogenes* exposed to preservatives in food and feed may not be sensitive to the normally lethal bile salts in the intestine of humans and animals. According to the WHO risk assessment report of *L. monocytogenes* in ready-to-eat-food, listeriosis cases can be reduced significantly if *L. monocytogenes* is kept below 1000 cfu/g food at the point of consumption (WHO risk assessment report, 2000).

L. monocytogenes in milk

Presence of *L. monocytogenes* in raw milk may be due to contamination from the environment or from udder infection (Larsen, 1966; Gitter *et al.*, 1980). It is observed that cows secrete *L. monocytogenes* in their milk as long as 13 days after abortion (Osebold *et al.*, 1960). The first milk-borne transmission of *L. monocytogenes* was described by Potel (1951), who isolated *L. monocytogenes* from stillborn twins of a woman who used to take milk from an infected cow. Outbreaks of listeriosis in Massachusetts suggested that the pasteurization process HTST (High Temperature

Short Time) was not sufficient to kill *L. monocytogenes* (Bearns & Girard, 1958; Fleming *et al.*, 1985) but later studies have proved that even heat-resistant strains of *L. monocytogenes* do not resist the temperature of HTST pasteurization. Multiplication of *L. monocytogenes* in milk depends upon the temperature at which milk is stored. *L. monocytogenes* has a unique property to multiply at 4°C. Usually the generation time of *L. monocytogenes* at 4°C is 30 to 45 hours. The fastest generation time is 30-40 minutes at 37°C, when pH is neutral and water activity ranges from 0.990-0.995 with sufficient nutrients in the medium.

L. monocytogenes in meat, egg and seafood.

Listeriosis associated with meat (turkey) consumption was first reported by Barnes *et al.* (1989). Egg transmission of *L. monocytogenes* is still not reported but there is one report of fatal *Listeria* meningitis of a man who worked in an egg products factory. Outbreaks of listeriosis due to fish or fish products were first reported from New Zealand (Lennon *et al.*, 1984). Listeriosis in man due to consumption of gravad rainbow trout has been reported (Ericsson *et al.*, 1997). It is not clear if the fish is contaminated in the water environment or in the seafood processing plant (Eklund *et al.*, 1995). Rocourt *et al.* (2000) classified seafood on the basis of risk factors as:

a) High risk - molluscs, oysters in shell, raw fish, lightly preserved fish products (NaCl <6% (w/w) in water phase, pH >5) salted, marinated, fermented, cold smoked fish, mildly heat-processed fish.

b) Low risk - semi preserved fish, (NaCl >6% (w/w) in water phase, pH<5), heat treated fish, dry salted and smoked fish, fresh fish.

Buchanan *et al.* (1997) predicted that there will be a 1 in 59 million chance of being infected from consumption of 50 g fish having 100 bacteria/ gram.

L. monocytogenes in vegetables.

L. monocytogenes has been isolated from a wide range of fresh and minimally processed vegetables such as bean sprouts (Francis & O'Beirne, 2001), cabbage (Beuchat *et al*., 1986; Conner *et al*, 1986) and shredded lettuce (Farber *et al.*, 1998). Contamination of vegetables can occur with the use of manure from *L. monocytogenes* carriers or diseased animals (Schlech *et al.*, 1983). Especially lettuce and cabbage that are held under conditions of cold storage and are not properly cooked can serve as source of *L. monocytogenes*.

Various factors responsible for contamination of food with *L. monocytogenes*.

- As a part of microbial ecosystem

L. monocytogenes has become part of microbial ecosystem of food processing environments because of its ability to form biofilms on surfaces leading to post-processing contamination of food.

- Advantage of survival and growth

Survival and growth of *L. monocytogenes* are determined by food substrate (pH and water activity [aw]). Especially cut vegetables and soft cheese provide a favourable combination of conditions for rapid growth of *L. monocytogenes*. Storage of contaminated food in refrigeration temperatures (4-10°C) does not inhibit the growth of *L. monocytogenes*.

- Post processing contamination.

