Listeria monocytogenes has been recognized as a human pathogen since 1929, food-borne outbreaks and ensuing concern about food safety have brought this bacterium into the spotlight. In this chapter the history of listeriosis is discussed, this is followed by discussion on etiology, listeric infection, virulence factors, epidemiology and isolation, transmission, prevalence, diagnosis, identification, pathogenicity and antibiotic sensitivity pattern of the organism.

2.1 Early History:

The official discovery of L. monocytogenes dates back to 1924, when Murray et al., (1926) isolated L. monocytogenes as the etiological agent of a septicemic disease affecting rabbits and guinea pigs in their laboratory at Cambridge in England. The first case of human listeriosis was reported in 1929 in Denmark (Nyfelt, 1929). However, the first recorded culture of L. monocytogenes was reported in France from a patient with meningitis (Dumont and Cotonii, 1921). L. ivanovii formerly known as L. monocytogenes serotype 5 was first isolated in Bulgaria in 1955 from lambs with congenital listeriosis (Ivanov, 1962). Human cases of L. ivanovii infection are rare (Cummins, 1994). The vast majority of reported isolation of these species is from abortions, stillbirth and neonatal septicemias in sheep and cattle (Alexander et al., 1992; Dennis, 1975). A third species L. seeligeri, although has been implicated in at least one case of human listeriosis (Rocourt et al., 1986) but is considered nonpathogenic (Rocourt and Grimont, 1983).

Infectious diseases are the leading cause of global disease burden with high morbidity and mortality especially in the developing world (Chugh, 2008). During the last three decades, L. monocytogenes has become increasingly important as a food-associated pathogen to human and animals, and most European Union countries have an annual incidence of human listeriosis ranges from 2 to 4 reported cases per million (Jemmi and Stephan, 2006). According to the reports of Center for Disease Control and Prevention, the
annual incidence of listeriosis in the United States declined from 7.9 cases per million in 1989 (or 1,965 cases and 481 deaths) to 4.4 cases per million in 1993 (or 1,092 cases and 248 deaths) (Tappero et al., 1995). Of more than 600 invasive listeriosis cases identified through this surveillance system, 32% occurred in association with pregnancy, affecting pregnant women, newborns, or both. No pregnant women died from listeriosis, but 22% of pregnancy-associated cases resulted in either fetal loss or neonatal death. Among nonperinatal cases, 28% were fetal. (Siegman-Igra et al., 2002)

Studies in United States, Europe and Australia demonstrated that the vast majority of patient with nonperinatal listeriosis have immunosuppressive conditions (Schuchat et al., 1992., Skogberg et al., 1992; Paul et al., 1994). A summary of 1,808 reported cases from 1067 to 1999 showed that 74% of the patients were immunosuppressed (Siegman-Igra et al., 2002). The most common predisposing conditions include immunosuppressive therapy, malignancy, diabetes mellitus, and organ transplantation and in some populations acquired immunodeficiency syndrome (Schlech et al., 1983). Although listeriosis is not common among patients with acquired immunodeficiency syndrome, such persons had approximately 280 times the risk for listeriosis of the general population in the area before highly active antiretroviral therapy (Schuchat et al., 1992).

Geographic differences have been observed in the incidence of perinatal listeriosis within the United States (Tappero et al., 1995; Schuchat et al., 1992). The rate of perinatal listeriosis in Los Angeles Country has been consistently higher than the rate in other surveillance areas. This geographic variation may reflect differences in dietary habits, host susceptibility or enhanced diagnosis by obstetric providers in Los Angeles. The Centers for Disease Control and Prevention surveillance project did not find clear evidence of a seasonal pattern, although the reports of Bayles et al., (1996) have observed a peak of listeriosis during the summer month.

Investigations of several outbreaks of listeriosis have enhanced understanding of the epidemiology of this disease. In 1981, an outbreak in Nova Scotia, Canada was caused by locally produced coleslaw. Investigation of this outbreak first established listeriosis as a food-borne illness (Schlech et al., 1992). The epidemiologic investigation of a 1983 outbreak of listeriosis in Massachusetts implicated a particular brand of pasteurized milk (Fleming et al., 1985). The largest outbreak in the United States involves 142 cases in Los Angeles in 1985, the result of consumption of Mexican-style cheese made from contaminated milk that was not adequately pasteurized. Several large outbreaks in Europe
were associated with ready-to-eat foods like soft cheese, pate, and pork tongue in jelly (McLauchlin et al., 1991; Goulet et al., 1993). One outbreak in 1997 in Italy resulted in gastroenteritis in 1,566 of 2,189 (72%) exposed by consumption of a contaminated corn salad; 292 (19%) were hospitalized (Aureli et al., 2000). Evidence shows that there are many cases of small and large outbreak or infection of listeriosis whole over the world in each and every year resulting by consumption of contaminated foods.

The disease has been reported in animals from Indian subcontinent, usually in the form of sporadic cases but occasionally as outbreaks. In India, works conducted by researchers showed prevalence of this pathogen in goat and sheep meat (Barbuddhe et al., 2000; Bhamu Rekha et al., 2006), poultry meat and poultry meat products (Mahmood et al., 2003; Barbuddhe et al., 2010), milk and milk products (Rawool et al., 2007a; D’Costa et al., 2010; Barbuddhe et al., 2010).

2.2 The Etiology:

The listeric infection, caused by microorganisms of the genus Listeria, occurs worldwide and in variety of animals including man. It comprises of six species namely L. monocytogenes (haemolytic), L. innocua (non-haemolytic), L. ivanovii (haemolytic), L. seeligeri (haemolytic) and L. welshimeri (non-haemolytic) and L. grayi (non-haemolytic) of which only L. monocytogenes and L. ivanovii are pathogenic (Liu, 2006). Recently two more species have paved their way in this genus i.e., L. marthii (Graves et al., 2009) and L. rocourtiae (Leclercq et al., 2009), however both of them are non-pathogenic. L. ivanovii, the other pathogenic species of genus Listeria has been thought to be frequently associated with abortions in sheep (Sergeant et al., 1991), and in cattle (Alexander et al., 1992; Gill et al., 1997). L. ivanovii infection in humans is although rare, but there are reports of isolation from cases of AIDS (Cummins et al., 1994) and abortion (Elischlerola et al., 1990). The remaining species are regarded as non-pathogenic (Low and Donachie, 1997).

2.3 Pathogenesis of Listeric infection:

L. monocytogenes is a facultative intracellular pathogen able to survive in macrophages and to invade a variety of nonphagocytic cells, such as epithelial cells, hepatocytes and endothelial cells (Cossart et al., 1995). The pathogenesis cycle of L. monocytogenes is portrayed in. In brief, L. monocytogenes uses the cellular machinery to move around inside the host cell. The most common mode of transmission is by ingestion,
beside there are other modes of transmission which includes handling of aborted fetus, nosocomial infection and vertical transmission to fetus. Upon ingestion by the host via contaminated food, the pathogen withstands exposure to host proteolytic enzymes, the acidic stomach environment (pH 2.0), bile salts and non-specific inflammatory attacks, largely through the actions of several stress-response genes (oupA, lmo1421 and bsh) and related proteins (Liu, 2006). Having survived this initial stage, the pathogen adheres to and is internalized by host cells with the assistance of a family of surface proteins called internalins (Gaillard et al., 1991). The most notable internalins are InlA and InlB. The InlA (an 88 kDa protein encoded by inlA) interacts with E-cadherin to mediate L. monocytogenes entry into epithelial cells, whereas, InlB (a 65 kDa protein encoded by inlB gene) recognizes C1q-R (or Met) to facilitate L. monocytogenes entry into a much broader range of host-cell types, including hepatocytes, fibroblasts and epithelioid cells(Liu, 2006).

