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1- History of *L. monocytogenes*

History of *L. monocytogenes* began in 1926, when Murray, Webb and Swann isolated a 1-2 μm long and 0.5 μm wide round-ended Gram-positive rod shaped bacterium in dead laboratory rabbits and guinea-pigs in Cambridge and named it *Bacterium monocytogenes* because of the large mononuclear leukocytes it caused. In 1927, Pirie described unusual deaths of gerbils (*Tetrabornus*), the disease caused was termed ‘Tiger River Disease’ as it was discovered near Tiger River in South Africa and named the discovered bacterium as *Listerella hepatolytica* the generic name being dedicated in honor of a British Surgeon, Lord Joseph Lister. Because the strains isolated by Murray *et al.* in 1926 and Pirie in 1927 showed great similarity, the bacterium was renamed as *Listerella monocytogenes*. Since, the generic name
Listeria had previously been used for a protozoan, and Pirie in 1940 proposed changing the name to Listeria monocytogenes. This name was accepted, even though it already existed in botanical taxonomy, an orchid named Listeria, and in Zoology, a Diptera called Listeria.

2- Classification of Listeria

The genera of Listeria and Brochothrix are members of the family Listeriaceae, the order Bacillales, the class Bacilli and the phylum Firmicutes. However in the recent classification system, the genus Listeria is placed in kingdom Prokaryota, Division II- Firmicutes, Section 14 - Regular, Non-sporing, Gram positive Rods, Order-Lactobacillales and family Listeriacea (Seeliger and Jones, 1986; Boerlin and Piffaretti, 1991; Rocourt et al., 1992).

The genus Listeria Pirie belongs to the Clostridium sub-branch together with Staphylococcus, Streptococcus, Lactobacillus and Brochothrix. This phylogenetic position of Listeria is consistent with it is low G+C DNA content 36 to 42 % (Allerberger, 2003). On the basis of DNA-DNA hybridization, multilocus enzyme analysis, and 16S r-RNA sequencing, the genus Listeria presently comprises six species divided into two lines of descent.

- Listeria monocytogenes and the closely related species which include Listeria innocua, Listeria ivanovii (sub species ivanovii and subspecies londoniesis), Listeria welshimeri and Listeria seeligeri.
- Listeria grayi (L. murrayi was recently included in this species).

Within the genus Listeria, only L. monocytogenes and L. ivanovii are considered to be pathogenic as evident by their 50 % lethal dose (LD50) in mice and their ability to grow in mouse spleen and liver (Rocourt and Cossart, 1997; Swaminathan, 2001). The most commonly occurring species in food are L. innocua and L. monocytogenes (Jay, 1996).
3- Cultural characteristics

*L. monocytogenes* grow well aerobically and anaerobically on a wide variety of laboratory media like blood agar and trypticase agar, with a temperature of 3 to 40°C for growth optimum being 30°C (Cheesbrough, 1993). On blood agar *L. monocytogenes* produces small, gray droplet like colonies surrounded by a small zone of β-haemolysis. A narrow zone of β-haemolysis around colonies is characteristic feature of *L. monocytogenes*, weak β-haemolysis is characteristic of *L. seeligeri* and wide or multiple zones of β-haemolysis are produced by *L. ivanovii*. The cultures emit characteristic sour or buttermilk like smell. Growth in broth is usually poor but enhanced by addition of 0.5 to 1% glucose. The bacterium multiplies rapidly in milk (Bhatia and Ichhpujani, 1994).

4- Morphological and biochemical characteristics

*Listeria* species are Gram-positive, non-spore forming, regular, short rods, 0.4-0.5 μm in length with rounded ends and some cells may be curved. Occur singly, in short chains or the cells may be arranged at an angle to each other to give “V” or “Y” shapes or in groups lying parallel along the long axis. In older or rough cultures, filaments of 6-20 μm or more in length may develop. It is motile by a few peritrichous flagella when cultured at 20-25 °C. *Listeria* species exhibit characteristic tumbling motility in a hanging drop. *Listeria* is positive for catalase, methyl red and Voges-Proskauer tests and negative for oxidase, urease, indol and H₂S production. All *Listeria* species hydrolyze esculin but do not reduce nitrate with the exception of *L. murrayi* which does reduce nitrate (Seeliger and Jones, 1986). Some of the biochemical characteristics of *Listeria* are presented in table -3.

5- Growth limits

The optimum temperature for *L. monocytogenes* is 30-37 °C but the growth temperature ranges from 0.5 to 45 °C (Petron and Zottola, 1989). *L. monocytogenes* can grow at temperatures as low as -2 °C in laboratory media broth (Bajard et al., 1996). In vacuum packed smoked blue cod, growth has been observed at -1.5 °C (Bell et al., 1995). *L.
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*monocytogenes* in milk does not survive heating at 69 °C and above for 16.2 seconds. In a majority of cases, heating at 70 °C for 2 min. inactivates *L. monocytogenes* in meat. Exposure of milk and meat to sub lethal temperatures of around 44-48 °C before the final temperature can enhance the thermo tolerance of the cells. This implies that temperature fluctuation can enhance the thermo tolerance of *L. monocytogenes* (Farber and Peterkin, 1991). *L. monocytogenes* can grow at very low levels of pH, as low as 4.3 when HCl is used as the acidulant (George *et al.*, 1996). However, in a study conducted by Tienungon *et al.* (2000) it was found that *L. monocytogenes* Scott A and L5 were able to grow at pH levels of 4.23 and 4.25 respectively.

