Characterization of *Listeria* spp. from clinical cases

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By

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DEDICATED

TO

MY PARENTS

Statement

This is to certify that the thesis entitled "**Characterization of *Listeria* spp. from clinical cases**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

All the corrections suggested by the external examiner were appended into the thesis.

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List of Abbreviations

µg : Microgram

µl : Microlitre

µM : Micromolar

AFLP : Amplified Fragment Length Polymorphism

AFNOR : Norme Francaise

ALOA : Agar *Listeria* according to Ottaviani and Agosti

AOAC : Association of Official Analytical Chemists

ATCC : American Type Culture Collection

BAM : Bacteriological Analytical Manual

BHI : Brain Heart infusion (broth)

BIS : Bureau of Indian Standards

Bp : Base pairs

BSH : Bile salt hydrolase

CAMP : Christie Atkins Munch Petersons test

CDC : Center of Disease Control and Prevention

CFU : Colony Forming Unit

CHEF : Contour Clamped Homogeneous Electrophoresis

Clp : Caseinolytic proteins

CLSI : Clinical and Laboratory Standards Institute

CNS : Central nervous system

CV : Crystal violet stain

DGGE : Denaturing Gradient Gel Electrophoresis

DNA : Deoxyribo nucleic acid

dNTP : Deoxy ribose nucleic acid

EDS : Energy dispersive spectroscopy

EDTA : Ethylene diamine tetraacetic acid

ELISA : Enzyme Linked Immunosorbent Assay

FAME : Fatty acid methyl ester

FAO : Food and Agriculture Organization

FbpA : Fibronectin binding protein

FDA : Food and Drug Administration

FSIS : Food safety and inspection service

FT-IR : Fourier Transform Infrared Spectroscopy

GABA : Gamma aminobutyrate

GAD : Glutamate decarboxylase system

GMP : Good Manufacturing Practices

HACCP : Hazard Analysis Critical Control Point

ILCC : Indian *Listeria* Culture Collection

ISO : International Organization of Standardization

LAMP : Loop mediated isothermal amplification

LAP : Listeria adhesion protein

LI : *Listeria ivanovii*

LLO : Listeriolysin O

LM : *Listeria monocytogenes*

LMWT : *Listeria monocytogenes*EGDe

MALDI-TOF MS : Matrix Assisted Laser Desorption & Ionization Time of Flight Mass Spectroscopy

Mg : Milligrams

MHFW : Ministry of Health and Family Welfare

MIC : Minimum Inhibitory Concentration

Min : Minute

Ml : Millilitre

MLEE: Multi Locus Enzyme Electrophoresis

MLST: Multi Locus Sequence Typing

mM : Millimolar

Mpl : Metalloprotease

MSG : Monosodium glutamate

MTCC : Microbial Type Culture Collection

MTWP : Microtiter well plate

PALCAM : Polymyxin Acriflavin Lithium-chloride Aesculin Mannitol (Agar)

PBS : Phosphate Buffered Saline

PC-PLC : Phosphatidylcholine Specific Phospholipase C

PCR : Polymerase chain reaction

PFGE : Pulsed field gel electrophoresis

PHAC : Public Health Agency of Canada

PI-PLC : Phosphatidylinositol Specific Phospholipase C

PI-PLC : Phosphotidylinositol Specific Phospholipase C

RAPD : Random Amplification of polymorphic DNA

REP-PCR : Repetitive Element Polymerase Chain Reaction

RFLP : Restriction Fragment Length Polymorphism

RTE : Ready-To-Eat (Food)

Spp.: Species

TBE : Tris–borate EDTA buffer

TE : Tris EDTA

TGGE : Temperature Gradient Gel Electrophoresis.

USDA : United States Department of Agriculture

UVM : University of Vermont media

WHO : World Health Organization

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CHAPTER 1

GENERAL INTRODUCTION

The members of the genus *Listeria* forms a group of low G+C content bacteeria closely related to the genera *Bacillus* and *Staphyloccus* .They show positive Gram staining reaction are facultative anaerobes and non-spore forming coccobacilli of 0.5 x 1–1.5 µm size. Genus *Listeria* has been further classified into a number of species namely, *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. grayi, L. welshimeri, L. rocourtiae* (Leclerq *et al*., 2010), *L. marthii* (Graves *et al*., 2010), *L. weihenstephanensis* (LangHalter *et al.,* 2013) and *L. fleischmannii* (Bertsch *et al*., 2014). Recently, five novel non-pathogenic species of *Listeria* namely, *L. floridensis, L. aquatica, L. cornellensis, L. riparia* and *L. grandensis* have been reported from environmental samples (den Bakker *et al*., 2014). Amongst all the above mentioned species *L. monocytogenes* and *L. ivanovii* are the known pathogenic species which cause listeriosis, of which *L. monocytogenes* affects humans and *L. ivanovii* causes infection in animals and occasionally in humans (Lopez, 2008). Listeriosis is an important bacterial disease of humans as well as of animals and the infection of *Listeria monocytogenes* causes listeriosis and therefore concerns economy and health. Wide variety of animals such as sheep, goats, cattle, buffaloes, dogs, horses, chickens, rabbits, wild animals and also human have been reported to get infected with *L. monocytogenes* (Low and Donachie, 1997). *Listeria* spp. are tolerant to great environmental variations such as pH, temperature and salt (Lungu *et al*., 2009) and occur in diverse surroundings. Through various ways the *L. monocytogenes* can enter into food processing environments and due to its innate ability to survive for longer periods of time in the environment (soil, plants, and water), this organism survives, grows and remains persistent (Swaminathan *et al*., 2001) making it a very versatile pathogen. It is no surprise that the incidence of *L. monocytogenes* is increasingly getting recognized with the refrigerated food. Generally, ready-to-eat (RTE) foods

needs minimal processing and get consumed without any significant bactericidal procedure; contamination of such food by *L. monocytogenes* is therefore concerning. In United States, a "zero tolerance" policy for RTE foods is followed according to which there should not be any *L. monocytogenes* contamination (Smoot and Pierson, 1997). *Listeria* is ubiquitous in nature and it is also found in wastewater, vegetation, grazing areas, sewage, animal droppings, stagnant water and animal feed. In addition, it can survive in the healthy humans and animals for long periods of time without causing any infections. *L. monocytogenes* has been reported to be present in upto 5% of normal healthy peoples gut therefore; exposure to the bacterium is inevitable (Ramaswamy *et al*., 2007).