Gaining entry to host cells enables L. monocytogenes to evade host immune surveillance functions (Vazquez-Boland et al., 2001). Following its uptake by host cells, L. monocytogenes is primarily located in single-membraned vacuoles. Two virulence-associated molecules are responsible for lysis of the primary single-membraned vacuoles and subsequent escape by L. monocytogenes: listeriolysin O (LLO) and phosphatidylinositol-phospholipase C (PI-PLC). The LLO (a 58 kDa protein encoded by hly) is a pore-forming, thiol-activated toxin that is essential for L. monocytogenes virulence (Portnoy et al., 1992). The PI-PLC (a 33 kDa protein encoded by plcA gene), acting in synergy with phosphatidylycholine-phospholipase C (PC-PLC, a 29 kDa protein encoded by plcB), aids LLO in lysing the primary vacuoles (Vazquez-Boland et al., 2001).

After lysis of the primary single-membraned vacuoles, L. monocytogenes is released to the cytosol, where it undergoes intracellular growth and multiplication. The intracellular mobility and cell-to-cell spread of L. monocytogenes require another surface protein, ActA (a 67 kDa protein encoded by actA), which is cotranscribed with PC-PLC and mediates the formation of polarized actin tails that propel the bacteria toward the cytoplasmic membrane. At the membrane, bacteria become enveloped in filopodium-like structures that are recognized and engulfed by adjacent cells, resulting in the formation of secondary double-membraned vacuoles. A successful lysis of the secondary double-membraned vacuoles signals the beginning of a new infection cycle, which is dependent on PC-PLC upon activation by metalloprotease (60 kDa encoded by mpl) (Vazquez-Boland et al., 2001). Once L. monocytogenes crosses the intestinal barrier, cells are
dispersed into the host carried by the lymph or blood, reaching the mesenteric lymph nodes, the spleen and the liver (Lecuit et al. 2005).

The minimum dose required to cause clinical infection in humans has not been determined but the large numbers of *L. monocytogenes* detected in food responsible for epidemic cases of listeriosis suggest that it is high, 106 CFU/g. Nevertheless, low doses can not be excluded in so far as they too may cause infection, especially in the high risk group. Levels of contamination as low as 102 or 103 CFU/g have been linked with clinical cases. The infection dose may vary depending on the virulence of the strain and the host factors.

Junittila et al., (1988) and Walker et al., (1996) reported that *L. monocytogenes* has a minimum growth temperature estimated to be just below 2°C. Leimeister-Wachter et al., (1991) found that low temperature have no significant effect on the production of virulence factor by *L. monocytogenes*. A major emphasis of recent studies has been placed on the increased expression on bacterial cold shock proteins (Csp's) in response to reduced temperatures (Panoff et al., 1998)

### 2.4 Virulence gene cluster of pathogenic *Listeria* spp:

There are six virulence genes (*prfA, plcA, hlyA, mpl, actA and plcB*), responsible for key steps of *L. monocytogenes* intracellular parasitism are physically linked in a 9-kb chromosomal island formerly known as the *hly* or *prfA*-dependent virulence gene cluster (Chakraborty, et al., 2000 and Kreft et al., 1989) and now referred to as LIPI-1 (*Listeria Pathogenicity island 1*) (Vazquez-Boland et al., 2001). Apart from the above mentioned virulence gene cluster, a major 60-kDa extra-cellular protein i.e., p60, encoded by *iap* (invasive associated protein) gene plays a vital role in intestinal invasion and *in vivo* survival (Hess, et al., 1996). Beside these, the internalin genes have also shown their diagnostic potential as virulence associated genes (Bierne et al., 2007).

#### 2.4.1 *mpl* (also called ORFD and *prtA*):

The first gene of the lecithinase operon (Vazquez-Boland et al., 1992) encodes a protein which contains significant amino acid homology to a family of metalloproteases, of which thermolysin is the prototype (Domann et al., 1991; Mengaud et al., 1991). A polypeptide corresponding to the mature form of the metalloprotease was detected with antiserum raised against thermolysin, but proteolytic activity has yet to be conclusively
demonstrated (Domann et al., 1991). Mutants with transposon insertions in \textit{mpl} are of reduced virulence and are reduced in lecithinase production (Mengaud et al., 1991, Raveneau et al., 1992). Interestingly, these mutants express only the 32-kDa form of the lecithinase polypeptide, suggesting that the metalloprotease may proteolytically process the lecithinase. Transposon insertions in \textit{mpl} exert a partial polar effect on the expression of \textit{plcB} because transcription in this operon also proceeds from a second promoter located downstream of \textit{mpl} (Vazquez-Boland et al., 1992).

### 2.4.2 \textit{actA} (also called \textit{prtB}):

\textit{actA} is the second gene of the lecithinase operon (Vazquez-Boland et al., 1992). The nucleotide sequence predicts a 639-amino-acid protein with a signal sequence and a membrane anchor. \textit{actA} mutants do not express lecithinase, do not form plaques in monolayers of mouse fibroblasts, and do not nucleate the polymerization of actin filaments (Domann et al., 1993). Gene disruption of \textit{plcB} and transformation of the \textit{actA} mutant strain with \textit{actA} on a plasmid have shown that the \textit{actA} gene product is a surface protein necessary for \textit{L. monocytogenes} actin assembly (Kocks et al., 1992). These data also indicate that the mature gene product is a 610-amino-acid protein with an apparent molecular mass of 90 kDa. Whether \textit{actA} encode an actin nucleator or another function is not yet known.

### 2.4.3 \textit{plcA} (also called ORFU and \textit{pic}):

Pathogenic \textit{Listeria} spp. produces three different enzymes with phospholipase C (PLC) activity, which is involved in virulence. Two of these enzymes namely \textit{plcA} and \textit{plcB} are present in \textit{L. monocytogenes} and \textit{L. ivanovii}; while the third one, \textit{sphingomyelinase C (smcL)} is specific to \textit{L. ivanovii}. The \textit{plcA} has only a minor individual role in virulence but acts synergistically with \textit{plcB} and \textit{mpl}, which themselves also have an accessory role in virulence to achieve, in conjunction with LLO, for optimal levels of escape from primary and secondary phagosomes (Vazquez-Boland et al., 2001).

\textit{plcA} gene is adjacent to \textit{hly} and transcribed divergently which is encoded as phosphatidylinositol-specific phospholipase C (PI-PLC) (Leimeister-Wachter et al., 1991; Mengaud et al., 1991a; Sun et al., 1990). The \textit{plcA} sequence predicts a protein with approximately 30% amino acid identify to \textit{Bacillus thuringiensis} and \textit{Bacillus cereus} PI-PLC. Interestingly, other gram-positive bacteria such as \textit{Staphylococcus aureus},
Clostridium novyi and Bacillus anthracis also secrete PI-PLC activity (Low, 1990). plcA insertion mutants of L. monocytogenes are clearly of reduced virulence (Camilli et al., 1991; Mengaud et al., 1991a), but the polar effect of these mutations on the downstream regulatory gene prfA (Mengaud et al., 1991) makes a definitive assignment of a role for PI-PLC premature. It should be noted that only pathogenic species in the genus Listeria reveals PI-PLC activity (Leimeister-Wachter et al., 1991; Mengaud et al., 1991a and Notermans et al., 1991b).

2.4.5 plcB (also called prfC):

L. monocytogenes isolates produce one or both of two distinct types of reaction on egg yolk agar, either a faint halo or a very dense zone of opacity surrounding the colony (Fuzi and Pillis, 1962). The former reaction may be due to the PI-PLC, as it is absent in plcA mutants, while the latter reaction is due to the secretion of a broad-spectrum phospholipase C which hydrolyzes phosphatidylcholine (lecithin), hence its designation as a lecithinase (Geoffroy et al., 1991; Leighton et al., 1975). Strains of L. monocytogenes which express high amounts of lecithinase activity secrete polypeptides of 29 and 32 kDa, both of which exhibit lecithinase activity on egg yolk overlays of renatured sodium dodecyl sulfate-polyacrylamide gels (Kathariou et al., 1990). The sequence of the gene encoding this enzyme, plcB (Vazquez-Boland et al., 1992), predicts a polypeptide of 289 amino acids with sequence similarity to the phosphatidylcholine-phospholipases C of B. cereus and Clostridium perfringens (alpha-toxin), with a signal sequence of 25 amino acids and by analogy with the B. cereus enzyme, a putative propeptide of 26 amino acids. plcB mutants have been constructed by interruption of the gene through the use of thermosensitive plasmids (Kocks et al., 1992). These mutants express no lecithinase activity and make small plaques on 3T3 fibroblast monolayers. Electron microscopic analysis of these plcB mutants suggest that the lecithinase might be involved in lysis of the double-membrane vacuole which is formed during cell-to-cell spread (Vazquez-Boland et al., 1992).