**Table - 3: Characteristics features of the genus Listeria.**

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Characteristics</th>
<th>Positive/Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catalase activity</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Oxygen requirement</td>
<td>F*</td>
</tr>
<tr>
<td>3</td>
<td>Growth at 35 °C</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Motility at 25 °C</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Motility at 37 °C</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Methyl red reaction</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Voges-Proskauer test</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>H²S production</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Acid from glucose</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Citrate utilization</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Urease activity</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Mannitol</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Nitrate reduction</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Gelatin hydrolysis</td>
<td>-</td>
</tr>
</tbody>
</table>

F* - Facultative

*L. monocytogenes* requires carbohydrate as the primary energy source for growth, with glucose being the preferred source (Premaratne *et al.*, 1991). *L. monocytogenes* grows well on nutritionally rich media such as brain heart infusion agar (BHIA) and tryptic soy agar (TSA). It can propagate up to 10% NaCl. However, *L. monocytogenes* is able to survive in salt concentrations as high as 25% at 4 °C (Hitchins, 1996). Metabolic end-products differ under aerobic and anaerobic conditions. Lactate and
acetate is produced under both aerobic and anaerobic conditions, but acetone is produced only under aerobic conditions (Romick et al., 1996). The organism can grow in the presence of CO₂ at low temperatures but CO₂ concentrations above 70% inhibit the growth of L. monocytogenes at temperatures < 7 °C. Even at high CO₂ concentrations (70%) and in the presence of low O₂ (5%), L. monocytogenes is able to grow (Wimpfheimer et al., 1990).

6- Serotyping of Listeria

Listeria species have number of serotypes based on somatic or ‘O’ antigens (1-4) and flagellar or H (a-e) antigens. There are sixteen serotypes known for the genus Listeria and thirteen serotypes known for L. monocytogenes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Table-4).

Table – 4: Serovar of the genus Listeria, (Seeliger and Jones, 1986).

<table>
<thead>
<tr>
<th>Designation</th>
<th>O antigens</th>
<th>H antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paterson</td>
<td>Seeliger</td>
<td>Donker-Voet</td>
</tr>
<tr>
<td>1</td>
<td>1/2a</td>
<td>I II (III)⁴</td>
</tr>
<tr>
<td></td>
<td>1/2b</td>
<td>I II (III)</td>
</tr>
<tr>
<td>2</td>
<td>1/2c</td>
<td>I II (III)</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>II (III) IV</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>II (III) IV</td>
</tr>
<tr>
<td></td>
<td>3c</td>
<td>II (III) IV</td>
</tr>
<tr>
<td>4</td>
<td>4a</td>
<td>(III) V VI VII IX X</td>
</tr>
<tr>
<td></td>
<td>4ab</td>
<td>(III) V VI VII IX</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>(III) V VI</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>(III) V VI</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>(III) V VI VII VIII</td>
</tr>
<tr>
<td></td>
<td>4e</td>
<td>(III) V VI (VIII) (IX)</td>
</tr>
<tr>
<td>5</td>
<td>(III) V VI (VIII) X</td>
<td>A B C</td>
</tr>
<tr>
<td>7</td>
<td>(III) XII XIII</td>
<td>A B C</td>
</tr>
<tr>
<td>6a (4f)</td>
<td>(III) V (VI) (VII) (IX) XV</td>
<td>A B C</td>
</tr>
<tr>
<td>6b (4g)</td>
<td>(III) V (VI) (VII) IX X XI</td>
<td>A B C</td>
</tr>
</tbody>
</table>

L. grayi        | (III) XII XIV | E |
L. murrayi      | (III) XII XIV | E |

( )³, not always present
For *L. monocytogenes*, two primary divisions have been delineated, with serovars 1/2b, 3b, and 4b falling into one division and serovars 1/2a, 1/2c, and 3a falling into the other based on multilocus enzyme electrophoresis (MEE) analysis, which defines and compares the genetic relationships among the electrophoretic types (Low and Donachie, 1997; Allerberger, 2003).

The identification of serotypes serves as a useful tool for epidemiological purposes. The epidemiological data suggests that serotype 1/2 causes approximately 60% of sporadic cases, and serotype 4b causes approximately 29% of cases (Schlech, 1996). Rocourt (1996), however, noted that serovar 4b being responsible for 45-70% of sporadic cases of listeriosis. Among *Listeria monocytogenes* strains, serotype 1/2a, 1/2b and 4b have been implicated in 90% of outbreaks of listeriosis (Gasanov et al., 2005). Results of serotyping *L. monocytogenes* strains have established that most strains accounting for human infection differ in certain respects from strains typically found in foods as contaminants (Rocourt, 1996; Gasonov et al., 2005).