Although the incidence of listeriosis is rare, the high case fatality rates (20-30%) makes *L. monocytogenes* one of the most lethal human food-borne pathogens. In addition, febrile gastroenteritis can also be observed after infection of healthy individuals with *L. monocytogenes* (Ooi and Lorber, 2005). The infection of *L. monocytogenes* is serious in immunocompromised individuals such as pregnant women, elderly patients, neonates, childrens and person whose immune system has been compromised. The infection in a pregnant woman initially causes either a placid, flu-like condition with fever, colds, headache, dizziness or gastrointestinal signs or an asymptomatic infection. This generally leads to abortion (second half to third trimester), stillbirth, premature birth or septicemia in the newborn. Newborns gets infected either *in utero,* from bacteria found in the vagina during delivery or from medical personnel and fomites in the neonatal unit (Farber *et al*., 1991; Rocourt and Brosch, 1992; Slutsker and Schuchat, 1999). Infection in newborns develops into septicemia, disseminated granulomatosis, respiratory disease or meningitis (Klarsfeld

et al., 1994). Symptoms are observed within few days to few weeks of delivery. If the infection is not treated or remains undiagnosed it may lead to meningitis followed by hydrocephalus (Evans *et al*., 1984).

In case of elderly but immunocompromised or debilitated persons, infection advances to meningitis, meningoencephalitis or less frequently, septicemia. The clinical signs such as confusion, seizures, cranial nerve deficits, ataxia, tremors or myoclonus resulting from infection in central nervous system (CNS) have been reported. In certain cases abscesses in brain can also be seen. Several other conditions such as endocarditis, septic arthritis, osteomyelitis, colecystitis, hepatitis, peritonitis, otitis, sinusitis, pleuritis, conjunctivitis and rare cases of pneumonia have also been reported (Blenden *et al*., 1987; Farber and Peterkin, 1991; Gallaguer and Watakunakorn, 1988; Gauto *et al*., 1992; Lorber, 1990; Low and Donachie, 1997; McLauchlin, 1990a; Rocourt, 1994; Slutsker and Schuchat, 1999).

Generally, healthy individual do not show any significant clinical signs. The people who are exposed directly to the *L. monocytogenes* (for e.g. handlers of infected newborns, aborted cows, or who perform necropsies on septicemic animals)*;* a cutaneous eruption characterized by a papular rash or pustules gets developed. Also, in some cases, fever, chills or generalized pain in addition to these rashes may be observed. This condition is commonly observed among the veterinarians and nurses (Allcock, 1992; McLauchlin and Low, 1994). *L. monocytogenes* has innate ability to resist the suboptimum conditions in the gastrointestinal tract. This organism possesses different accessories as well as mechanism in order to overcome changes in acidity, osmolality, oxygen tension, antimicrobial peptides and bile. Also, *L. monocytogenes*

has been reported to colonize and persist in the gallbladder suggesting the chronic infection and surviving ability in different microenvironment (Begley *et al*., 2009).

Though the listeriosis has been studied and reported in developed countries, in case of developing countries, it is largely underestimated. The main reason behind such misjudgment is symptoms common to other bacterial infections. The infection of pathogenic salmonellas and clostridia's exhibit highly similar symptoms therefore creates confusion. In addition, due to rare occurrence of listeriosis it is less likely to get suspected. However, if the mortality data from last two decades is considered, *L. monocytogenes* comes third among the bacterial food borne infection (Rossi *et al*., 2008). According to study performed by Mead *et al*. (1999), the prevalence of listeriosis is 0.1% in developed countries and is less than that of all food-borne illnesses, but high mortality rate (20 to 30% deaths), high hospitalization rate (91%), makes *L. monocytogenes* a significant pathogen. In addition, from certain outbreaks, the mortality of listeriosis has been reported up to 70% (Jemmi and Stephen, 2006). Such high level mortality is observed from untreated neurologic disease. In case of pregnancy related death, combined perinatal and neonatal mortality rates varies from 19% to 63% while newborns case fatality rate is approximately 50% (Jemmi and Stephen, 2006).

In animals, listeriosis is most frequently reported from domesticated animals such as small ruminants and bovines. Unlike humans, the infection of *L. monocytogenes* in animals is detected when infection reaches to its severe stages such as abortion, neonatal septicemia and meningoencephalitis (Campero *et al*., 2002). Listeriosis in small ruminants may present as encephalitis, abortion, and septicemia or

endophthalmitis, but mainly takes the form of meningoencephalitis called circling disease in its most common form. Affected animals circle in one direction only and display unilateral facial paralysis, difficulty in swallowing, fever, blindness and head pressings. Paralysis and death follow in 2 to 3 days (Songer and Post, 2005). Usually, solitary form of manifestation is observed in a cluster of affected animals. Septicemia is often encountered in neonates and can also occur in adult sheep (Quinn *et al*., 2002). In pregnant animals, *L. monocytogenes* may localize in the placentas and cross over to amniotic fluid. It multiplies there and is ingested by the fetus, eventually causing fetal death and abortion. Listerial abortion occurs in late gestation (Songer and Post, 2005).

Although the *Listeria* species are widely spread in nature, clinical diseases in animals occur mainly in the northern and southern latitudes and are much less common in tropical and subtropical than in temperate climates (Radostits *et al*., 2007). In the northern hemispheres, listeriosis has a distinct seasonal occurrence, probably associated with seasonal feeding of silage, with the highest prevalence in the months of December through May (Radostits *et al*., 2007; Hirsh *et al*., 2004).

Soil contamination and ingestion of contaminated feed such as poor quality silage are the main modes of transmission of *Listeria* and hence it is often called as the silage disease (Hirsh *et al*., 2004). Lambs with septicemia may acquire it from the surface contamination of the teat, from the milk, through the navel, from the environment or as congenital infection (Radostits *et al*., 2007). Encephalitis due to *Listeria* can be due to abrasion of buccal mucosa during feeding or browsing or from

infection of tooth cavities whereas spinal myelitis is due to growth in spinal nerves (Radostits *et al*., 2007, Hirsh *et al*., 2004).