2.4.6 prfA (Master Regulator of Virulence):

prfA is the only regulator identified to date in Listeria spp., and is directly involved in the control of virulence gene expression. This protein is the switch of a regulon including the majority of the known listerial virulence genes. However, the expression of
some listerial virulence genes is totally independent of prfA, as in the case for the smcL
gene of *L. ivanovii* and the inl GHE internalin locus of *L. monocytogenes* (Vazquez-
Boland et al., 2001). The hly gene, which is primarily prfA dependent, has an alternative
means of expression via a weak prfA independent promoter (Domann et al., 1993). This
explains why *L. monocytogenes* prfA deletion mutants have weak hemolytic activity
(Domann et al., 1993 and Ripio et al., 1997) and low-level expression of hly is detectable
in *L. monocytogenes* in conditions of prfA down regulation, such as extracellular growth
in broth culture.

A spontaneous non-hemolytic mutant of *L. monocytogenes* was shown to have a
deletion in a region downstream of plcA (Gormley, et al., 1989; Leimeister-Wachter and
Chakraborty, 1989). Mutants with transposon and site-specific integration mutations in the
prfA gene or its promoter region were defective in the expression of the plcA, plcB, hly
and mpl gene products (Chakraborty et al., 1992 and Mengaud et al., 1991b).

2.4.7 hly (also called hlyA and lisA):

Listeriolysin O (LLO), a bacterial pore-forming toxin essential for lysing the
vacuolar membrane and allowing *L. monocytogenes* to escape into the cytoplasm of the
cell, is encoded by the hly gene (Ellin Doyle, 2001). However, spontaneous loss of
hemolysin production results in avirulence (Hof, 1984) and led various other groups to
generate isogenic hemolysin mutants of *L. monocytogenes* by transposon mutagenesis.
These mutants were much less virulent in mice (Gaillard et al., 1986; Kathariou et al.,
1987 and Portnoy et al., 1988). Moreover, LLO–defective, hlyA negative and LLO
defective wild type mutants could not survive in macrophages (Cossart et al., 1995;

A recent report has also shown that *L. monocytogenes* mutants expressing a PEST-
deleted hly allele, although fully hemolytic, are strongly impaired in the ability to escape
from the phagocytic vacuole. PEST-like sequence present at the N terminus of LLO and
absent in PFO is responsible for the rapid degradation of the listeriae toxin in the host cell
cytosol (Decatur and Portnoy, 2000). This suggests that the PEST motif in LLO may also
play a specific role in the disruption of the phagosomal membrane e.g., by serving as a
ligand for targeting to that membrane other factors required for its efficient disruption
(Lety et al., 2001).
2.4.8 *iap* (invasive associated protein):

All isolates of *L. monocytogenes* secrete a protein of 60 kDa as a major extracellular product (Kuhn and Goebel, 1989) encoded by the *iap* (invasive associated protein) gene. Spontaneous rough mutants of *L. monocytogenes* show reduced expression of p60 and form long chains, which possess double septa between the individual cells. These rough mutants show a decrease in invasiveness but are relatively normal in intracellular growth and polymerization actin filaments (Sun et al., 1990).

The expression of *iap* is independent of *prfA* (Bubert et al., 1997 and 1999) and is controlled at the post transcriptional level (Kohler et al., 1990). The p60 homologous protein are synthesized by all *Listeria* spp. but in each species there are specific differences in amino acid sequence that can be used for identification purposes in PCR based and immunological assays (Bubert et al., 1994).

2.4.9 *Inl* (Internalins):

Internalins are family of virulence associated genes in *Listeria* spp. (Vazquez Boland et al., 2001). The *InlA* and *InlB* characterized first, encoded by *InlAB* operon identified in *L. monocytogenes*. An element common to all Internalins is a leucine rich repeat (LRR) domain consisting of leucine residues in fixed position. LRR protein, less frequent in prokaryotes and most are virulent factors like *TopM* of *Yersinia pestis* and *Y. pseudotuberculosis*, *IpaH* of *Shigella flexneri*, and filamentous hemagglutinin of *Bordetella pertussis* (Vazquez Boland et al., 2001). The *InlC* also is known as *IrpA* (Internalin related protein A). It is a 30 KDa small secretory protein which is linked to virulence of *L. monocytogenes* and *L. ivanovii*. The *InlC* gene was discovered in a *prfA* enriched/*prfA* regulated gene cluster knockout of *L. monocytogenes* (Engelbrecht, 1996). The purification and extraction method for *InlC* protein had been available (Lingnau et al., 1996); however, the receptor for this protein and its exact function is not clear till date. Mass production of recombinant protein of InlC helped in revealing the basic structure of *InlC* by crystallography studies, which showed that it is small, flatter and more hydrophilic protein consisting of α-helical cap, a leucine rich repeat (LRR) and immunoglobulin like domain (Ooi, 2006). It is less polymorphic than other internalins proving its conserved regions valuable for diagnostics. Besides this, *InlC* protein has been found to elicit humoral immune system in human (Engelbrecht, 1996). Recently, a *InlC*-based multiplex PCR has been developed, which helped in identification of virulent strains of *L.
monocytogenes and L. ivanovii recovered from clinical cases (Liu et al., 2007). In another study on ewes experimentally infected with $10^{10}$ CFU dose of L. monocytogenes, the IniC-based ELISA had shown two-fold antibody response against IniC than that detected against the LLO by LLO-based ELISA, indicating that IniC is more immunogenic than LLO (Zundel et al., 2007).

2.5 Work on Epidemiology and Isolation:

2.5.1 Distribution and host range:

Surveillance of foodborne diseases is of an increasingly high priority in the public health arena worldwide (Suryawanshi, 2011). Bacterial contamination of food represents one of the major public health problems. In the food industry, L. monocytogenes represents an important health risk. L. monocytogenes can grow and multiply under harsh conditions, which explain its ubiquity. It has been isolated from a wide variety of produce of animal origin (Farber and Peterkin, 1991).

Ryser et al., (1996) isolated L. monocytogenes from ground beef, pork sausage, ground turkey, and chicken (160 samples) by primary enrichment in University of Vermont Broth (UVM) and Listeria Repair Broth (LRB) (30°C for 24 h) followed by secondary enrichment in Fraser broth (35°C for 24 and 40 h) and then plating on modified Oxford agar. All Listeria isolates were found to be identical on DNA analysis, and belonged to serotype 4b. Experimental contamination of sterile samples of the implicated foods showed that L. monocytogenes grew on corn when kept at least 10 hrs. at 25°C.

L. monocytogenes was frequently isolated from unfermented dairy products, cheese and other dairy products (Ryser, 1999), meat products (Farber and Peterkin, 1999), poultry and egg products (Cox et al., 1999), and fish and seafood products (Jinneman et al., 1999). Bhilegaonkar et al., (1997) screened 121 milk samples comprising of 66 raw cattle milk, 35 pasteurized bulk tank milk and 20 non-pasteurized bulk tank milk samples and recovered 16 listerial isolates. Of which 7 were L. monocytogenes.

Chaudhari (1997) processed 130 raw buffalo meat samples collected from slaughterhouse for prevalence of L. monocytogenes by employing two step enrichment in UVM-I, UVM-II and subsequently DRIA as selective media. The worker reported 3.08% prevalence of L. monocytogenes.

were recovered of these, 26 (67%), 08 (21%), 03 (8%) and 02 (5%) were *L. monocytogenes*, *L. seeligeri*, *L. grayi* and *L. welshimeri*, respectively.

Kaur et al., (2010) processed 333 samples (blood, faecal and genital swab) collected from 111 female animals (cattle, buffaloes, ewes and goats) employing combination of UVM (I, II) and DRIA. The workers reported isolation of *Listeria* spp. from 19.8% samples whereas two isolates of *L. seeligeri* were obtained from 23 goats.