L. monocytogenes in feed

Improperly fermented silage was associated with listeriosis among sheep for the first time in Iceland; the disease was called silage sickness (Gray, 1960). Listeriosis due to consumption of silage has been reported in goats (Wiedmann *et al*, 1996 and 1999), dairy cattle (Fenlon, 1986) and silage is considered the prime source of infection in sheep (Gitter *et al.*, 1986; Grønstøl, 1979; Ryser and Marth, 1991; Nash *et al.*, 1995). *L. monocytogenes* is commonly present in silage but it does not multiply to any significant extent in effectively preserved silage which is characterized by anaerobic storage, a high concentration of organic acids and a pH below 4.5. The critical pH of silage depends upon its dry matter (McDonald *et al.*, 1991). Silage contamination is directly related to its hygienic quality, as indicated by the presence of *Enterobacteriaecae* (Fenlon *et al*., 1996).

Table 5. Food-borne human listeriosis since 1975

Country	Year	Food associated	Number of cases	Serovar	References
USA	1979	Vegetables	20	4b	Ho *et al.* (1986)
New Zealand	1980	Shellfish, raw fish	29	1/2b	Lennon *et al.* (1984)
Canada	1981	Coleslaw	41	4b	Schlech *et al.* (1983)
USA	1985	Cheese	142	4b	James *et al.* (1985), Linnan *et al.* (1988)
Austria	1986	Milk, vegetables	28	1/2a, 4b	Allerberger and Guggenbichler (1989)
Switzerland	1983-87	Milk, cheese	122	4b	Bille (1990)
USA	1986-87	Ice-cream, salami	36	1/2a, 1/2b, 3b	Schwartz *et al.* (1989)
U.K	1987-89	Pate´	>300	4b	McLauchlin *et al.* (1991)
France	1993	Rillettes	279	4b	Goulet *et al.* (1998)
France	1994	Raw milk cheese	20	4b	Goulet *et al.* (1995)
Sweden	1994-95	Rainbow trout	8	4b	Ericsson *et al.* (1997)
USA	1997	Chocolate milk	45	1/2b	Dalton *et al.* (1997)
Finland	NK	cold-smoked rainbow trout	5	1/2a	Miettinen *et al.* (1999)
Australia	1991	Smoked mussels	2	NK	Misrachi *et al* (1991)
Italy	1997	Corn and tuna	1566	4b	Aureli *et al.* (2000)
Canada	1996	Imitation crab meat	NK	1/2b	Farber *et al.* (2000)
USA	2001	Delicatessen meat	16	1/2a	Frye *et al.* (2002)
New Zealand	2001	Ready-to-eat meats	31	1/2	Sim *et al.* (2002)
Sweden	2001	Raw milk cheese	120	1/2a	Danielsson-Tham *et al.*, (2004)

Nk- not Known

L. monocytogenes in Environment

L. monocytogenes has been isolated from waste water (Geuenich *et al.*, 1985), surface water (Combarro *et al.*, 1997) and sludge

(Watkins *et al.*, 1981) and is able to survive sewage treatment (Al-Ghazali and Al-Azawi, 1986). De Luca *et al.* (1998) showed that sewage sludge can be a source of *L. monocytogenes* and use of sludge as fertiliser could increase the risk of crop contamination. Thus, sewage sludge can be a potential source for the indirect transmission of *L. monocytogenes* between humans and animals.

Table 6. Flow chart showing interaction of *Listeria* with environment (Mc Lauchlin, 1998; modified by V.S.Parihar)