The main reservoirs of the infectious agent are soil, water, healthy carriers, animals, birds, fish and crustaceans (Fenlon, 1999). *L. monocytogenes* have also been reported from aborted fetuses and in the nasal discharges and urine of symptomatic animals. Fecal contamination of silage and fodder is also an important factor in its transmission. Most infections are foodborne and due to direct ingestion, but inhalation and direct contact as well as venereal transmission is also possible (Shakespear, 2002).

In most of the cases, *L. monocytogenes* enters through food. Depending upon the virulence capability and number of cells, the pathogenesis of different strains varies. The report from New York State Department of Health (2011) states that the incubation period for *L. monocytogenes* could be from 3 to 70 days. The invasive listeriosis may be asymptomatic till 35 days (Linnan *et al*., 1988). On the other hand, gastrointestinal symptoms may be observed within 24 hours (Ooi and Lorber, 2005; Dalton *et al*., 1997). *L. monocytogenes* first encounters the conditions in the stomach and intestinal tract. As it possesses well adapted mechanisms to overcome barriers such as low stomach pH, bile, altered molarity and low oxygen pressure, the cells easily survive (Cotter *et al*., 2000; Ferreiea *et al*., 2010). Previous studies have shown that glutamate decarboxylase (GAD) system of *L. monocytogenes* gets activated in acidic environment which provides resistance ability to the cells. Absence of GAD system causes lack of tolerance ability in *L. monocytogenes* (Cotter *et al*., 2000). The GAD system has been reported from many foodborne pathogenic bacteria, apparently

as an accessory system for pathogenesis. In addition, the acid adapted bacteria have been shown to infect more Caco-2 cells as compared to non-adapted cells (Conte *et al.* 2000).

Similar mechanism helps *L. monocytogenes* cells for its existence in the different environmental situation as well as in food processing establishments. This ability to tolerate pH has been considered as one of the important ability that allows *L. monocytogenes* cells to grown in acidic food where other bacteria cannot. In case of osmolality, *L. monocytogenes* cells possess capability to vary the cytoplasmic solute concentration that helps to tackle the decreased or increased osmolarity in environment as well as in host (Sleator and Hill, 2002). Unlike other bacteria, *L. monocytogenes* can also tackle bile. The resistance to bile was revealed when a lot of *L. monocytogenes* cells were observed in gall bladder of experimentally infected mice (Hardy *et al*., 2004). It was worth noting the presence of cells in the gall bladder where concentrated bile gets stored. Further study revealed the presence of *bsh* and *bilE* genes that confers the resistance to bile (Begley *et al*., 2009). The *bsh* and *bilE* genes together are responsible for the bile salt hydrolase activity. After successful entry the carnitine uptake ability helps survival and colonization of *L. monocytogenes* (Schuppler and Loessner, 2010).

Once colonized, *L. monocytogenes* starts invading epithelial cells of intestine. Several internalins identified in *L. monocytogenes* allow cells to enter into epithelial cells (Vazquez-Boland *et al*., 2001). The virulence in *L. monocytogenes* has been well described by the presence of a group of virulence genes known as '*Listeria* pathogenicity Island-I' (LIPI-I). The LIPI-I contains a cluster of six genes in 9 Kb

segment. These six genes are as – (i) phosphatidylinositol-specific phospholipase C (*plc*A), (ii) haemolysin (*hly*), (iii) Zinc metalloproteinase precursor (*mpl*), (iv) actin (*act*A), (v) phosphatidylcholine-specific phospholipase C (*plc*B) and (vi) a hypothetical protein. The whole virulence cluster get regulated by a single promoter - positive regulatory factor A (*prf*A) immediately upstream to the LIPI-I (Vazquez-Boland *et al*., 2001). The phosphatidylinositol-specific phospholipase C helps *L. monocytogenes* for intracellular survival. The haemolysin gene is responsible for blood lysis. Though the role of Zinc metalloproteinase precursor has not been fully understood, the absence of mpl gene causes decreased virulence. The *act* gene encodes for actin filament that helps for intracellular movement. The virulence factors overall contribute in explicit ways in the disease process (Kuhn and Goebel, 1998).

Pathogenic *Listeria* enters the host primarily from the intestine and localizes in the liver where it multiplies until it is controlled by cell mediated immune response (Werbrouck *et al*., 2006). In patients with lowered immunity, the unhindered increase of the organism in the liver may lead to lesser bacteremia which may advance to invade secondary target organs namely, brain and the gravid uterus leading to clinical illness (Longhi *et al*., 2004).

The epidemiological studies with *L. monocytogenes* have helped to trace the sporadic, outbreak and epidemic strains. Several techniques have been employed to differentiate the *L. monocytogenes* that are actually involved in the clinical cases. Traditionally, *L. monocytogenes* have been divided into 13 serotypes. A study with 1363 patients performed by McLauchlin (1990b) showed that out of 13 serotypes serovar 4b was the most common, while other serovars were reported in less than

29% cases. Another approach is the differentiation based on specific genetic markers. Based on the genetic markers, three lineages were defined in case of *L. monocytogenes* (Jadhav *et al*., 2012). However, different subtyping methods allows *L. monocytogenes* now to be divided into at least 4 progressive lineages, I to IV which have diverse but partly covered ecological niches (Orsi *et al*., 2011). Lineage I, II and II have serotypes 4b, 1/2b, 3b, 4d and 4e; 1/2a, 1/2c, 3a and 3c, and 4a, 4c and atypical 4b, respectively. Lineage IV strains are rare. Lineage I serotypes are mostly implicated in human outbreaks of listeriosis. Strains belonging to lineage II are most often found in food, natural and farm environments, cases of animal listeriosis and also in sporadic human listeriosis cases. Strains belonging to lineages III and IV are rare and mostly isolated from animal clinical cases. Recently, based on some phenotypic differences, lineage 4 has been introduced. The members from lineage 4 represent distintinguishable ecology, genetic and phenotypic characteristics which influence their ability to be transmitted through foods and causing human diseases (Orsi *et al*., 2011).