### 7- Ecology and food vehicles of *Listeria*

*Listeria* species are widely distributed in the environment and the occurrence of *L. monocytogenes* in raw and processed foods has been extensively studied. *Listeria* species have been isolated from a large variety of sources including soil, rotting vegetation, sewage water, rivers and salt estuaries (Ryser, 1999). It has been suggested that the bacteria exists in a saprophytic environment involving plants and soil, which serves as a reservoir for later infection transmitted to animals and humans via faecal material, foods, insects etc. (Farber and Peterkin, 1991). Some of the possible routes for transmission of *L. monocytogenes* to humans are depicted in figure-1.

Ben Embarek (1994) summarized many reports relating to raw and processed sea foods in which prevalence up to 75% in lightly preserved (cold-smoked, hot-smoked, marinated) fish products are noted. Beuchat (1996) reported the prevalence of *L. monocytogenes* in raw vegetables including bean sprouts, cabbage, cucumber, potatoes, pre-packed salads, radish, salad vegetables, and tomatoes, ranged from 1.1
to 85.7%. Because of their widespread occurrence, *Listeria* species have many routes to enter food production and processing environment due to their psychrotrophic nature, they are then able to grow in food, even at temperatures such as those of refrigerators. In addition, 2-6 % of healthy individuals are reported to be a symptomatic faecal carrier of *L. monocytogenes* (Rocourt, 1996). It has been estimated that 90 % of adults possess immune lymphocytes to pathogenic *Listeria* (Hof, 2003).

Fig-1: Possible routes for transmission of *L. monocytogenes* to humans (Alexsson and Sorin, 1998).

8- Epidemiology

The incidence of human listeriosis has been growing in the last few years, whereby most cases are sporadic but some are occasionally also epidemic. Contaminated food has been suspected as the source of infection caused by *L. monocytogenes* but food borne transmission was not confirmed until 1981 when an outbreak in the maritime
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provinces of Canada was shown to be caused by ingestion of contaminated Coleslaw (Schlech et al., 1983). In this incident, 41 people became infected and 18 deaths resulted. The cabbage used to make the Coleslaw was fertilized with manure from sheep with listeriosis. The cabbage was held in cold storage over the winter and then used to prepare the Coleslaw. The same serotype of *L. monocytogenes* (4b) was isolated from both the Coleslaw and patient’s blood, which corroborated food-borne transmission. A second food-borne outbreak occurred in Massachusetts in 1983 where listeriosis was epidemiologically linked to whole and 2% pasteurized milk (Fleming et al., 1985), in this outbreak 49 people became ill and 14 patients died. *Listeria* was found in the raw milk but not in the final pasteurized products.

Mexican style cheese was the food implicated in the last major outbreak of listeriosis in North America in 1985 (Anon., 1985). This incident infected 142 people with a total of 47 deaths. Raw milk was used to prepare the cheese and *L. monocytogenes* was isolated from the plant environment. Since 1985, *L. monocytogenes* has been isolated from all types of foods including French and domestic soft cheeses, ice cream, Pate, meat and sea foods (Farber and Peterkin, 1991). Major food-borne outbreaks in the past decade indicate that, like other pathogens, *L. monocytogenes* poses a serious health risk to certain members of the population (Dever et al., 1993).

9- Reservoirs

The role of silage in the transmission of animal disease was documented as early as 1960 and *L. monocytogenes* strains were isolated from natural decaying vegetation in 1968 and thereafter (Weis and Seeliger, 1975). *L. monocytogenes* has been isolated from the faeces of many healthy animals and birds. Humans exhibiting symptoms of listeriosis and asymptomatic carriers shed the organism in their faeces. *L. monocytogenes* can be spread from the environment to animals and humans and back to the environment (Figure-1). The host range of *L. monocytogenes* includes at least 42 kinds of domestic and wild animals, 22 avian species, fish, ticks, flies and crustacean (Bahk and Marth, 1990). Two properties of *L. monocytogenes* in particular contribute to its widespread distribution; (i) despite not forming spores, it can survive for long periods of time in many different environments, and (ii) it is psychrotrophic.
Food can therefore become contaminated at any step of the food chain, and cold storage does not inhibit the growth of *Listeria*. These studies clearly suggest that, *L. monocytogenes* spreads through number of routes.

10- Virulence factors

10-1 Virulence factors involved in adhesion and invasion

There are different proteins of *L. monocytogenes* involved in adhesion and invasion of host cells. Internalin A (InlA) and B (InlB) were the first proteins to be identified and are expressed on the surface (Vasquez-Boland *et al*., 2001).

(a) *Internalin A (InlA)*: InlA is composed of 800 amino acids, promotes entry into epithelial cells and plays a major role in crossing the intestinal barrier. Its receptor is E-cadherin, which is a transmembrane glycoprotein and the interaction between InlA and E-cadherin is species specific. The clinical strains of *L. monocytogenes* express a full length InlA far more frequently (96% of the cases) than strains recovered from food products (65%), suggesting that InlA has a critical role in pathogenesis of human listeriosis, and can be used as a marker of virulence in food assessments (Dramsi *et al*., 1995).

(b) *Internalin B (InlB)*: InlB is composed of 630 amino acids, promotes entry into many different cell lines, including hepatocytes and non epithelial cells. Hepatocyte growth factor or Met has been recognized as the main receptor for InlB on target cells (Dramsi *et al*., 1995).