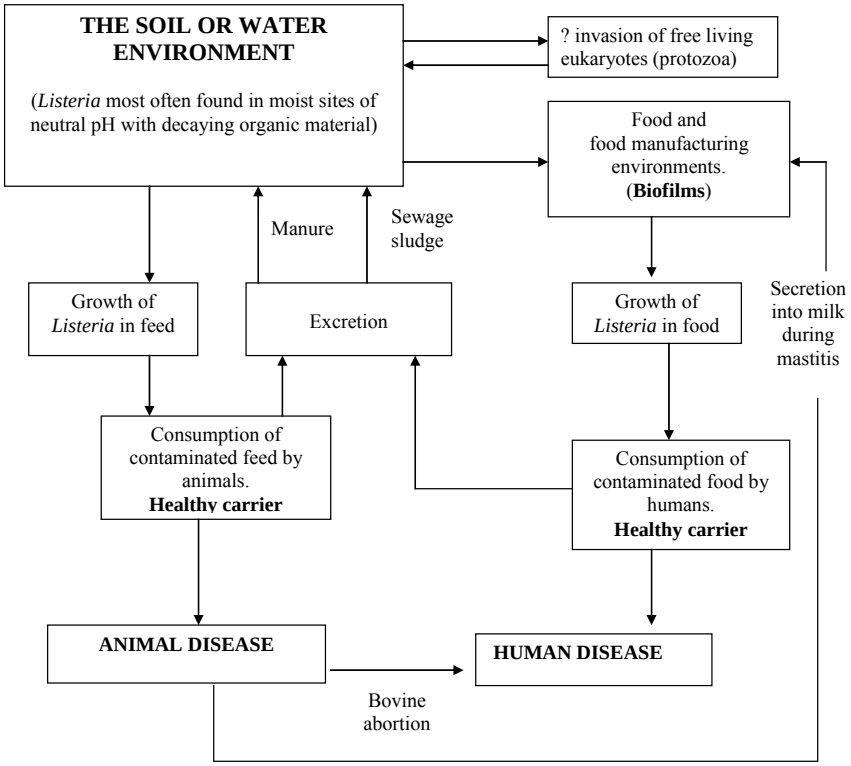

Fig. Interactions of *Listeria* with Environment

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Research Report

Molecular characterization of *Listeria monocytogenes* isolates from different animals in Sweden.

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Abstract

Animal clinical isolates of *L. monocytogenes* 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 previously characterized. 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

Key words: *L. monocytogenes*; Zoonoses; Animal

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1. Introduction

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. The most important clinical manifestations among both animals and humans include meningitis, septicaemia, abortion and febrile gastroenteritis. Thus, both the peroral infectious route and the clinical picture in animals and humans are similar and well documented (Roberts & Wiedmann, 2003). Knowledge of the direct or indirect transmission of *L. monocytogenes* between animals and humans, via e.g. foods, is however, 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.

2. Materials and methods

Each strain of *L. monocytogenes* was characterized by restriction enzyme analysis (REA) using *Asc* I and *Apa* I followed by pulsed-field gel electrophoresis (PFGE). The PulseNet standardised protocol by Graves and Swaminathan (2001), with some modifications, was used. Altogether 104 animal strains were analysed.

2.1. Preparation of DNA

L. monocytogenes strains were cultured onto Blood agar (Oxoid CM55, 5% horse blood) and incubuated at 37°C for 24h. One, single, well-isolated colony was inoculated into 5 ml Brain Heart Infusion Broth (Oxoid CM225) and incubated at 37°C for 24 h. The culture was cooled (4-8°C) and then centrifuged (Wifug, 5500 rpm) for 5 min. Pellet was washed twice in 5 ml TN buffer (10 mM Tris HCl, pH 8.0, 5M NaCl) and resuspended in 0.7 ml lysozyme solution

(1mg lysozyme/ml TN buffer) and then incubated at 37°C for 30 min.

2.2. Preparation of agarose

Fifteen ml 1.2% low melt agarose (SeaKem Gold, SKG), stored in 55°C water bath, was supplemented with 1.67 ml ESP (1 g N-lauroylsarcosin, Merck + 100 ml 0.5 M EDTA, pH 8 + 200 mg pronase, Roche). Of the above solution 1 ml is added to each culture with lysozyme and kept in water bath at 55°C. The mixture was poured into the slots, each 115 µl, of a plastic mould (Gene Navigator Pharmacia-Biotech, USA). The agarose plugs so formed were transferred to Eppendorf tube and soaked twice in ESP at 55°C in water bath during 2 h, resuspended in fresh ESP and reincubated at 55°C over night (15-20h). Finally, old ESP was replaced by fresh ESP, thus plugs could be stored in refrigerator for up to two years.

2.3. Restriction

DNA from each strain, represented by a longitudinal half plug, was transferred to an Eppendorf tube containing 0.5 ml PEFA (3.5 mg PEFA block in 10 ml TE-solution [10 ml 1 M Tris HCl, pH 8.0 + 2 ml 0.5 M EDTA, pH 8.0 and aqua dest. up to 1000 ml]) which was incubated at 37° in waterbath for 40 min. Old PEFA was replaced with the same amount of fresh PEFA and the sample reincubated at 37°C for another 40 min. PEFA was replaced by TE and sample incubated at 55°C in waterbath for 40 min. During incubation TE was replaced twice with fresh TE. Finally, TE was removed. The solution used for restriction with *Asc* I contained 870 µl aqua dest., 108 µl NE 4 buffer (10 x conc., New England Biolabs, Beverly, MA, USA), 10 µl acetylated BSA (bovine serum albumin 10 mg ml-1, Promega), 12 µl of *Asc* I (10 units/ml, New England Biolabs). Restriction was carried out at 37°C overnight. The solution used for restriction with *Apa* I contained 870 µl aqua dest, 98 µl Buffer A (10 x conc., Boeringer Mannheim), 10 µl acetylated BSA, 22 µl of *Apa* I (10 units/ml, Boeringer Mannheim). Restriction was carried out at 30°C overnight. Restriction solution for both *Apa* I and *Asc* I plugs was replaced with 200 µl 0.5 x TBE buffer (9 ml aqua dest. and 1 ml 5xTBE [54 g 0.45 M Trisbase, Amersham Biosciences, 27.5 g 0.45 M Boric acid, 20 ml 0.5 M EDTA, pH 8.0 and aqua dest. up to 1000 ml]). Samples were incubated at room temperature for more than 30 minutes.