Listeriosis has been recognized as a disease of mammals and birds, and as a potential zoonosis (Vaquez-Boland *et al*., 2001; Low *et al*., 1997). The countries where industrially processed ready–to-eat foods are being consumed in larger amount, 2 to 10 cases of listeriosis per million have been reported per year (Goulet *et al*., 2008). Considering the scenario of listeriosis, American and European countries have stringently regulated and supervised the occurrence of *L. monocytogenes* in food and food processing environments. Hazard Analysis and Critical Control Points (HACCP) programmes have been employed throughout the food industry from these countries (Goulet *et al*., 2001; Tappero *et al*., 1995). To the effort, *L. monocytogenes* has been

controlled to a great extent but could not be irradicated as a whole. Therefore, since the last two decades, there is some control over incidences of listeriosis in American and European countries. However, as a result of the detection of *L. monocytogenes* in food and food processing environments there have been several food recalls and therefore, heavy economic losses to these countries. Unfortunately, many countries do not have a surveillance system in order to estimate the incidences of listeriosis. Most of the developing countries including India do not have any strong monitoring systems to observe the incidences of listeria. However, the incidences of listeriosis in such countries cannot be denied. In addition, export and import of food from countries where *L. monocytogenes* is not stringently controlled apparently contributes in spreading the strains.

The first human case of listeriosis can be traced back to 1929 by Nyfeldt *et al*. (1929). The incidence of human listeriosis varies from 5.4 to 9.4 per million of human population annually in France and USA. Since, the first documented confirmed foodborne listeriosis outbreak in 1981, there has been a number of listeriosis outbreaks globally (Martin and Braden, 1999). Recently, outbreaks of listeriosis were reported in in the US and in Spain (CDC, 2012). In US, an outbreak from contaminated cantaloupes from Jensen Farms has been reported. These cantaloupes were distributed to 28 states, where Listeriosis cases occurred. The outbreak was responsible for 30 deaths (CDC, 2011). A report related to consumption of Latin style fresh cheese was reported from Spain in August 2012 (deCastro *et al*., 2012) and in September 2013, the FDA and the CDC investigated a multi-state outbreak of *L. monocytogenes* infections linked to cheese (CDC, 2013).

In India, there were no case reports on *Listeria* until the pioneering work on listeriosis was carried out involving women with bad obstetric history (Krishna *et al*. 1966). Prevalence rate of 3% and 10% was seen in women with history of abortions (Bhujawal *et al*., 1973, Stephen *et al*., 1978), work on pediatric listeriosis (Gupta *et al.*, 1997) and neonatal listeriosis (Srivastav *et al.*, 2005) was also carried out. Kaur et al. (2007) reported isolation of *L. monocytogenes* from 3.3% cases having abad obstetrics history (Kaur *et al*., 2007). The latest reports from India are of a perinatal listeriosis in a 2 day old baby and mother from Himachal Pradesh (Mokta *et al.*, 2010), meningoencephalitis in a 20 month old immunocompetent child from a hospital in Srinagar (Peer *et al.*, 2010), neonatal meningitis in a 2 week old child from a hospital in South India (Khan *et al*., 2011), neonatal septicemia at Bankura (Ray *et al*., 2012), listeriosis with disseminated tuberculosis in an HIV positive patient (Joel *et al*., 2013). Recent reports from the sub Himalayan belt and other areas of India indicate that the disease caused by *Listeria* may be more prevalent in the country than was previously thought (Srivastava *et al.*, 2005).

In India, the principal manifestations among the sporadic cases of listeriosis in animals include reproductive disorders including endometritis, subclinical mastitis and meningoencephalitis (Barbuddhe *et al*., 2012). The first report of animal listeriosis from India was from a sheep showing symptoms of the circling disease (Mahajan, 1936). Subsequent work was carried out on sheep (Vishwanathan and Iyyar, 1950), on goats (Katiyar *et al*., 1960, Brahmbhatt and Anjaria, 1993), on outbreak in chickens (Nagi and Verma, 1967; Vijaykrishna *et al*., 2000). Isolation of *Listeria* spp. and detection of antibodies to listeriolysin O (LLO)in buffaloes (Barbuddhe *et al*., 2002), detection of *L. monocytogenes* in buffaloes with a history of

reproductive disorders (Shakuntala *et al.*, 2006) and isolation of *Listeria* from sheep in Gujarat (Yadav and Roy, 2009) have been reported.

Listeriosis outbreaks similar to massive outbreaks observed in Europe and North America have probably not occurred in India yet. One of the reasons may be the difference in the lifestyle and food habits amongst the population, but with urbanization and change in the lifestyle and the propensity to consume ready to eat and processed foods may prove to be hazardous unless adequate preventive measures are taken. There is lack of extensive knowledge of listeriosis outbreaks both of sporadic as well as of epidemic nature in India. Awareness about the disease is also lacking in the medical community as well as in the general population. Hence, no systematic data is available with respect to the occurrence of the disease, prevalence of the pathogen, variations occurring in it at a genomic level based on which distribution of its subtypes in India can be traced. However, inspite of the poor surveillance and epidemiology of diseases in the Indian subcontinent, a few sporadic cases reported for both animal and human listeriosis could be traced which dates back to the 1930's. Of late, there has been an increase in the sporadic cases of listeriosis in the country (Janakiraman, 2008). Few reports are available from India dealing with *Listeria* isolated from clinical cases, hence this study would give valuable insight about prevalence of *Listeria* in clinical cases both of human as well as animal origin.

The present study was proposed with the following objectives.

1. To isolate and characterize *Listeria* isolates from clinical samples.
2. To characterize *Listeria* by phenotypic and genotypic methods.
3. To investigate the genetic diversity of *L. monocytogenes* strains with different virulence potentials using various molecular tools.

CHAPTER 2

REVIEW OF LITERATURE

2.1 *Listeria* and Listeriosis

Listeria monocytogenes is a Gram positive bacterium which was first described in 1926 in Cambridge in laboratory rodents (Murray *et al*., 1926). In 1929, Nyfeldt isolated the bacteria from blood culture of patients, in 1936 listeriosis as sepsis and meningitis was described in the USA and in 1919 *L. monocytogenes* was isolated from the cerebrospinal fluid collected from a soldier in France (Nyfeldt, 1929; Gray and Killinger, 1966; Cotoni, 1942). Listeriosis as a disease was largely ignored since then, however, disease outbreaks were reported in Germany in 1966, in the Anjou region of France between 1975-1976 and in early to mid 1980's the number of animal and human listeriosis in Europe and North America increased. In 1926, it has been stated by Pirie that disease could be produced by inoculation subcutaneously or by feeding and thought that the disease might spread by feeding in nature.