The available current literature shows that *L. monocytogenes* has been reported from a wide variety of animals and their product from various part of the world (Farber et al., 2000 and Dhanashree et al., 2003). Reports also indicate that listeriosis is dreadful emerging disease in under developed countries rather than in developed countries (WHO, 1988).

Frederick et al., (1996) reported the occurrence of *L. monocytogenes* in soil and water. Vegetable can become contaminated from soil and from contaminated manure used as fertilizer. Warke et al., (2010) analyzed a total of 417 samples comprising of eleven different types of fresh leafy vegetables collected from supermarkets and local markets in and around Nagpur. The workers stated prevalence of *L. monocytogenes* to the tune of 7.31% among the samples.

Animals can carry the bacteria without becoming ill and can contaminate the foods of animal origin such as meat and dairy products. These bacteria have been found in a variety of raw foods, such as uncooked meats and vegetables, as well as in the processed foods that become contaminated after processing, such as soft cheese and cold cuts at the counters. Raw milk or products made from unpasteurized milk may contain the bacteria. Gogate and Deodhar, (1981) also suggest that babies could be born with listeriosis, if the mother had eaten contaminated food during pregnancy.

Surveillance of foodborne diseases is of an increasingly high priority in the public health arena worldwide (Suryawanshi, 2011). *L. monocytogenes* can grow and multiply under harsh conditions, which explain its ubiquity. It has been isolated from numerous sites, including soil, sewage water and decaying plant material (especially poorly fermented silage). The exposure to this bacterium is unavoidable and it has been found even in the gut of normal healthy people up to five per cent or more (Association of Medical Microbiologists, 1995). The reported incidence of human listeriosis has, of late, increased in developed countries due to factors such as increased awareness, diagnosis and better monitoring systems in these countries. The prevalence of listeriosis is greater among
individuals with immunocompromised status, young age, elderly and persons who are immune-suppressed as a result of medication or illness (WHO, 1988). Listeriosis has been reported to occur in children suffering from malnutrition, congenital heart disease and digestive failure (Gupta et al., 1997).

Bacterial contamination of food represents one of the major public health problems. In the food industry, L. monocytogenes represents an important health risk. The public health significance of L. monocytogenes as a major pathogen lies in its ubiquitous nature (Low and Donachie, 1997) and wide host range, which includes 40 mammals, 20 birds, crustaceans, ticks and fishes (Sonnenwirth, 1980). L. monocytogenes has been isolated from various animals like sheep, goat, cattle, swine, dog, cat and horses besides rodents and birds (Gray and Killinger, 1966; Topley and Wilson, 1990). However, many wild and domestic animal species such as sheep, goat, buffalo, cattle, pig, poultry, bird and fish are intestinal carriers of listeriae.

2.6 Transmission:

The faeces of healthy animals often contain L. monocytogenes, so cross contamination with pathogenic species is likely to occur. Excretion of the organisms in the faeces of sheep and goats has been reported from days 2 to 28 after experimental infections (Gupta et al., 1980). Application of faeces or dung slurries of infected animals onto agriculture land as manure can serve as source of the organism (Chukwu et al., 2006; Nicholas et al., 2000). It could be transmitted to healthy animals while browsing and human through contaminated vegetables or fruit (Schlech et al., 1983; Chukwu et al., 2004).

Milk can also be source of contamination for environment and other milk products. Female sheep and goats have been reported to excrete the organisms through vagina after abortion and also in milk for 2 days. (Gupta et al., 1980). However, the main route of transmission of pathogen is by ingestion of food or water contaminated with faeces, urine, soil and/or aborted material from infected animal (Sharma et al., 1996)

In humans, food borne transmission is the prominent means of infection, poor hygiene and environmental conditions, or continuous exposure to domestic animals at abattoirs, may act as a source of infection (Barbuddhe et al., 1999). The disease has been reported from women with poor obstetrics, history of abortions (Bhujwala and Hingorani, 1975).
2.7 Prevalence of listeriosis:

2.7.1 Human:

*L. monocytogenes* is one of the most important food borne bacterial pathogen due to its high mortality rate of 20-30% and severity of disease, particularly among the pregnant women, neonates and immunocompromised adults (Esteban et al., 2009). The mortality rate may even be higher, and reach up to 30-40% among neonates and immunocompromised individuals (Farber and Peterkin, 1991). Almost all cases (about 98%) of human listeriosis and 85% of animal cases are due *L. monocytogenes* (Mc Lauchlin, 1987), although rarely, *L. seeligeri* and *L. ivanovii* have also been implicated (Palmer et al., 1998). In humans, *L. ivanovii* infection is rare and has been reported from a patient with AIDS (Cummins et al., 1994) and in one case of abortion from Slovakia wherein small wild animals were thought to be the reservoirs (Elischerola et al., 1990).

*L. monocytogenes* infection in human results in meningitis or encephalitis. Usually prodromal symptoms such as headache, vomiting, fever and malaise may occur before the appearance of focal signs of central nervous system (CNS) infection. In pregnant women, the pathogen can cause bacteremia, and stillbirth or premature birth (Vazquez-Boland et al., 2001).

Unlike the *L. monocytogenes* serovars 4a and 4c, which are rarely associated with outbreaks of disease, *L. monocytogenes* serovars 1/2a, 1/2b and 4b are responsible for 98% of documented human listeriosis cases (Mc Lauchlin, 1987; Wiedmann et al., 1996; Jacquet et al., 2002). Of these, the *L. monocytogenes* serotypes 1/2a and 1/2b have been linked to only sporadic cases whereas *L. monocytogenes* serotype 4b has been isolated from approximately 40% of sporadic cases as well as many epidemics (Wiedmann et al., 1996; Rocourt and Bile, 1997).

Reports on human listeriosis in India are scanty with only two recorded cases of listeriosis in humans (Rocourt, 1991) and less than ten published papers on the isolation of the pathogen from women with spontaneous abortions, miscarriage or bad obstetric history (Malik et al., 2002; Kaur and Malik, 2007a; Kaur et al., 2007b; 2009). The pathogen has been recovered from 02/61 (3.20%) cases of spontaneous abortions in women, who showed a seropositivity of 16% in LLO-based ELISA (Kaur et al., 2006). The disease seems to be grossly under-diagnosed primarily due to the lack of awareness about the disease, failure to appreciate it as a probable cause of abortion and meningitis, and
inadequate diagnostic facilities equipped with rapid and reliable diagnostic tests (Barbuddhe and Malik, 2009). The seroprevalence against listeriosis in humans has been reported to range from a low of 4.38% in a general population by trypsinized antigen based-tube agglutination test (Dutta and Malik, 1978) to a high of 49.2% in abattoir personnel by LLO-based indirect-ELISA (Barbuddhe et al., 1999).

2.7.2 Animal:

Overall mortality in animals is as high as 30% in listeriosis accounting for huge loss to animal husbandry (OIE, 2008). Economic losses of listeriosis in United States of America amount to $ 480 million per year. L. ivanovii, the other pathogenic species of genus-Listeria has been thought to be frequently associated with abortions in sheep (Sergeant et al., 1991), and in cattle (Alexander et al., 1992; Gill et al., 1997).

L. monocytogenes is a well-recognized cause of abortion, encephalitis and septicaemia in animals (Radostits et al., 2000). L. ivanovii has also been implicated as a cause of abortion in sheep and cattle but occurs less frequently than L. monocytogenes (Ivanov, 1962; Macleod et al., 1974; Sergeant et al., 1991; Alexander et al., 1992) and is extremely rare as a cause of other conditions (Low and Donachie, 1997).

2.8 Abortions:

Listeric infections and abortions usually develop in the late winter or early spring. Abortions are most commonly recognized in the last trimester of pregnancy, frequently in the absence of other clinical signs (Kapur and Sadana, 1974; Jubb et al., 1985).