(c) *P60 (Invasion associated protein - iap)*: Spontaneously rough mutants (R-mutants) produce reduced level of a 60-KDa extra cellular protein termed p60. It is a major 60-KDa surface protein that was first described as a protein involved in the invasion of host cells by *L. monocytogenes*. p60 (also known as *iap*) has a murein hydrolase activity that is implicated in cell division. A recent report suggested that this protein is directly involved in binding intestinal Caco-2 cells (Kuhn and Goebel, 1998).
(d) **Listeriolysin O (LLO):** LLO is a pore forming protein involved in helping *Listeria* escape from primary and secondary phagosomes. In addition, LLO triggers many cellular responses, such as interleukin-1 secretion in macrophages, apoptosis, cell adhesion, and protein expression in infected epithelial cells. Many of these functions are Ca\(^{+2}\) dependent (Gedde *et al.*, 2000).

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

### 10-2 Virulence factors involved in intracellular life cycle

(a) **Listeriolysin O (LLO) and two phospholipases C:** LLO together with two phospholipases play a critical role in the bacterial escape from primary and secondary intracellular vacuole. The two phospholipases include phosphatidylinositol-specific phospholipase C (PI-PLC) and Phosphatidylecholine specific phospholipase C (PC-PLC) (Vasquez-Boland *et al.*, 2001).

(b) **Act A:** Act A is a protein with 639 amino acid and it induces polymerization of the actin filaments promoting movement of bacteria in the cytoplasm of infected cell and cell-to-cell spread (Vasquez-Boland *et al.*, 2001).

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

(d) **Metalloprotease:** Phosphatidylinositol-specific phospholipase C (PI-PLC) is synthesized as an active form whereas as phosphatidylcholine-specific phospholipase C (PC-PLC) is produced as an inactive precursor. A bacterial zinc-dependent metalloprotease and a host cell cysteine protease are required to cleave off part of the precursor and activate the phospholipase. Metalloprotease encoded by *mpl* aids maturation of PCPLC, which is synthesized as a proenzyme (Portnoy *et al.*, 1992; Dussurget *et al.*, 2004).
11- Food-borne *L. monocytogenes* infection

*L. monocytogenes* was recognized for the first time as a human pathogen in 1929, but the route of infections was unknown until the 1980s, when outbreaks of listeriosis were associated with consumption of contaminated food (Linnan et al., 1988). Most cases of listeriosis outbreaks are attributed to foods that were generally designed as ready-to-eat (RTE) foods. The presence of *L. monocytogenes* in RTE foods is widely believed to occur due to post process procedures rather than being due to survival during the processing itself (McLachlin, 1987). Food items that have been linked to the outbreak of listeriosis include dairy, soft cheeses, coleslaw, meat, sea foods, and vegetable based products (Elliot and Kvenberg, 2000). There are several reasons for the late establishment of the food-borne nature of listeriosis.

- The symptoms of invasive listeriosis are severe and differ from classical food-borne diseases.
- Listeriosis is a rare disease, occurring mainly among a high-risk group of people and majority of cases are believed to be sporadic.
- The long incubation time hinders tracing of vehicles of infections and often no food is left for microbiological analysis.

The first confirmed evidence of food-borne transmission of *L. monocytogenes* was presented by Schlech et al. (1983) who demonstrated Coleslaw as a vehicle of infection in an outbreak of listeriosis in Nova Scotia, Canada. Between March and September 1981, of the 34 prenatal cases, there were 9 stillbirths, 23 live births of an ill infant with a subsequent 27% mortality rate, and 2 live births of a well infant. The adult mortality rate was 28.6%. The epidemiological study of these cases is a model of its kind. As a result of analysis of case-control survey, the illness was associated with Coleslaw. Coleslaw obtained from the refrigerator of one of the patients was shown to contain *L. monocytogenes* type 4b, the epidemic strain. The Coleslaw had been prepared by a regional manufacturer, and distribution was confined to the Maritime Provinces (Farber and Peterkin, 1991). Several epidemics have subsequently been described and various kinds of food products linked with the outbreaks. A list of food-borne outbreaks of human listeriosis is shown in table-5.
Because, *Listeria* can survive and grow under many adverse conditions including low pH, refrigeration temperatures and high salt concentrations, they can easily contaminate food. This is of great concern to the food industry, since low inoculums can translate into a substantial dose of *Listeria* for the consumer, depending on the shelf life and handling of a particular product (Farber, 1993). Although, other modes of transmission exist, foods have been clearly identified as the single most important source of infection. The high prevalence of *L. monocytogenes* in foods in general in combination with a high mortality rate of listeriosis makes *L. monocytogenes* a very important and complex problem for microbiological risk assessment and risk management (Norrung *et al*., 1999).

12- Pathophysiology of *Listeria*

12-1 Infective dose and high case fatality rate

Dose-response relationships are much less well understood for human *L. monocytogenes* infection (Mclauchlin *et al*., 2004). The infective dose of *L.
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*Listeria monocytogenes* depends on many factors including the immunological status of the host (Hof, 2003). In addition to host factors and exposure to particular foods, it is likely that microbial characteristics are important risk factors for disease. The occurrence and the course of infection may well depend on virulence factors and infective dose. The severity of the disease is such that tests with human volunteers are impossible. Studies in monkeys and in mice suggest that reducing levels of exposure will reduce clinical disease (Vasquez-Boland *et al.*, 2001). However, these experiments do not help to determine the minimal infective dose for humans. The published data indicate that the number of *L. monocytogenes* in contaminated food responsible for epidemic and sporadic food-borne cases were more than 100 CFU/g. However, because enumeration procedures are not fully reliable and the time between consumption and analysis of the contaminated food results may not always be indicative of the numbers consumed.