2.4. Electrophoresis

Electrophoresis for plugs restricted with *Asc* I
The plugs were cast in a 1.17 % agarose gel (SKG). Pharmacia Gene Navigator (Pharmacia, Sweden) was used for electrophoresis; the migration period being 24 h with initial and final pulse times of 4.0 and 40 sec. Circulating buffer (0.5 x TBE) was kept at 8°C.
Electrophoresis for plugs restricted with *Apa* I
The plugs were cast in a 0.99 % agarose gel (SKG).
CHEF MAPPER XA (BIO-RAD) was used for electrophoresis; the migration period being 20 h with initial and final pulse times of 1.0 and 15.0 sec. Circulating buffer (0.5 x TBE) was kept at 8°C.

2.5. Staining and interpretation

The gels were stained with ethidium bromide (1 µg ml-1) for 20 minutes, washed in 0.5 x TBE and photographed over a 312 nm transilluminator. The photographs were analyzed visually. Lambda ladder PFG Marker NO 340 S (New England Bio-Labs, Inc., Beverly, MA, USA) was used as molecular weight markers. Strains were considered to belong to the same clonal type if patterns obtained with both enzymes were indistinguishable. The strains were considered closely related when a three-fragment difference was not exceeded with one or both enzymes. ‘‘Different strains’’ had a seven-fragment difference or more (Tenover *et al.*, 1995).

3. Results

Table 1. Classification of animal *L. monocytogenes* isolates in comparison with previously characterized human isolates.

Group	Group description	Strains	Clonal types
A	Identical with *Asc* I and identical with *Apa* I	33	14
B	Identical with *Asc* I and closely related with *Apa* I	10	9
C	Identical with *Asc* I and different with *Apa* I	15	4
D	Closely related with *Asc* I and closely related with *Apa* I	4	4
E	Different with *Asc* I and different with *Apa* I	42	35
	Total	104	66

Table 2. Distribution of animal species and *L. monocytogenes* strains/clonal types into groups (For group description see table 1.)

Group	Animal species	Strains	Clonal types
A	Cattle (12), Fallow deer (4), Roe deer (4), Goat (4), Sheep (3), Chinchilla (2), Ape (1), Horse (1), Pig (1), Elk (1)	33	14
B	Cattle (4), Panda (1), Horse (1), Goat (1), Duck (1), Fallow deer (1), Chinchilla (1)	10	9
C	Fallow deer (8), Sheep (5), Roe deer (2)	15	4
D	Cattle (2), Sheep (1), Fallow deer (1)	4	4
E	Sheep (12), Cattle (13), Fallow deer (10), Horse (1), Goat (2), Duck (2), Ape (1), Chinchilla (1)	42	35
	Total	104	66