2.8.1 Sheep and goats:

World over, abortions in sheep and goats are usually associated with L. monocytogenes and L. ivanovii, the latter being implicated as the most frequent cause in case of sheep (McLauchlin, 1987), with abortion rates ranging from 1.65% (Alexander et al., 1992) to 12% (Sergeant et al., 1991). L. monocytogenes infection in these species is normally low but may reach as high as 15% (Blood et al., 1979). Cases of listeric abortions in sheep are high during February and March due to seasonal lambing. In U.K., listeric abortion is most common in sheep (Anon, 1992) and in exceptional cases approximately 50% of the flock has been affected (Low and Renton, 1985).

In India, the first authentic case of listeric abortion in any species of domesticated animal was reported by Dhanda and co-workers (1959) in an ewe. Under experimental
conditions the \textit{L. monocytogenes} serotype 4 has been shown to cause abortion and mortality up to 33% in sheep and 3.33% in goats (Gupta et al., 1980). In Himachal Pradesh, migratory flocks of 216 sheep and 331 goats with history of abortions revealed \textit{L. monocytogenes} from vaginal swabs of 8 sheep and 11 goats whereas \textit{L. ivanovii} could only be isolated from 2 sheep but not from goats (Sharma et al., 1996). \textit{L. ivanovii} has also been isolated from an outbreak of abortions in a flock of 254 crossbred ewes (Chand and Sadana, 1999). In Himachal Pradesh, screening of goats with abortion revealed association with \textit{L. monocytogenes} and \textit{L. ivanovii} in 8.3% cases for each, while ewes showed such association in 9.1% and 4.6% cases, respectively (Nigam, et al., 1999a).

\subsection*{2.8.2 Bovines:}

\textit{L. monocytogenes} is a well-recognized cause of abortion, encephalitis and septicaemia in cattle. In France, recovery of the pathogen has been reported from 4.31% cases (Guilloc, 1979), and also from 3% of foetal and 6% of placentals samples out of 89 cases of bovine abortions (Ramisse et al., 1986). Out of 30,000 bovine abortions occurring annually in U.K., 0.05 to 0.13% have been attributed to listeriosis alone (Anon, 1994). Bovine abortion due to \textit{L. ivanovii} has been reported in 4 cases from U.S.A. (Alexander et al., 1992), and Australia (Gill et al., 1997).

In India, the isolation of \textit{L. monocytogenes} has been reported from aborted fetuses from a cow (Dutta and Malik, 1981) and two cases in buffaloes (Dutta and Malik, 1981; Shakuntala, 2004). Of late, isolation of \textit{L. ivanovii} from a case of abortion in a cow has been reported from Bareilly, U.P. (Thakur, 2000).

\subsection*{2.8.3 Equines:}

Cases of equine abortion attributable to \textit{L. monocytogenes} are relatively rare and include one report due to serotype 4b from U.S.A. (Welsh, 1983). However, no such report from India is available.

\subsection*{2.8.4 Porcine:}

In India, few epidemics of listeriosis in swine herds leading to abortions and circing syndrome have been reported (Dutta and Malik, 1981; Rahman et al., 1985 and Dash et al., 1998).
2.9 Repeat breeding/Infertility cases:

2.9.1 Sheep and goats:

Information on listeric infection as cause of repeat breeding or infertility in sheep and goats is largely lacking barring few reports such as isolation of *L. ivanovii* from endometritis cases in sheep and goats from Himachal Pradesh in India (Mahajan and Katoch, 1997). The seroprevalence for listeriosis in infertile does and ewes in this region has been found to be 24.07 and 18.30%, respectively (Nigam et al., 1999b).

2.9.2 Bovines:

In India, *L. monocytogenes* has been isolated from 6% of repeat breeding cows and buffaloes from Mhow (Sharda et al., 1991). Two strains of *L. monocytogenes* serotype 1c and 4b were isolated from the endometrium of infertile cows (Srivastava et al., 1985). In a recent study, haemolytic *L. monocytogenes* isolates (6) could be recovered from cases of repeat breeding (4.4%), abortion (6.6%) and endometritis (6.25%) in female buffaloes (135) with reproductive disorders in Bareilly region, however, only one of these isolates from abortion and none from retained placenta cases turned out to be of pathogenic nature (Shakuntala et al., 2006). The isolation of *L. ivanovii* from a repeat breeding buffalo has also been documented for the first time in Himachal Pradesh (Nigam et al., 1998).

2.10 Septicaemia:

The septicaemic form of listeriosis, though uncommon, tends to precede the intrauterine infection. The organism can be isolated from various lesions. It has been suggested that the pathogen may be confined to the lymph node system and Kupffer cells of the liver in later stages of the infection, resulting in negative blood cultures (Lhopital et al., 1993). Septicaemic dissemination of the organism following oral infection of buffalo calves and recovery of pathogenic *L. monocytogenes* from 1.5-1.6% blood samples of buffaloes slaughtered in Bareilly, Uttar Pradesh has been reported (Chaudhari, et al., 2004).

2.11 Faecal carriage:

The faeces of healthy animals often contain *L. monocytogenes* (Gray and Killinger, 1966; Husu, 1990) and the faecal carriage rate of *Listeria* spp. has been reported to be in the range of 3.1% to 45.8% (Gronstol, 1979; Loken et al., 1982; Husu, 1990). The faecal excretion of *L. monocytogenes* is probably through biliary route (Briones et al., 1992). The
tendency for excretion rates has been observed to be lower in grazing animals (Husu, 1990). In another study conducted in Germany, out of 1872 faecal samples, *L. monocytogenes* was isolated from 46 (33.3%) of 138 cattle, 8 (8%) of 100 hens, 8 (8%) of 100 sheep, 2 (5.9%) of 34 pigs, 19 (4.8%) of 400 horses, 4 (1.3%) of 300 dogs and 3 (0.9%) of 350 pigeons (Weber et al., 1995).

In India, 4% of the faecal swabs collected from slaughtered buffaloes showed the presence of pathogenic *L. monocytogenes* (Chaudhari, et al., 2004).

### 2.12 Diagnosis of Listeriosis:

#### 2.12.1 Isolation of *L. monocytogenes*:

Listeriae are presumptively identified based on their morphology (Gram positive cocccobacilli), reaction in catalase and oxidase tests, haemolysis on sheep blood agar (SBA), and characteristic tumbling motility at 25°C (Farber and Peterkin, 1991). But the most authentic diagnosis of listeriosis is made by isolation of the pathogen (Rocourt et al., 1983). The recovery of *L. monocytogenes* and *L. ivanovii* from food, animal/human and environmental samples requires the use of enrichment cultures followed by selective plating and where injured organisms are likely to be present, a pre-enrichment step is required (Curtis and Lee, 1995). The pathogen can be isolated from clinical specimens, such as blood, cerebrospinal fluid (CSF), the meconium of the newborn or foetus in abortion cases, faeces, vomitus, food stuffs/animal feed and/or vaginal secretions of infected individuals. Diagnosis should be made from aborted fetal tissues or blood from febrile period (Gupta et al., 2003). Higher percentages of isolations are obtained within the first 10 days after birth. The endocervical exudate provides 33% more isolations than the vaginal (Gomez-Mampaso et al., 1980).

Various enrichment and selective media for detection of *Listeria* spp. have been reviewed by Curtis and Lee (1995). To isolate the organism, two methods were developed in United States, the Food and Drug Administration (FDA) method (Lovett et al., 1987; Lovett and Hitchens, 1988) and United States Department of Agriculture (USDA) method (Mc Clain and Lee, 1988).

However, a third method, Netherlands Government Food Inspection Service (NGFIS) was developed which involves 48 hours enrichment in L-PALCAMY (Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk) enrichment broth at 30°C followed by plating on to PALCAM agar (Hayes et al., 1992).
The recovery rates of the USDA and NGFIS were 74% each while FDA showed 65% recovery. However, the combination of USDA and NGFIS gave greatest recovery (91%) (Farber, 1993).

2.12.1.1 Enrichment procedure/media:

Earliest method available was the cold enrichment technique (Gray et al., 1948). Its chief drawback was the need to make subcultures from the broth at intervals up to several months to even a year. Early attempts at isolation of *Listeria* within days resulted in tryptose phosphate broth with polymixin (Bojsen-Moller, 1972) and Levinthal broth with trypaflavine and nalidixic acid (Ralovich et al., 1972).