The cases of human listeriosis are increasing globally and many outbreaks have been reported in countries around the world. Some of the major global outbreaks are as follows. In 1981 an outbreak of listeriosis due to coleslaw occurred in Canada involving 41 cases and resulting in 18 deaths (Schlech *et al*., 1983), and outbreak due to pasteurized milk occurred in 1983 in the USA involving 49 cases and resulting in 14 deaths (Flemming *et al*., 1985). In Switzerland an outbreak linked to soft cheese occurred in the period 1983-1987 leading to 122 cases and 31 deaths (Bille *et al*., 1990). An outbreak linked to Mexican style cheese occurred in the year 1985 in USA involving 142 cases and 48 deaths (Linnan *et al*., 1988). In the period between 1987 - 1989 an outbreak due to pate occurred in UK and Ireland involving 355 cases and

resulting in 94 deaths (McLauchlin , 1991). In France jellied pork in 1992 was responsible for 279 cases of listeriosis and 85 deaths (Goulet *et al*., 1993) and in Canada in 2008 an outbreak due to RTE delimeat involved 57 cases and 23 deaths (PHAC, 2008). Recently an outbreak linked to cantaloupes occurred in Colorado involving 28 different states and 30 deaths (CDC, 2012). In September 2013, a multistate outbreak of listeriosis was caused by cheese in Wisconsin and resulted in one death and one miscarriage (USFDA, 2013).

Massive listeriosis outbreaks like those in Europe and North America have not occurred in India; however, some sporadic cases of both human and animal listeriosis have been reported from the Indian subcontinent. In India pioneering work was carried out involving women with bad obstetric history (Krishna *et al*., 1966; Malik et al., 2000). *Listeria monocytogenes* was isolated from spontaneous abortion cases (Kaur *et al*., 2007) and in infections in children born from infected in utero (Gogate and Deodhar, 1981). Isolation of *L. monocytogenes* was reported from paediatric cases having underlying conditions such as congenital heart disease and severe malnutrition (Gupta *et al*., 1997). A case of listeriosis with perinephric abscess was reported in a 5 year old malnourished child (Gomber *et al*., 1998); the pathogen was also been detected from cerebrospinal fluid (Pandit *et al*., 2005). The most recent reports from India include perinatal listeriosis in a 2 day old baby (Mokta *et al*., 2010), meningoencephalitis in a child (Peer *et al*., 2010), neonatal meningitis (Khan *et al*., 2011), neonatal septicemia (Ray *et al*., 2012), listeriosis with disseminated tuberculosis in a HIV patient (Joel *et al*., 2013). Based on these recent reports it is clear that the disease is more prevalent than previously thought (Srivastav *et al*., 2005).

Animals act as a reservoir and are responsible for dispersal of *L. monocytogenes* that includes subtypes previously reported from human listeriosis cases and outbreaks into the farm environment. In India, sporadic cases as well as outbreaks of listeric infections have been reported from the animal population. The clinical manifestations commonly seen were spontaneous miscarriages, subclinical mastitis, meningoencephalitis and endometriosis (Barbuddhe *et al*., 2012). *L. monocytogenes* had been isolated from different domesticated animals in India. The first case of abortion related to *Listeria* was reported from a ewe (Dhanda *et al*., 1959). *Listeria* species were also isolated from flocks of migratory sheep and goats (Sharma *et al*., 1996); *L. ivanovii* also caused an outbreak of abortions in sheep (Chand and Sadana, 1998). Isolation of *L. monocytogenes* related to endometriosis cases was reported from buffaloes, infertile cows, sheep and goats (Shah and Dholakia, 1983; Srivastav *et al*., 1985; Mahajan and Katoch, 1997). *L. ivanovii* was also isolated from aborted; mastitic and healthy goats as well as seropositivity to the pathogen were also seen in these animals (Elezebeth *et al*., 2007). Listerial meningoencephalitis was reported in a piggery and in another case circling movements, upward deviation of the head as well as epileptic convulsions were reported in diseased pigs (Rahman *et al*., 1985; Dash *et al*., 1998). Listeriosis manifested as neurological symptoms was seen in broiler chickens with a mortality rate over 40% (Vijaykrishna *et al.*, 2000). Recent occurrences of listeriosis in sheep (Yadav and Roy, 2009) as well as in poultry and in livestock (Sarangi and Panda, 2012) have also been reported.

The presence of *L. monocytogenes* in the animal population can prove to be a source for human infections through contaminated food and dairy products as some

subtypes of the pathogen isolated from farm animals are also implicated in human outbreaks (Barbuddhe *et al*., 2012). Therefore adequate care should be taken during the processing of farm produce and strategies should be implemented to contain and get rid of the pathogen at source.

Listeria monocytogenes has been revealed as leading cause of death among the food borne diseases (Buzby *et al*.,1996 ; Donnelly, 2001). The persons at high risk to get infected with listeriae include the persons above 60 years of age, immuno-compromised individuals, pregnant women and neonates causing a serious manifestations such as septicemia, meningitis, abortion, stillbirths and in neonates, granulomatous infantiseptica (Vázquez-Boland *et al*., 2001). Rhombencephalitis, an unusual form of listeriosis also has been reported (Armstrong and Fung, 1993). Other manifestations such as eye infections, skin infection, inflammation of lymph glands, hepatic, splenic and brain abscesses, cholecystitis, peritonitis, pneumonia, infection of joints, bone marrow infection, pericarditis, myocarditis, arteritis, necrotizing inflammation of fascia and endophthalmitis have been described (Allerberger and Wagner, 2010).

In severe form, listeriosis may cause an invasive disease with the highest case fatality rate of foodborne pathogens which is 20-30% for both epidemic and sporadic cases (Rocourt *et al*., 2001; Watson *et al*., 2009). In persons with competent immune systems, it can also cause severe as well as self- limiting benign infections like gastroenteritis (Salamina *et al*., 1996).

Advances in food processing technology and changing demands of Ready-to-eat food have increased the number of incidences of food borne illness (Oldfield, 2001). The growth ability of *L. monocytogenes* at wide temperature range including low temperature upto 4^{o}C makes it difficult to control in food processing area (Salyers and Whitt, 2002). Policy makers and public safety officers have forced to regulate the occurrence of *L. monocytogenes* at due to these abilities. In addition, a low level or undetectable level of *L. monocytogenes* at food processing industry may increase in number by the time product reaches to consumer. According to one surveillance, approximately 76 million cases of foodborne diseases are estimated to take place in the U.S.A. every year, of which about 2,500 (<1%) are cases of listeriosis. While, ~27.6% of the total deaths are due to *Listeria* as compared to a fatality rate of only 0.04% in case of foodborne salmonellosis (Mead *et al*., 1999).