Fig. 1. *Apa* I profiles of *L. monocytogenes* isolates of animal origin

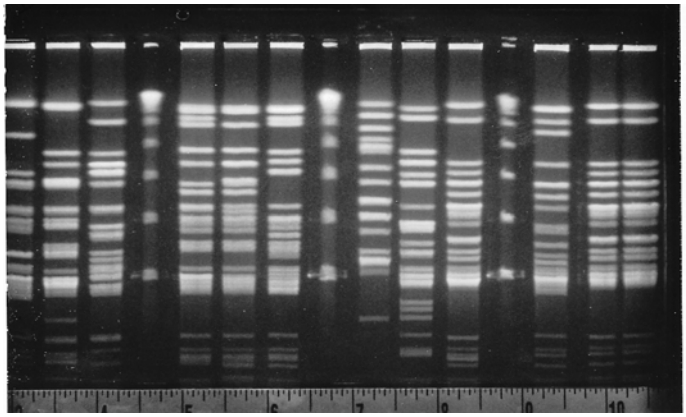
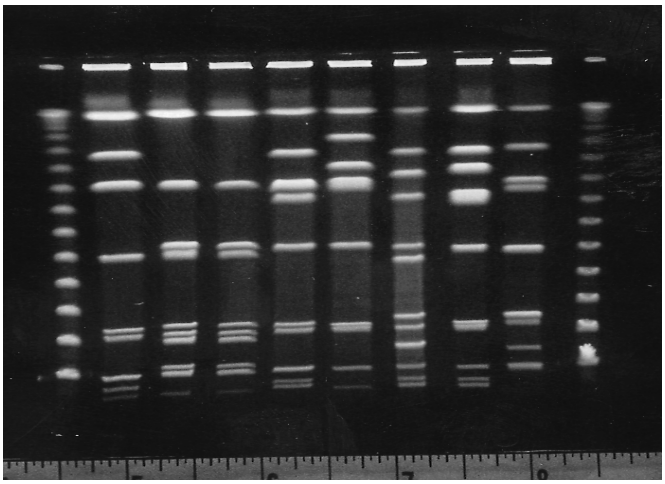


Fig. 2. *Asc* I profiles of *L. monocytogenes* isolates of animal origin.



4. Discussion

Animal clinical isolates of *L. monocytogenes* were characterized by use of restriction enzymes *Asc* I and *Apa* I followed by PFGE. Out of 104 animal strains, 33 belonged to clonal types also seen among clinical human strains previously characterized. In addition, 14 animal strains were closely related to human clonal types. Vela *et al.* (2001) report the findings of the same *L. monocytogenes* PFGE clonal types (pulsotypes) causing disease in both humans and sheep. An interesting observation in our investigation is that no animal species is more associated to the human clonal types than any other. The simplest explanation to the findings of common clonal types could be that they are widely distributed in the environment and thus could be picked up both by man and animal. The clonal types shared by animals and humans may also indicate that there is an exchange of *L. monocytogenes* strains between these two groups. The exchange may be due to direct or indirect transmission. Direct transmission of *L. monocytogenes* from animals to humans is reported among professionals such as animal handlers (Cain and McCann, 1986) and veterinarians (Owen *et al*., 1960), having close contact with diseased animals or healthy carriers.

Indirect transmission may occur simply by consumption of food products from diseased animals, for example, Danielsson-Tham *et al.* (2004) reported that on-farm manufactured raw milk cheese partly made of milk from a goat with an udder infection caused an outbreak with febril gastrointestinal listeriosis involving 120 people. This incident highlights a rarely reported but very short route of indirect transmission. The goat, the cheese and the visiting consumer

were all at the same place (summer farm) at the same time. The other way of indirect transmission has a more complicated rout, in that the distance between the original source of infection and the presumptive patient may be much longer. Such transmission deals with contamination of the environment in which the food product is processed. For example: in the slaughterhouse, faecal contamination of meat during evisceration; in the dairy, the reception of faecally contaminated milk; and in fish processing plants; the incoming raw fish. Once introduced into the food processing environment *L. monocytogenes* may persist there for longer periods by forming biofilms. Unnerstad *et al.* (1996) reported the survival of an unusual *L. monocytogenes* clonal type (serovar 3b) in a dairy for at least seven years. Thus, a considerable time may have elapsed from the initial contamination of the food processing plant with a certain strain of *L. monocytogenes* until the consumption of a contaminated food item, with the same strain.

In the present investigation 42 animal strains belonged to clonal types that were unfamiliar to our collection of human strains. Those clonal types might not yet have been transmitted to the human population. Another, explanation might be that certain clonal types of *L. monocytogenes* are adapted to specific hosts (Boerlin.and Piffaretti, 1991; Wiedmann *et al.*, 1997; Nightingale et al., 2004), *i.e.*, some of the 42 strains belong to clonal types with limted virulence for man.

Another interesting finding in our study is that 15 animal isolates distributed into eight clonal types yielded *Asc* I profiles familiar to our human clonal types. The *Apa* I profiles from the 15 animal isolates, however, have not been seen among our human strains. Among our ca. 400 human isolates, the allocation of an isolate to a certain clonal type by use of *Asc* I is only exceptionally inconsistent with information given by *Apa* I. The 15 strains were isolated from fallow deer (8), roe deer (2) and sheep (5). If small ruminants constitute a niche for *L. monocytogenes* isolates that are difficult to characterize by use of PFGE remains to be investigated. According to Chasseignaux *et al.*, (2001) divergent results may be due to a mutation only recognized by one of the two enzymes. Nightingale *et al*., (2004) report that *L. monocytogenes* ecology differ between bovines and small ruminants.