Increased interest in *L. monocytogenes* as a food pathogen led to the development of many selective enrichment broths like *L-PALCAMY* (Van Netten et al., 1989), (Lovett et al., 1987) University of Vermont-I (UVM-I) (Donnelly and Baigent, 1986), University of Vermont-II (UVM-II) (McClain and Lee, 1988) and Fraser broth (Fraser and Sperber, 1988).

The use of complete UVM, a two step procedure employing UVM-I and UVM-II has found to be more effective than that of Holman medium or UVM-I alone (Ralovich, 1989). UVM and Listeria enrichment broth (LEB) broth allow better recovery of heat-injured cells of *L. monocytogenes* (Bailey et al., 1990).

2.12.1.2 Selective or differential plating media:

The first significant step in producing a selective agar for *Listeria* was by incorporating potassium tellurite to inhibit Gram-negative organisms (Gray et al., 1950). Subsequently in 1960, Mc Bride and Girard who added lithium chloride, glycine and blood to phenyl ethanol agar developed another selective agar.

Ralovich et al. (1971) published the first truly selective agar using trypaflavine and nalidixic acid in a serum agar base. Various selective agents have been proposed for isolation of *L. monocytogenes* from food samples (Donnelly, 1988). Listeria selective agar of Domínguez-Rodriguez (LSAM) has been found superior with 100% recovery of *Listeria* over plating on McBride agar, which has a recovery rate of only 50-60% (Fernandez-Garyazabal, 1990).
2.12.2 Differentiation/Identification of *Listeria* species:

In the interests of public safety and for consideration of public health microbiology, all *L. monocytogenes* should be regarded as potentially pathogenic (McLauchlin, 1997). Food regulatory agencies in a number of countries have adopted standards for the absence of *L. monocytogenes* in specific food products (Shank et al., 1996). Hence, it is important to be able to correctly identify different *Listeria* species, or at least differentiate *L. monocytogenes* from the remainder of the genus (McLauchlin, 1997).

Conventional methods for the identification of *Listeria* spp. have relied on the results of fermentation of sugars and hemolytic reactions (Seeliger and Jones, 1986), and the API *Listeria* identification kit based on some of these tests is commercially available (Bille et al., 1992). Problems have been described in the interpretation of hemolytic reaction, particularly CAMP test (Schuchat et al., 1991; Fernandez-Grarayzabal et al., 1996), and although generally working well, the API *Listeria* kit has been reported to produce occasional discrepant results (Bille et al., 1992), API 20E, AP 20 STREP, API 50CH (Rocourt et al., 1983; Kerr et al., 1990) have been evaluated. These strips often yield good results regarding genus identification, but these were not found fully adequate at the species level (Bille et al., 1992; Chen and Chang, 1996).

A rapid and inexpensive identification scheme has been proposed by Lachica (1990), which relies on the detection of hemolysin and on two carbohydrate (L-rhamnose and D-xylose) fermentation tests done on agar plates with one isolated colony growing on selective lithium chloride-ceftazidime agar plate after 40 hrs of incubation. Similarly, use of agar-based media in place of conventional liquid media has been proposed (Evanson et al., 1991), however, haemolysis can be difficult to assess, and various atypical *L. monocytogenes* strains (non-haemolytic, catalase-negative, rhamnose-negative, non-motile) would not be identified (Bille et al., 1992). Till date, only genomic methods can firmly identify various isolates of *Listeria* (Leimeister-Wachter et al., 1990; Jacquet et al., 1992), however, they require special equipments.

DIM (differentiation of *innocua* and *monocytogenes*) is a new test based on the detection of acrylamidase which is present in *L. innocua* strains and in majority of other non-*L. monocytogenes* listerial strains but absent in *L. monocytogenes*. The test can easily and clearly differentiate *L. innocua* and other *Listeria* strains from *L. monocytogenes* (Bille et al., 1992). It has also been noticed that all species of *Listeria* except *L. monocytogenes* produce amino acid peptidase activity on alanine substituted substrates
(Kampfer et al., 1991; Kampfer, 1992). This reaction has been modified by using DL-alanine β-naphthylamide (DLABN) as the substrate and has successfully been carried out for the identification of Listeria within 5 h (Clark and McLauchlin, 1997; McLauchlin, 1997). However, despite the availability of alternative identification techniques, conventional and hemolytic reactions are most commonly used (Chen and Chang, 1996; McLauchlin, 1997).

2.13 Established molecular techniques:

2.13.1 Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR) has been used increasingly as an additional tool for the identification of microorganisms because of its rapid, sensitive and specific detection. This facilitates exponential amplification of a pre-selected region of DNA (Mullis and Faloona, 1987) and thus represents a highly specific and sensitive detection technique. Amplification of specific DNA sequences is possible from purified plasmid, chromosomal DNA and also crude DNA samples (Kawasaki, 1990). Thus, PCR has allowed direct identification of organisms from complex substrates without prior purification (Ou et al., 1988; Steffan and Atlas 1988; Olive, 1989; Williams et al., 1990).

In the first reported PCR for the detection of L. monocytogenes (Besseansen et al., 1990), the haemolysin (hlyA) sequence published by Mengaud et al., (1988) was used. It has remained one of the most frequently used sequences for the identification of L. monocytogenes (Besseansen et al., 1990; Border et al., 1990; Furrer et al., 1991). Subsequently, an array of PCR-based procedures for identification of Listeria species were developed by targeting the genes for 16s rRNA, 23 rRNA, 16S/23S rRNA intergenic regions hly, plcA, plcB, actA, prfA, InlA, InlB, InlC, InlJ, iapA, fhp, flaA, pepC, IpE, Imo0733etc for L. monocytogenes (Liu, 2006). Similarly 16s RNA, 23s rRNA, 16S/23S rRNA intergenic regions, iap, liv 22-228 for identification of L. ivanovii (Liu, 2006).

Balamurugan et al., (2004) standardized PCR by targeting the hlyA gene of L. monocytogenes, which produced a 731 bp product. The amplicon was further confirmed by nested PCR and Hinf I digestion. The primers specifically amplified only L. monocytogenes isolates. The minimum detection level was found to be $10^5$ cells/ml with pure culture. The internal fragments (ca. 418 to 469 bp) of 3 virulence genes (prfA, inlB and InlC) and other 3 virulence-associated genes (dal, lisR and clpP) have been sequenced and analyzed (Zhang et al., 2004). Modified PCR techniques like multiplex PCR (Furrer et
al., 1991) nested PCR (Jaton et al., 1992), magnetic immunopolymerase chain reaction assay (Fluit et al., 1993) and RT-PCR (Klein and Juneja, 1997) have been used.

PCR has been developed for identification of *L. monocytogenes* in food samples (Rossen et al., 1991; Niederhauser et al., 1992) and in vegetables (Torriani and Pallotta, 1994). Using a two-step PCR with nested primers, one colony-forming unit (CFU) of *L. monocytogenes* could be directly detected in 25 ml of raw milk (Herman et al., 1995). PCR detection of *Listeria* in foods directly without enrichment has several problems besides the presence of PCR inhibiting substances in foods and inadequate lysis of bacteria present in foods to release the DNA (Karunasagar and Karunasagar, 2000). However, among various treatments of enriched cultures tested for the use in PCR, centrifugation and washing the pellet twice with PCR buffer was found to be the best method to concentrate the target organisms and remove PCR inhibiting substances in meat samples (Balamurugan, 2002).

Multiplex PCR involving simultaneous detection of different foodborne pathogens has evolved as an area of great potential, as it is more cost effective and time saving than conventional PCR. Multiplex PCR is a way to amplify two or more amplicons in a single PCR reaction. Multiplex PCR assay has been used to selectively amplify a shared *iap* gene and facilitate the differentiation of all six *Listeria* species in a single test (Bubert et al., 1999). However, caution should be exercised to ascertain the sizes of the amplified products, which may show minute size differences among various *Listeria* species.