2.2 Routes of transmission

The main source of *L. monocytogenes* infection is contaminated food (Minea *et al*., 2005). *Listeria* can survive and proliferate in wide variety of food products such as meat, milk, milk related product, vegetables, fruit-salad etc. (Kerouanton *et al*., 2010). The first outbreak of listeriosis reported in Canada in 1981 (Schelch *et al*., 1983), since then several large scale outbreaks have been reported due to consumption of various contaminated foods. Industrially processed and ready-to-eat food such as milk, milk-originated products, different types of industrially processed meats, fishes, vegetables, seafood, and salads have been linked to listeriosis outbreaks (Jadhav *et al*., 2012). Industrially processed and ready-to-eat food that are generally kept at refrigerated conditions are more likely to transmit the *L. monocytogenes.* (Gravesen *et al.,* 2000).

2.3 Isolation & Identification methods

To isolate *Listeria* including *L. monocytogenes* the samples need to be enriched in selective media followed by plating on selective agar. Based on typical colony morphologies, tentative identification of *L. monocytogenes* or other *Listeria* species is being carried out (Jadhav *et al*., 2012).

2.3.1 Culturing methods

Traditionally, *L. monocytogenes* was detected based on its ability to proliferate at low temperatures. The suspected samples used to be inoculated in nutrient broth followed by incubation for 3 to 4 weeks. The method was followed till 1960. However, as it used to take long duration of time, the method got replaced subsequently with *L. monocytogenes* enriching medium.

According to the norms prescribed by regulatory agencies, the method to isolate *L. monocytogenes* should be enough sensitive to detect the organism per 25 g of food-products. The only way to achieve higher sensitivity is to enrich the samples. Antimicrobials agents are incorporated in these enrichment media in order to suppress competing microflora. To isolate *L. monocytogenes,* selective agents such as acriflavine (which inhibits the growth of other Gram-positive bacteria); nalidixic acid (as an inhibitor for Gram-negative bacteria); and cycloheximide (as an inhibitor of fungi) are used. The antimicrobials having broad spectrum of activity are commonly employed as inhibitors which include the ceftazidime, moxalactam and lithium chloride. Esculin is hydrolysed by all *Listeria* spp. In the presence of ferric ions, it gives black color due to the formation of a complex with ferric iron and 6, 7-dihidroxycoumarin, a product of β-D-glucosidase cleavage of esculin . Hence esculin

and ferric ions are also incorporated in many enrichment and isolation media (Fraser and Sperber , 1988).

Several traditional methods to isolate *Listeria* spp. from different sources have been recommended for international regulatory purposes. Cold enrichment was frequently used for isolation of *Listeria* spp. in early days of clinical bacteriology (Gray *et al*., 1948). For microbiological diagnosis of clinical cases of animal listeriosis, plating of specimens on blood agar directly or its enrichment in other enrichment media concomitantly with the use of the 'cold enrichment' was employed. The inoculated samples were subcultured weekly for up to 12 weeks (Gasanov *et al*., 2005). Established techniques such as the ISO 11290 Standards (ISO, 1996, 1998, 2004), the USDA-Food Safety and Inspection Service (FSIS) method (USDA-FSIS, 2002), 'the Association of Official Analytical Chemists (AOAC) official method (AOAC, 2000) have been used for international regulatory purposes. The United States Food and Drug Administration (FDA) method (Hitchins, 1998), and the French Standards (AFNOR, 1997, 2000) have been recommended for the isolation of *L. monocytogenes*. Depending on the type of the sample (food, environment, clinical) the isolation method of *L. monocytogenes* varies. ISO: 11290 part 1 and 2 have been recommended for the recovery of *L. monocytogenes* from raw-foods as well as RTE food products, while FDA and AOAC methods have been recommended specifically for dairy related products. The USDA-FSIS method have been recommended for the isolation of *L. monocytogenes* from meat products such as red meat and poultry (raw or cooked ready-to-eat), egg and its products and environmental samples. In case of clinical samples a modified AOAC method is recommended (Eld *et al*., 1993),

however, they also state that the standard methods may not be appropriate in every instance.

In case of FDA-BAM method, samples are enriched for 1-2 days at 30^{o}C in *Listeria* Enrichment broth (LEB). The enriched broth is then streaked onto selective media such as Oxford, PALCAM, MOX or LPM (Gasanov *et al*., 2005). Two step enrichment technique has been followed in ISO-11290 method. Initially, the samples are inoculated in half Fraser broth and later after incubation, an aliquot is transferred to full-strength Fraser broth for further enrichment. After respective incubation, enriched broths are plated onto selective agar plates such as Oxford and PALCAM agars (Gasanov *et al*., 2005).

The USDA method also uses a two-stage enrichment process using primary inoculation in UVM I for 24h and subsequent transfer of an aliquot into UVM II for further enrichment, followed by plating onto PALCAM agar (Gasanov *et al*., 2005)

In order to identify *Listeria* colonies conclusively the suspected colonies should be checked for purity. Generally, suspect positive cultures were plated onto Brain-Heart Infusion broth and observed for colonies by oblique lighting. A typical listerial colony appears to be reticulated with a distinct blue-green cast (Welshimer, 1981). The isolates which are usually classified as *Listeria* display Gram-positivity, grow in the presence of air, do not form spores, show catalase positivity and oxidase-negativity, and ferment sugars producing acid without gas. Most strains show motility at 25 ^{0}C and are non-motile at 37^{0}C (Welshimer, 1981). These cultural

methods are reliable and give good results; therefore are recommended by several regulatory agencies.

In addition to these conventional methods several commercial kits also are available. These kits utilize highly specific characteristics of the *L. monocytogenes.* These kits are employed widely as alternatives to the established biochemical testing. The traditional time consuming methods are comparatively tedious and takes over a week for discrimination of species. On the other hand, the commercially available test kits such as API Listeria (Bio-Merieux, France) and Micro-IDe (Remel, USA) have been expansively corroborated, fast, reliable, accurate and therefore are now used as a part of standard methodology (Hitchins, 2001; USDA, 2002).