The minimum infective dose has not been reliably established, however published information on the numbers of *L. monocytogenes* in contaminated foods during outbreaks indicate that levels of between $10^3$ - $10^4$ cells were responsible for illness (Duffy *et al.*, 1999). Infection of pregnant women in the early stages usually leads to spontaneous abortion. Overall lethality is usually around 20-30% for both epidemic and sporadic cases (Goulet *et al.*, 1995). Higher rates have also been published, for instance of about 51% in Barcelona in 1990 (Nalla-salas *et al.*, 1993). Lethality in immuno-compromised or elderly patients and among patients suffering from central nervous system infections is higher, at 38-45% (Bula *et al.*, 1995).

12-2 Route of entry and colonization of host tissues

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood. Most of the available information is derived from interpretation of epidemiological, clinical, and histopathological findings and observations made in experimental infections in animals, particularly in the murine model. As contaminated food is the major source of infection in both epidemic and sporadic cases (Farber and Peterkin, 1991), the gastrointestinal tract is thought to be the primary site of entry of pathogenic *Listeria* organisms into the host. The clinical course of infection usually begins about 20h after the ingestion of heavily contaminated food in cases of
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gastroenteritis (Dalton et al., 1997), whereas the incubation period for the invasive illness is generally much longer, around 20 to 30 days (Riedo et al., 1994). Similar incubation periods have been reported in animals for both gastro-enteric and invasive disease (Vazquez-Boland et al., 2001).

Before reaching the intestine, the ingested Listeria cells must withstand the adverse environment of the stomach. Oral infective doses are lower for cimetidine-treated experimental animals than for untreated animals (Schlech et al., 1993) and the use of antacids and H2-blocking agents has been reported to be a risk factor for listeriosis (Schuchat et al., 1992). This indicates that gastric acidity may destroy a significant number of the Listeria cells ingested with contaminated food. There is controversy concerning the point of entry and the mechanism of intestinal translocation used by L. monocytogenes. In an early study with guinea pigs infected intra-gastrically with $10^{10}$ cells of L. monocytogenes, detailed histological analyses revealed that all the animals developed enteritis (Vazquez-Boland et al., 2001) and suggested that L. monocytogenes penetrates the host by invading the intestinal epithelium. In other studies using mice inoculated with $10^8$ to $10^9$ cells, suggested that L. monocytogenes uses the membranous cell (M-cell) epithelium as an entry portal, as reported for other bacterial pathogens (Siebers and Finlay, 1996).

Direct evidence that L. monocytogenes may indeed penetrate the host via the M cells overlying the Peyer's patches has been provided by a recent study using a murine ligated-loop model and scanning electron microscopy (Jensen et al., 1998). The Listeria cells that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver (Pron et al., 1998). As stated above, this initial step of host tissue colonization by L. monocytogenes is rapid. The unusually long incubation period required by L. monocytogenes for the development of symptomatic systemic infection after oral exposure in relation to that for other food-borne pathogens is therefore puzzling and indicates that listerial colonization of host tissues involves a silent, sub clinical phase, many of the events and underlying mechanisms of which are unknown (Figure- 2).
Fig- 2: Schematic representation of the pathophysiology of *Listeria* infection (Vázquez-Boland *et al.*, 2001).

12-3 Intracellular infectious cycle

The cycle begins with adhesion to the host cell and subsequent penetration. Once the *Listeria* invade cells, macrophages or non-phagocytic cells, such as epithelial cells, fibroblasts, hepatocytes, endothelial cells and nerve cells, they undergo an intracellular life cycle (Figure- 3). *L. monocytogenes* invades non-phagocytic cells by a zipper type mechanism, in which the host cell engulfs the bacteria and the cell membrane surrounds the bacterial cell forming a phagocytic vacuole. After 30 min. the phagosome membrane is lysed by the bacteria and a fine, fibrillar material composed of actin filaments immediately surrounds bacterial cell in the cytoplasm. These filaments form an actin tail up to 40 μm long at one pole of the bacterium that propels the bacterial cell into the cytoplasm at an average velocity of 10-15 μm/min. but can be as fast as 36 μm/min. In contact with the cell membrane, a finger-like protrusion is formed with the bacterium at the tip. Uninfected neighbouring cells are penetrated by these protrusions, which are engulfed by phagocytosis forming a secondary phagosome. The bacteria are covered with two cell membranes, the donor cell forms an inner membrane and the newly infected cell forms an outer membrane. Bacteria rapidly dissolve these membranes (within 5 min.) and initiate another round
of intracellular proliferation in the cytoplasm, and thus, spread from cell to cell (Vázquez-Boland et al., 2001; Cossart et al., 2003).