Recently, Rawool et al., (2007a) developed a multiplex PCR assay targeting various virulent genes (*hlyA*, *plcA*, *prfA*, *ActA*) of *L. monocytogenes* in a single tube reaction and employed for *in vitro* assessment of the pathogenicity of *Listeria* isolates recovered from cases of mastitis (Rawool et al., 2007b) and spontaneous abortion in women (Kaur et al., 2007b, 2009). Another virulence genes-based multiplex PCR targeting internalin genes (*InIA, InIC* and *InII*) has also been reported for such purposes (Liu et al., 2007).

Several virulence-associated genes have been sequenced and used as PCR-targets for the detection of *L. monocytogenes*, for example, the listeriolysin O (*hlyA*) gene (Besselsen et al., 1990; Border et al., 1990; Rossen et al., 1991), the Dth 18 gene (Wernars et al., 1991), the *iapA* gene (Jaton et al., 1992; Kohler et al., 1990) and the *plcA* gene (Notermans et al., 1991b). Listeriolysin O (LLO) encoded by *hlyA* gene has been regarded as the most important virulence factor.
It has been reported that prfA and hlyA are detected by PCR distinctively in L. monocytogenes suggesting the usefulness of PCR amplification of these genes for the identification of L. monocytogenes from various food samples (Niederhauser et al., 1992; Wernars et al., 1992).

The expression of hlyA has been reported to be under the control of positive regulatory factor gene, prfA (Chakraborty et al., 1992; Leimeister-Wachter et al., 1990) that is located upstream of hlyA but is transcribed in the reverse direction. Three other genes located adjacent to hlyA have been cloned, namely, plcA encoding phosphatidylinositol-specific phospholipase C (Leimeister-Wachter et al., 1991; Menguad et al., 1991), mpl encoding metalloprotease (Domann et al., 1991; Menguad et al., 1991) and plcB gene encoding phospholipase C (PLC) or lecinhase C (Raveneau et al., 1992; Vazquez-Boland et al., 1992). The mutants defective for each of above three genes were reported to be less virulent than the parental strain, suggesting that all five of these genes are associated with the virulence expression in L. monocytogenes.

Further ActA protein, encoded by actA gene, one of the virulence factors, plays an important role in intracellular bacterial movement in the host cytosol following escape into the host cytoplasm (Domann et al., 1992; Friederich et al., 1995; Kocks et al., 1992; Vazquez-Boland et al., 1992). Twenty four strains of L. monocytogenes isolated from healthy human beings and patients with listeriosis were found to have two different types of actA gene among the clinical isolates as well as isolates from healthy humans (Moriishi et al., 1998).

The invasion associated protein (iapA) gene of Listeria spp. encodes protein p60 which is a major extracellular product secreted by all isolates of L. monocytogenes (Kohler et al., 1990). The PCR primers specific for iapA yielded PCR products, which represented essentially the repeat region of the iapA gene (Bubert et al., 1992). The species-specific identification of L. monocytogenes by PCR using primers against iapA gene has been reported by various workers (Bubert et al., 1992; Jaton et al., 1992; Jeyasekaran et al., 1996).

Detection of only one virulence-associated gene by PCR is not always sufficient to identify L. monocytogenes, and that PCR amplification of genes other than hlyA and plcA may not be adequate for the detection of virulent strains of L. monocytogenes (Nishibori et al., 1995). In order to avoid the possible failure in the detection of virulent L. monocytogenes, a one step procedure that enabled demonstration of three virulence-
associated genes, prfA, hlyA and plcB simultaneously in a single PCR mixture was developed for a large-scale survey, aiming at the detection of virulent strain of L. monocytogenes without failure (Cooray et al., 1994). Recently, multiplex PCR targeting virulence genes of L. monocytogenes (plcA, prfA and hlyA genes) and of L. ivanovii (plcA, prfA and actA genes) has been employed (Rawool, 2004; Shakuntala, 2004).

Beside 16s rRNA (Border et al., 1990; Wiedmann et al., 1993) gene sequences have also been used for the identification of L. monocytogenes. In addition, more L. monocytogenes virulence protein should be tested in order to elicit the pathogenicity of L. monocytogenes strains depending on the human clinical manifestations, as well as the ecology of L. monocytogenes in food and environment.

Norton (2002) has reviewed nucleic acid amplification-based methodology, specifically polymerase chain reaction (PCR)-based assays, for the detection of L. monocytogenes in food and environmental samples. The development of ribonucleic acid (RNA) amplification-based assays may increase in importance, particularly if end product testing is prioritized by regulatory agencies, as messenger RNA appears to serve as an accurate indicator of cell viability. Further, the increase in target copy number may improve assay sensitivity.

In Listeria spp. the presence of plasmids was first reported by Pérez-Díaz et al. (1982). Mainly plasmids in Listeria are cryptic but numerous authors have recently shown the involvement of plasmids and transposons in cadmium and antibiotic resistance in Listeria (Poyart-Salmeron et al., 1990; Lebrun et al., 1992; Poyart-Salmeron et al., 1992; Hadorn et al., 1993; Lebrun et al., 1994). Margolles and de los Reyes-Gavilan (1998) reported the isolates of L. monocytogenes serogroup 1 harbouring a single plasmid, pLM33 (33.2 kbp), whereas the serogroup 4 isolates did not contain plasmids.

Kolstad et al., (1992) screened three hundred and seven Listeria monocytogenes isolates from various origins (clinical sources, raw chicken, seafoods, dairy and meat products and processing environments) for plasmids. The workers reported overall frequency of L. monocytogenes isolates containing plasmids to the tune of 77%. The highest percentages of plasmid positive isolates were found from meat (89%), chicken (81%) and dairy products (64%), while clinical isolates had the lowest plasmid percentage (28%). Seven sizes of plasmids (21, 24, 27, 35, 40, 47 and 52 MDa) were distinguished. All sizes were represented in the meat isolates; clinical isolates contained only two of the
plasmid sizes, while several different sizes of plasmids were found in the isolates from other origins.

2.13.2 RAPD analysis:

Since 1989, various molecular typing methods have been applied to L. monocytogenes, including multilocus enzyme analysis, ribotyping, DNA microrestriction and macrorestriction profile analysis and random amplified polymorphic DNA (RAPD) assay (Bibb et al., 1990). In epidemiology, the RAPD assay is appropriate for screening large panels of strains. Many researchers have used this assay for typing L. monocytogenes isolates from different sources (Wagner et al., 1996). Numerous reports can be found on the presence of L. monocytogenes in fish and fish product by this method (Ericsson et al., 1997).

Ertas and Seker, (2005) isolated L. monocytogenes from intestine of 150 fishes of Keban Dam Lake and carried randomly amplified polymorphic DNA (RAPD) analysis. They concluded that the isolates might have originated from different source, and fish contaminated with L. monocytogenes may cause serious health problems in human. According to Czajka et al., (1993) and Fekete et al., (1992) RAPD analysis provides a fast ans simple method for differentiating bacterial strains.

2.13.3 PCR serotyping:

L. monocytogenes strains are serotyped according to their somatic and flagellar antigens. Although 13 serovars have been described; only three of this serovers, specifically 1/2a, 1/2b and 4b, account for the majority of clinical cases (Liu, 2006). Doumith et al., (2004) affirms that at least 95% of strains isolated from contaminated foods and infected patients were of serotypes 1/2a, 1/2b, 1/2c and 4b. Also suggest while serovars 1/2a is the most frequently isolated strains from contaminated foods, the majority of epidemic listeriosis were caused by the type 4b strain.

For L. monocytogenes serotyping, a PCR- based method was developed to probe four gene targets. Multiplex PCR is a variant of traditional PCR techniques that introduces two or more sets of primer pairs with specificity for different genes or gene regions. The four major serotypes 1/2a, 1/2b, 1/2c and 4b can produced four distinct PCR profiles with properly desined primer pairs. This technique can be easily adapted to different laboratories, is quick and reproducible (Doumith et al., 2005). The multiplex
PCR analysis is utilized to detect the presence of virulence-associated genes of *L. monocytogenes* (Kaur et al., 2007). It was developed for repeat speciation and virulence determination of *L. monocytogenes* (Liu et al., 2007).