2.3.2 Chromogenic media

Except *L. monocytogenes*, other members of listeria species possess ability to grow faster and therefore growth of *L. monocytogenes* gets suppressed. Therefore previously (before 1999) medias employed to isolate *Listeria* lacked the ability to separate out *L. monocytogenes* and other listerial species. Even the randomly selected suspected colonies were likely to be detected as non-pathogenic *Listeria* species, as they may supercede the growth of *L. monocytogenes*. Agar Listeria Ottaviani and Agosti (ALOA) (Biolife, Milan, Italy), RAPID'*L.mono* (BioRad, France), BCM *L. monocytogenes* detection system (Biosynth International, USA) and CHROM agar (Mast Diagnostics, Germany) are chromogenic media which allow fast and accurate detection. The chromogenic media utilizes the specific virulence related characteristics of pathogenic species of *L. monocytogenes.* All *Listeria* species exhibits β-glucosidase activity while phospholipase enzyme is synthesized exclusively

by pathogenic members such as *L. monocytogenes* and *L. ivanovii*. Taking the advanages of these properties, Agar Listeria Ottaviani and Agosti (ALOA) media has beend designed. β-glucosidase enzyme which is produced by all *Listeria* species cleaves chromogenic substrate producing blue/green colonies, while, phospholipase enzyme from pathogenic members hydrolyzes lecithin producing an opaque halo around the colony distinguishing these species as pathogenic *Listeria*.

However, as both *L. monocytogenes* and *L. ivanovii* produce phospholipase, ALOA can not differentiate between them (Restaino *et al*., 1999, Istafanos *et al*., 2002, Jinnemann *et al*., 2003, Vlaemynck *et al*., 2000, Bauwens *et al*., 2003). Therefore, another chromogenic test came in to practice that employs alanyl peptidase enzyme. *L. monocytogenes* does not produce alanyl peptidase as other *Listeria* species do. A simple colour reaction developed due to the hydrolysis of DL-alanine-b-naphthylamide and D-alanine-p-nitroanilide are used (Clark and McLaughlin, 1997). This test is commercialized as Monocytogenes ID Disc (Biolife, Milan, Italy). These media based on chromogenic substrates are relatively simple, cheaper and easy to use and interpret (Jeyaletchumi *et al*., 2010; Gasanov *et al*., 2005).

2.3.3 Hemolysis & CAMP test

Hemolysis reactions are generally shown by pathogenic species. The pathogenic *Listeria* species show hemolysis. Exceptionally, some strains of *L. seeligeri* also exhibit the hemolysis (Robinson *et al*., 2000).

Those species which do not exhibit the extensive hemolysis, are hard to distinguish. Groves and Welshimer (1977) devised a test – 'The Christie, Atkins,

Munch–Petersen' (CAMP) test in order to differentiate hemolytic *Listeria* species. The test uses β-hemolysin-producing *Staphylococcus aureus* and *Rhodococcus equi*. The two strains are streaked on 5% sheep blood agar plate and the listerial cultures are streaked at right angles. The principle of this test is that in presence of strong hemolysin of *S. aureus* the hemolysis by *L. monocytogenes* and *L. seeligeri* is enhanced, while in case of *L. ivanovii,* hemolysis is enhanced in the presence of the *R. equi* factors. At several instances differentiation of *L. monocytogenes* and *L. ivanovii* becomes diffiuclt by CAMP test (Vazquez-Boland *et al*., 1990). A commercial version of *b*-lysin discs based CAMP is available wich is much more user friendly and therefore recommended by USDA. (Hitchins, 2001; USDA, 2002).

2.4 Pathophysiology of listeriosis

Despite antibiotic treatment, listeriosis exhibits 20 to 30% or higher mortality rate in humans (McLauchlin, 1990a; Rocourt and Brosch, 1992; Schuchat *et al*., 1991). The clinical symptoms of listeric infection are very similar in all susceptible hosts. The disease can be broadly categorized as perinatal and non-perinatal. In case of feto-maternal and neonatal listeriosis, the fetus is infected via the placenta and results in either abortion or birth of a baby with sepsis or stilbirth of the fetus with granulomatosis infantiseptica or late neonatal listeriosis (Farber *et al*., 1991; Rocourt and Brosch, 1992; Slutsker and Schuchat, 1999).

Manifestations of listeriosis in adults which mostly occur in immune-compromised individuals includes meningoencephalitis (Lorber, 1996; Nieman and Lorber, 1980), bacteremia, gastroenteritis, endocarditis, inflammation of myocardium , arteritis, infection of lungs, pleuritis, liver infections , localized abscesses, , joint

infections,bone marrow infections, sinusitis, otitis, and infections involving eyes (Blenden *et al*., 1987; Farber and Peterkin, 1991; Gallaguer and Watakunakorn, 1988; Gauto *et al*., 1992; Lorber, 1990; 1996; Low and Donachie, 1997; McLauchlin, 1990a; Rocourt, 1994; Slutsker and Schuchat, 1999). Studies carried out on recent foodborne outbreaks have proved that febrile gastroenteritis is emerging as the main clinical manifestation of Listeriosis especially in immunocompetent individuals (Daniels *et al*., 2000; Dalton *et al*., 1997; Miettinen *et al*., 1999; Salamina *et al*., 1996; Slutsker and Schuchat, 1999).

In animals listeriosis is manifested as outbreaks of diarrhea, gastroenteritis, abortion storms, conjunctivitis, mastitis, hepatitis, pneumonitis and rhomboencephalitis (Ryser and Marth, 2007; Brugere-Picoux, 2008; Kumar *et al*., 2007; Kah and Scott, 2010; Vazquez-Boland *et al*., 2001).