**Fig- 3: Stages of listerial intracellular parasitism. Scheme of the intracellular life cycle of pathogenic *Listeria* species.** 1- entry into the host cell by induced phagocytosis, 2- transient residence within a phagocytic vacuole, 3- escape from the phagosome into the cytoplasm, 4- cytosolic replication and recruitment of host cell actin onto the bacterial surface, 5- actin-based motility, 6- formation of pseudopods, phagocytosis of the pseudopods by neighboring cells, 7- formation of a double-membrane phagosome, 8- escape from this secondary phagosome, and reinitiation of the cycle (Vázquez-Boland et al., 2001).

**12-4 Clinical features**

The clinical signs of *L. monocytogenes* infection are very similar in all susceptible hosts. Two basic forms of presentation can be distinguished, prenatal listeriosis and listeriosis in the adult patient. In both instances, the predominant clinical forms correspond to disseminated infection or to local infection in the central nervous system (CNS) (Schuchat et al., 1991). Listeriosis is usually a very severe disease with a mean mortality rate in humans of at least 20-30% despite early antibiotic treatment.
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(Schuchat et al., 1991). It has bimodal distribution of severity with most cases being either mild or severe (Anon., 1994). Mild cases are characterized by a sudden onset of fever, severe headache, vomits, and other influenza-type symptoms (Table- 6). Severe manifestations of listeriosis correspond to disseminated infection or to local infection in the central nerve system, including septicemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd / 3rd trimester) or stillbirth (McLauchlin, 1990). The incubation period for listeriosis varies from 4 days to several weeks and the duration of the illness from a few days to several weeks (Anon., 1994).

Table- 6: Types of illnesses caused by *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Type of illness</th>
<th>Incubation time</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoonotic infection</td>
<td>1-2 days</td>
<td>Localized skin lesions that are mild and self resolve</td>
</tr>
<tr>
<td>Neonatal infection: Newborn babies infected from mother during birth or due to cross infection from one neonate in the hospital to other babies.</td>
<td>1-2 days (early onset) usually from congenital infection prior to birth</td>
<td>Can be severe, resulting in meningitis and death.</td>
</tr>
<tr>
<td>Infection of the mother during pregnancy acquired from contaminated food. Infection is more common in third trimester.</td>
<td>Varies from 1 day to several months</td>
<td>Mild flu like illness or asymptomatic in the mother but serious implications for the unborn infant including spontaneous abortion, stillbirth and meningitis.</td>
</tr>
<tr>
<td>Listeriosis acquired by non-pregnant people from contaminated food.</td>
<td>Varies from 1 day to several months.</td>
<td>A symptomatic or mild illness which may progress to central nerve system infections such as meningitis. Most common and most severe in immunocompromised or elderly people.</td>
</tr>
<tr>
<td><em>Listeria</em> food poisoning caused by consumption of food containing very high level (&gt;10³ per gram) of <em>L. monocytogenes</em>.</td>
<td>&lt; 24 hours</td>
<td>Vomiting and diarrhoea, sometime progressing to bacteraemia but usually self-resolving.</td>
</tr>
</tbody>
</table>

Source: (Anon., 1994).

13- *L. monocytogenes* infections

(a) In animals: Nearly all domestic animals are susceptible to *Listeria* infections. The main clinical manifestations of ruminant listeriosis are encephalitis, septicemia and abortion (Low and Donachie, 1997). *L. monocytogenes* may also be shed in milk by
both diseased and healthy animals, and prolonged excretion of bacteria has been described. Seasonal variation both in prevalence of listeriosis and in prevalence of *L. monocytogenes* in raw milk has been reported. The higher incidence in winter months has been suggested to be associated with indoor housing and silage feeding (Jemmi and Stephan, 2006).

**b) In humans:** *L. monocytogenes* can cause both invasive and non-invasive infections in humans. Invasive listeriosis is a severe disease mainly associated with a specific risk group of people and the case fatality rate is high, whereas fairly mild non-invasive infections can also occur in healthy people (Crum, 2002). Invasive listeriosis is rare, with an annual incidence rate of 2-9 cases per million, but it is one of the most severe food-borne infections and has a high, approximately 30%, case fatality rate (Mclauchlin et al., 2004). Listeriosis characteristically occurs in persons with a predisposing condition or disease, such as pregnancy, neonatality, malignancy, transplantation, alcoholism, immunosuppressive therapy, diabetes, old age and AIDS (Schuchat et al., 1992). The complication of listeriosis includes central nerve system (CNS) infections, septicemia, abortions, neonatal infections and stillbirth.