### 2.14 Pathogenicity Tests:

#### 2.14.1 *In vitro* tests:

Virulence attenuation in *L. monocytogenes* has been detected and characterized using *in vitro* cell culture and animal models. The *in vitro* assays can be designed to detect phenotypic or genotypic markers of virulence attenuation (Roberts et al., 2005). Molecular techniques, such as mixed-genome microarrays, can detect genetic markers specific to clonal groups of *L. monocytogenes* that have various associations to human listeriosis (Call et al., 2003).

*L. monocytogenes* is heterogeneous in respect of its virulence (Hof and Rocourt, 1992), which is thought to be a multifactorial phenomenon (Ralovich, 1984). Differentiation between hemolytic and pathogenic species (*L. monocytogenes* and *L. ivanovii*) and non-hemolytic and apathogenic species (*L. innocua* and *L. welshimeri*) becomes possible by the hemolytic activity of the former. Positive haemolysis demonstration of virulent *Listeria* can be done either by simple agar slab technique or the Christie Atkins Munch Petersen (CAMP) test. A positive CAMP reaction, fermentation of rhamnose and non-fermentation of xylose are the three *in vitro* tests used to identify pathogenic *Listeria* spp. However, there is one species, i.e., *L. seeligeri*, which is haemolytic but apathogenic (Seeliger, 1981). Hence, CAMP test needs further animal pathogenicity testing in order to demonstrate the virulence clearly.

A microplate technique for the routine determination of haemolytic activity with erythrocyte suspension has been used as a reliable method yielding semi quantitative results (Rodriguez et al., 1986). Its combination with D-xylose, L-rhamnose and α-methyl-D mannopyranoside fermentation has been found to differentiate *L. monocytogenes, L. ivanovii* and *L. seeligeri* (Farber and Peterkin, 1991). The Voges-Proskauer (VP) and Methyl red (MR) reactions have been more frequently negative among avirulent *Listeria* strains than the virulent ones (Ralovich, 1989).

The production of cytopathic effect (CPE) in human enterocyte like cell line CaCO 2 has been observed only with pathogenic *Listeria* (Pine et al., 1991) and only hemolytic strains could multiply within murine hepatocyte cell line (ATCC TIB73) although non-
hemolytic strains could invade (Wood et al., 1993). Continuous cell lines have been suggested as a test vehicle for pathogenic *Listeria* species (Pine et al., 1991).

None of the conventional selective plating media recommended in official international standard methods can distinguish between *L. monocytogenes* and other *Listeria* species. Agar Listeria according to Ottaviani and Agosti (ALOA) is a chromogenic medium, which is not only selective for the isolation of *Listeria* species but also allows the direct differentiation of pathogenic *L. monocytogenes* and *L. ivanovii* in the presence of other *Listeriae* and various background flora. The ALOA medium comprises of a chromogenic compound 5-bromo-4-chloro-3-indoxyl-β-d-glucopyranoside, which acts as a substrate for the detection of β-d glucosidase that occurs in all *Listeria* species. The differential detection of *L. monocytogenes* and *L. ivanovii* is based on the production of PI-PLC by the pathogen which can hydrolyze the substrate L-α-phosphatidylinositol and produce opaque clear-cut halo surrounding the colonies (Ottaviani et al., 1997; Reissbrodt et al., 2004).

The chromogenic media allows the detection of *L. monocytogenes* more rapidly and with higher levels of specificity and sensitivity (Sacchetti et al., 2003). The detection of *Listeria* species on ALOA medium is as good after 24 hrs of incubation of plates as after 48 hrs in contrast to oxford agar. There is no significant difference in recovery of *L. monocytogenes* when compared with the conventional media (Greenwood et al., 2005). Combination of chromogenic media with aesculin containing conventional media should be used to detect or enumerate atypical strains of *L. monocytogenes* (Leclercq, 2004).

Of late, phosphatidylinositol-specific phospholipase C (PI-PLC)-based assay has been reported to be more reliable to discriminate pathogenic and haemolytic *Listeria* spp., i.e., *L. monocytogenes* and *L. ivanovii* from nonpathogenic but haemolytic spp., i.e., *L. seeligeri* (Notermans et al., 1991). However, in order to judge the potential of an *in vitro* pathogenicity test it needs to be compared with *in vivo* pathogenicity tests.

### 2.14.2 *In vivo* tests:

Virulence of *L. monocytogenes* for man has been correlated with pathogenicity in mice (Mainou-Fowler et al., 1988), particularly in immuno-compromised mice (Stelma et al., 1987). However, death in normal mice has been found to give clear differentiation between virulent and non-virulent strain following single inoculum (Tabouret et al., 1991).
All the virulent strains tested showed 100% mortality by aerosol infection, while virulent and avirulent strains were indistinguishable by gastric intubation (Bracegirdle et al., 1994).

On inoculation of Listeria species in chick embryo through chorioallantoic (CAM) route, pathogenic strains cause death of embryo within 72 hours while nonpathogenic fail to do so (Terplan and Steinmeyer, 1989) and the test has been reported to agree with mouse bio-assay (Notermans et al., 1991a). Yolk sac route inoculation has been found to be less suitable than the CAM challenge for assessing virulence because of non-specific deaths encountered in the former route (Notermans et al., 1991a).

2.15 Antiogram of Listeria:

Ideal antibiotic therapy is based on determination of the etiological agent and its relevant antibiotic sensitivity. Though these antibiotics are considered to be very important and necessary drugs required to treat severe and life threatening bacterial infections, the associated antibiotic abuse, has contributed to the problem of bacterial resistance. (Suryawanshi, 2011)

Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. Therefore, antibiotic resistance poses a significant problem. Considering that L. monocytogenes is slowly becoming antibiotic resistant by acquisition of known antibiotic resistance genes from gram-positive bacteria, a continued surveillance of emerging antimicrobial resistance of this pathogen is important to ensure effective treatment of listeriosis (Suryawanshi, 2011).

L. monocytogenes and the strains of other Listeria spp. are susceptible to a wide range of antibiotics except cephalosporins fosfomycin (Hof and Nichterlein, 1997). The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole can be a second-choice therapy (Chanpentier and Courvalin, 1999).

Ruiz-Bolivar et al., (2011) studied one hundred eight L. monocytogenes food isolates from four cities in Colombia. Isolates were evaluated against 17 antimicrobials. The worker reported cent-per-cent susceptibility to ampicillin, amoxicillin/clavulanic acid, and chloramphenicol, whereas it was 98% for other antimicrobials such as Trimethoprim/sulfamethoxazole, 97% for azithromycin, 92% for vancomycin, 90% for erythromycin, 86% for tetracycline, 84% for penicillin, 70% for ciprofloxacin, 57% for rifampin, 56% for meropenem, and 32% for clindamycin, and the primary drugs of choice against listeriosis remain effective for most of the isolates (84%).
Zade and Karpe (2010) studied antibiotic sensitivity pattern of *Listeria* isolates from foods of animal origin. Overall sensitivity was recorded towards ampicillin, doxycycline hydrochloride and tetracycline (88.88% each); amoxyclov, cephotaxim, gentamicin and neomycin (77.77 each); erythromycin and streptomycin (44.44% each); amikacin and penicillin-G (33.33% each) and azithromycin (22.22%). While all isolates were cent-percent resistant against nalidixic acid and trimethoprim followed by azithromycin (77.77%); ceftazidime and streptomycin (55.55 % each); erythromycin (44.44 %); penicillin-G (33.33 %); amoxyclov, amikacin and gentamicin (22.22 % each), ampicillin, doxycycline hydrochloride, neomycin and tetracycline (11.11 % each).

Ennaji Hayat et al., (2008) screened 426 samples of raw meats from butcheries and supermarkets in Casablanca, Morocco and isolated ten strains of *L. monocytogenes*. On testing the antibiotics susceptibility, activities of the 21 antibiotics; all isolates were susceptible to for gentamicin and chloramphenicol. Likewise, penicillin and ampicillin showed good activity against *L. monocytogenes*. Of the other antibiotics, trimethoprim and trimethoprim in combination with sulfamethoxazole, except for one strain (meat) was resistant to both antimicrobial agents. For the activity of the first generation cephalosporins tested, only one strain (meat) was resistant to cephalotin and cefazolin and one other strain showed moderate susceptibility to cefazolin.