5. Conclusion

In this small study we showed that about half of the clinical animal strains characterized by PFGE were familiar to clonal types seen among clinical human strains previously characterized. Further

studies are needed to highlight routes of transmission between animals and humans, *e.g*., via food (Farm – to – table approach).

Acknowledgements

The author would like to thank "The Swedish Foundation for International Co-operation in Research and Higher Education (STINT)" for awarding research funding.

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Acknowledgement

To all Gods (Bhagvan, Allah, Jesus, etc…) and goddesses, for giving me the opportunity for being a Veterinarian and for great support in life.

The study and the research under current project were performed at the Department of Food Hygiene, Faculty of Veterinary Medicine and Animal Science, SLU, Sweden.
The author (Vishal Singh Parihar) was financially supported by the Swedish Foundation for International Co-operation in Research and Higher Education (STINT).

I also want to express my sincere thanks to all the following people who were involved in this study physically or mentally answering my silly questions

Prof. Wilhelm Tham, my core supervisor, for the valuable knowledge and sagacious guidance about *Listeria* science. I thank you for checking my thesis several times and to make it best with recent knowledge. I enjoy this (MSc) part of my professional study

with you very much. I like when you say “I have to sleep on that thought”. Please do not forget to say “WHEN”.

Prof. Marie-Louise Danielsson-Tham, head of the Department Food Hygiene for being my co-supervisor and to welcome me at the Department. I appreciate the way you teach me the microbiological science. I will not forget the difference and meaning of somatic antigen (o) and flagella antigen (H), which you told us. I am highly influenced by the patience you have and the way you explain something.

Dr. Viveka Båverud for accepting me as your Msc student and allowing me to use you collection of *Listeria* strains. I am also thankful to you for the pain you have taken to culture the Listeria for pure isolation. I think you have great smile and great knowledge.

For all the staff involved in SIPAR who are involved directly and indirectly. Prof. Karin östenson for giving me the great opportunity to study in Sweden at SLU and to guide me like a child. I have seen that you are a very good administrator and is more students orientated. Thanks for all the effort you have taken to get me out of the trouble of ECTS grading. I also want to extend the great appreciation to Marie Sundberg for making this MSc comfortable in study and private life.

Dr. Karel Krovacek for giving good advices and some laughing moments. I like your knowledge about women and practical life.

Dr. Amitha Reddy to give all support in science and private life. I highly apologize for always being late during lunch but i enjoyed the food very much in the aquatic climate of Sweden. I am really sorry that I did not accompany you enough. I wish you good luck for your further studies.

Prof Karl-Erik Johansson for sharing valuable knowledge of biochemistry.

Prof. Ulf Olsson for introducing me to the world of statistics and letting me know how easy this subject is. I have seen your pain taking effort for students by making notes easy and interesting. I have no hesitation to designate Prof. Ulf as Father of Modern Statistics. I would like to attend all the classes that you want to give at SLU during my study period in Sweden.

Dr. Sølvi for giving good advice on laboratory work and letting me do work at your laboratory for sometime. I am impressed the way you handle the things in laboratory and the way you stain bacteria with great perfection. I wish you good luck with new work.

To all other peoples of this Department, Dr. Henrik Ericsson, Susanne Broqvist, Helena Höök and others

To my family Papa, Mummy, Didi, Tony to encourage me in this nasty world of science and private life and to organize my wedding ceremony without my presence. Sister, I am really sorry that I have not attended your marriage ceremony, please forgive me.

To all my fiends here in Uppsala (Sweden), Dr. Suresh Yamanni, Dr. Carlos E. Hernández Verduzco, Dr. Mai, Dr. Tuempong Wongtawan for great company during my stay in Sweden.

Er. Avinash Kotwal of Adobe India for valuable guidance on computer software and Dr. Pushkar Kulkarni in scanning graphs.

Nigel Rollison for linguistic editing and formatting thesis

To Lord Krishna, for giving the true knowledge to mankind even in this nasty world. I always try to remember what you said to Arjun "teachers can be good or bad but they are always respectful".