**c) Host defenses:** *L. monocytogenes* is an intracellular parasite, and it is in this environment that the pathogen gains protection and evades some of the host defense. However, animals defend themselves from bacterial attack using three strategies, general non-specific resistance factors, cell-mediated immunity, and antibody-mediated immunity. Antibody-mediated immunity does not appear to play a significant role in protection from listeriosis. Non-specific host defenses for food-borne pathogens include stomach acidity, which is lethal to many bacteria in food, and the normal gut micro flora which are usually so numerous that they occupy all available niches in the intestine and consume essential nutrients, thereby preventing incoming bacteria from getting established. *L. monocytogenes* is known to be a poor competitor; this probably explains why the majority of adults exposed to this bacterium do not become sick (Okamoto et al., 1994). Infection of epithelial and macrophage cells by *L. monocytogenes* strongly modulates the complex host cell signaling systems affecting the production and activity of a number of non-specific proteins and cells which counteract or support listerial infections (Kuhn and Goebel, 1998). Cell-mediated immunity is probably the main host defense against listeriosis.
with CD4+ T helper lymphocytes activating macrophages (Czuprynski, 1994). Activity of immune system cells and proteins against *L. monocytogenes* will be described below. The effectiveness of immune reactions in different individuals is modified by genetic and nutritional factors. For example, dietary selenium and fatty acids have been shown to affect the ability of mice to react to listerial infections. Pregnancy and aging also affect the effectiveness of the immune response (De Pablo *et al.*, 2000).

14- Treatment for *L. monocytogenes* infection

Practically all common antibiotics, except cephalosporin, are active against *L. monocytogenes in-vitro* (Hof, 1991). However, *in-vivo* a low efficiency may be expected and is probably in part due to the organism’s intracellular location. Experimentally, ampicillin and amoxicillin are the most active and tetracycline and chloramphenicol are reportedly not the therapeutic agents of choice (Hof, 1991; Low and Donaghie, 1997). Antimicrobial resistance to chloramphenicol, erythromycin, streptomycin and tetracycline, has been recorded (Poyart-Salmeron *et al.*, 1990). In the treatment of human listeriosis, either ampicillin or amoxicillin in combination with gentamicin is the primary choice of therapy (MacGowan *et al.*, 1990).

15- Isolation methods for *Listeria*

Isolation of *L. monocytogenes* from material expected to be heavily contaminated with other bacteria, e.g. faecal samples and some food samples, is more difficult than isolation from normally sterile clinical specimens, like blood and cerebrospinal fluid. Historically, it has been challenging to isolate *Listeria* from food or other samples and this explains why it remained unnoticed as a major food-borne pathogen until recently.

In early studies it was noted that *Listeria* is able to grow at low temperatures and this feature has been used to isolate these bacteria from clinical samples by incubation for prolonged periods at 4 °C on agar plates until the formation of visible colonies. This method of isolation takes up to several weeks and usually does not allow for the
isolation of injured *Listeria* cells, which will not survive and grow when stressed. These two key issues enrichment, isolation time and the recovery of stressed *Listeria* cells must be addressed if methods of enrichment and isolation are to provide meaningful results that can be used to control *Listeria* contamination and food-borne outbreaks (Gasanov, 2005). Tests considered for approval by regulatory agencies must be able to detect one *Listeria* organism per 25g of food. Generally, this sensitivity can only be achieved by using enrichment methods in which the organism is allowed to grow to detectable levels of \( \approx 10^4\text{-}10^5 \text{ CFU ml}^{-1} \). *Listeria* cells are slow growing and can be rapidly out-grown by competitors, and hence bacteriostatic agents, such as acriflavin and nalidixic acid that specifically act to suppress competing micro flora, have been introduced into enrichment media or selective agar media (Welshimer, 1981). These two agents are incorporated into all standard methods used to isolate *Listeria* from food and environmental samples. The list of antimicrobial agents that are commonly used for isolation of *Listeria* in selective broth and agar media are presented in *table - 7*.

**Table- 7: Antimicrobial agents used in *Listeria* selective broth and agar media.**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Antimicrobial agent</th>
<th>mg/L</th>
<th>Bacterial inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acriflavine</td>
<td>5-25</td>
<td>Gram-positive, including <em>Lactobacillus bulgaricus</em> and <em>Streptococcus thermophilus</em></td>
</tr>
<tr>
<td>2</td>
<td>Ceftazidime</td>
<td>50</td>
<td>Broad spectrum</td>
</tr>
<tr>
<td>3</td>
<td>Cycloheximide</td>
<td>50</td>
<td>Fungi</td>
</tr>
<tr>
<td>4</td>
<td>Lithium chloride</td>
<td>0.5</td>
<td>Gram-negative, except <em>Pseudomonas</em>.</td>
</tr>
<tr>
<td>5</td>
<td>Moxalactam</td>
<td>20</td>
<td>Broad spectrum</td>
</tr>
<tr>
<td>6</td>
<td>Nalidixic acid</td>
<td>20-40</td>
<td>Gram-negative except <em>Pseudomonas</em> and <em>Proteus</em>.</td>
</tr>
</tbody>
</table>

Source: (Alexsson and Sorin, 1998).

In food industry, standard culture procedures are used as reference methods for regulatory purposes and for validation of new technology. These methods are sensitive but often time consuming and may take 5-6 days. Numerous methods have been developed over the years for isolating and detecting *Listeria* from food, clinical and environmental samples (*Figure- 4*). Three general types can be distinguished they
are; direct plating, enrichment followed by plating and enrichment followed by a rapid method (Donnelly et al., 1992). Two of the most widely used culture reference methods for detection of Listeria in all foods are the Food and Drug Administration (FDA) [Bacteriological and Analytical Method (BAM)] and International Organization of Standards (ISO) 11290 method. Both of these methods require enrichment of 25 g of food sample in selective broth, designed to slow the growth of competing organisms, prior to plating onto selective agar and biochemical identification of typical colonies. The detection of L. monocytogenes according to ISO 11290-1 requires the following successive stages:

(a) Pre-treatment of each food type and reference materials (RM) according to a standardized procedure to obtain an initial sample for primary enrichment.

(b) Primary enrichment in a selective liquid enrichment medium with reduced concentration selective agents (half-Fraser broth) with incubation at 30 °C for 24 h.

(c) Secondary enrichment of a culture obtained from (b) in a selective liquid enrichment medium with full concentration of selective agents (full strength Fraser broth) with incubation at 37 °C for 48 h.

(d) Plating of cultures obtained in (b) and (c) and identification on either Oxford or PALCAM media with incubation at 30 °C (Oxford) (Curtis, 1989) or 37 °C (PALCAM) and examination after 24 h and, if necessary, after 48h to check for the presence of characteristic colonies which are presumed to be Listeria species.

(e) Confirmation of the presence of L. monocytogenes by means of appropriate morphological, physiological and biochemical tests carried out on five presumptive colonies (Scotter et al., 2001).

The solid Listeria selective media commonly used are Oxford (Curtis et al., 1989) and PALCAM (Van Netten et al., 1989), both of which contain aesculin with ferric ammonium citrate as indicatory substrates to differentiate Listeria from other bacteria. Combined use of these media provides high recovery rates, as they differ in their selectivity. To distinguish L. monocytogenes from other Listeria species, they contain chromogens as in ALOA agar (Vlaemynck et al., 2000) or enhanced haemolysis agar, EHA, (Cox et al., 1991) and Listeria monocytogenes blood agar (LMBA) (Johansson, 2001).
1998). Typical colonies grown on selective media are confirmed as *Listeria* species by Gram staining, catalase reaction and motility test at 25 °C. Gram-positive, catalase positive rods showing tumbling motility at 25 °C are further verified as *L. monocytogenes* by detection of β-haemolysis, by testing the fermentation of rhamnose and xylose, and by CAMP test. Several commercial rapid tests for identification of *Listeria* species and *L. monocytogenes* have been developed and are considered suitable alternatives to conventional tests. However, all these methods suffer from sensitivity and methods are labour intensive.

**Fig-4**: Schematic representation of protocols for isolation and detection of *Listeria*. The size of arrows denotes an estimated popularity of various methods (Alexsson and Sorin, 1998).

**16- Importance of the current research**

Studies from India indicated the incidence of *L. monocytogenes* in pregnant women, with bad obstetric history, to be in the vicinity of 1.34% and 4% (Stephen et al., 1978). Incidence of neonatal listeriosis in India was reported by Gupta et al. (1997). A review of literature indicated that there have been occasional reports of listeriosis in humans and animals in India (Karunasagar and Karunasagar, 2000). The incidence of
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*L. monocytogenes* in clinical and food samples was reported in India, a total of 633 clinical and 320 food samples have been collected and analyzed, *L. monocytogenes* was isolated in two clinical and two food samples and the incidence of *Listeria* species found to be 0.3% in clinical and 17.5% in food samples respectively (Dhanashree *et al.*, 2003; Gupta *et al.*, 2003).

India at present is the second largest milk producing country in the world and Indian dairy products ranging from liquid milk to value added products has tremendous export potential, which has not been fully exploited (Pednekar *et al.*, 1997). Data on the incidence of *Listeria* species in Indian foods is limited to a few recent reports, so that it becomes necessary to carry out more such studies on Indian dairy products (Pednekar *et al.*, 1997). Incidence of *L. monocytogenes* in milk and dairy products have been reported and sometime even co-related to food poisoning events in different parts of the world (Warke *et al.*, 2000). *L. monocytogenes* have been isolated from fish and fishery products from different parts of the world (Karunasagar and Karunasagar, 2000). The earlier study from India showed 38 samples of fish and their products were contaminated with *Listeria* species (Kamat and Nair, 1994). The reported incidence of *L. monocytogenes* in fish and fishery products from temperate parts of the world varies from very low to 50% (Dillon and Patel, 1992; BenEmbarek, 1994). Fuchs and Surendran (1989) could not detect *L. monocytogenes* in fresh fish samples from India although 33% of the samples harbored *Listeria* species. However, Jeyasekaran *et al.* (1996) reported a 13.8% incidence of *L. monocytogenes* in fish and shell fish from India.

The isolation and identification of *Listeria* from samples contaminated with multiple species relies on selective enrichments and subsequent biochemical analysis. These are difficult, laborious and time consuming (Bubert *et al.*, 1997). To avoid these cumbersome methods faster diagnostic method such as polymerase chain reaction (PCR) protocols are useful and they are more rapid than conventional methods. In addition to this, there is scope for automation (Cheesbrough, 1993). In view of all these in the present research work molecular method such as PCR has been proposed to determine the per cent incidence of *L. monocytogenes* with the following objectives.
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- To know the per cent incidence of *Listeria monocytogenes* in milk and dairy products.
- To know the per cent incidence of *Listeria monocytogenes* in sea foods.
- To standardize PCR technique for quick detection of *Listeria monocytogenes* in dairy and sea foods.
- To test the diagnostic kit for routine screening for *Listeria monocytogenes* in dairy and sea foods.