Pathogenic *Listeria* can persuade phagocytosis in host cells followed by cell to cell transfer and since the pathogen remains within the host cells it is protected from the host defense mechanisms such as antibodies and complement. In the gastrointestinal tract the *Listeria* enters through enterocytes or M cells near small intestine's Peyers' patches. Once entered *L. monocytogenes* multiply in the primary phagocytic cells. Eventually, within the macrophages, these *L. monocytogenes* cells reach to the liver and spleen. Depending upon the individial hosts immune capability *L. monocytogenes* cells get killed by the neutrophils along with Kupffer cells. If person in immunecomporomised some cells get killed while some may persist. This explains why *L. monocytogenes* infects only the individuals whose immue system is weak. If the T cell mediated immune reaction is poor, the *Listeria* multiplies in the

hepatocytes and macrophages. Therafter, these cells spread by hematogenous route to other parts of the body particularly, the brain and pregnant uterus crossing the blood brain and placental barriers. The invasion process is mediated by a complex series of virulence factors produced by *Listeria* (Vazquez-Boland *et al*., 2001).

These virulence factors are encoded by genes which are present within the pathogenicity island of *Listeria* and are regulated by the *prf*A gene (Renzoni *et al*., 1999; Kuhn and Goebel, 1999; Cossart and Lecuit, 1998).

Several putative viruelnce factors play an important role in pathogenesis of *L. monocytogenes*. Surface protein p104 which helps in adhesion to intestinal cell (Pandiripally *et al*., 1999). Internalins are cell surface protein that contributes significantly in the virulence. Internalins allows the entry of *L. monocytogenes* cells to the epithelial cells of host (Gaillard *et al*., 1991). There are several types of internalins such as A, B, C, D and E which have been shown to be involved in the entry of *L. monocytogenes* in host cells (-Schuppler and Loessner, 2010).

Listeriosysin O (LLO) is a poreforming toxin that is only produced by pathogenic species of *Listeria* , lyses the vacuolar-membrane and allows the bacteria to enter into the cytoplasm (Low *et al*., 1992). LLO can also induce apoptosis and act as an inflammatory stimulus (Schuppler and Loessner, 2010).

Two phospholipases namely, phosphatidyl inositol specific phospholipase C (PI-PLC) and phosphotidyl choline specific phospholipase C (PC-PLC) play an

important role in the invasion and spread of *Listeria*. PI-PLC helps in cell to cell spread (Vazquez-Boland *et al*., 2001).

2.5 Isolation of *Listeria* from clinical cases

Listeriosis has been occurring in sporadic as well as outbreak forms all over the world, in humans as well as in animals and in many instances animals may act as reservoirs for the pathogen. Animals add to intensification and spreading of *L. monocytogenes* in the farm premises, and as a result cross-contamination can occur through produce generated on the farm. If, proper measures are not taken, this can lead to outbreaks in animal as well as human populations. Nowadays, epidemiological data pertaining to listeriosis is available from different countries including India which may be a result of increased awareness of the disease among the population.

Listeriosis as a rule is easily contracted by immunosuppressed individuals such as the elderly, neonates, pregnant women and patients with underlying morbidities resulting in malfunctioning of the immune system (McLauchlin, 1991).

A retrospective study conducted in 28 Turkish institutions for the elderly showed an infection rate of 8.8% in case of *L. monocytogenes* (Erdem *et al*., 2010). In Chile a 15 fold increase in the rate of occurrence of neonatal listeriosis has been reported for the year 2007-2008 and all the cases were reported in case of first pregnancies which could be due to similar lifestyles and food habits (Miguel *et al*., 2008). Occurrence of maternal–fetal infections for the time period 1994-2005 have also been reported from Denmark, however the survival rate was good in case of both mother as well as infant (Smith *et al.*, 2009). A collaborative surveillance study of

Listeria infections in Europe led by France Institute de Veille Sanitaire and Institut Pasteur for the year 2000 reported an incidence of 0.15 cases of listeriosis per 100,000 inhabitants for Spain, whereas, in Navarre a city in Spain the incidence was 0.91, similarly in 2004 a higher rate was reported in Navarre which could be due to mandatory reporting and active surveillance (Garrido *et al*., 2009). This study emphasizes the need for mandatory reporting in surveillance of listeriosis. The latest outbreak of listeriosis due to consumption of sour cream cheese occurred in the USA , eight individuals were infected and resulted in one death. Five cases were of feto-maternal nature (CDC, 2014).

In India, genital form of listeriosis has been reported most frequently and initial work was also carried out in patients with poor obstetric history. In the first study performed (Krishna *et al*., 1966), 14% patients with poor obstetric history were reported to be positive for *L. monocytogenes*. In another study, of the 670 women with a poor obstetric history, 9 (1.3%) were reported to be positive for *L. monocytogenes* (Bhujwala and Hingorani, 1975). *L. monocytogenes* was also isolated from 3.3% cases having a history of miscarriages (Kaur *et al*., 2007). Isolation of *L. monocytogenes* from clinical cases of abortions in South India was also reported by Dhanashree *et al*. (2003). Perinatal listeriosis in a two day old infant and mother was reported from Himachal Pradesh (Mokta *et al*., 2010), meningoencephalitis caused by *L. monocytogenes* in a twenty day old child has been reported from Srinagar (Peer *et al*., 2010). The most recent report is of listeriosis with disseminated tuberculosis in an HIV positive patient (Joel *et al*., 2013). It can be concluded that with increased awareness and availability of diagnostic techniques the rate of detection and diagnosis of listeriosis has increased.

Listeriosis in animals can be caused either by *L. monocytogenes* or *L. ivanovii*. Enteric listeriosis has been reported from a 10-11 month Freisian jersey heifers on a farm in New Zealand resulting in mortality (Fairley and Colson, 2013). Bovine listeriosis associated with encephalitis was reported in two steers from a farm in Parana, Southern Brazil, resulting in death of the infected animals (Headley *et al*., 2014). Natural infections of cattle associated with *L. innocua* is not common, however an atypical cerebral listeriosis associated with *L. innocua* has been reported in a beef bull (Rocha *et al.*, 2013). *L. monocytogenes* has been reported as the causative agent of sepsis and necrotizing typhocolitis in a 5 week old filly who later sucumbed to the infection (Warner *et al*., 2012). An outbreak of listeric infection was documented in a dairy cattle herd of 315 animals of which 9 animals displayed symptoms of encephalitic listeriosis and resulted in death of 3 animals with euthanization of 4 more due to severity of the symptoms (Bundrant *et al*., 2011), the *L. monocytogenes* serotype 4b strain which was isolated was identified as belonging to lineage III and displayed an extra band on carrying out serotyping. Cerebral listeriosis has also been reported in an adult Freiberger gelding which was later euthanised (Rutten *et al*., 2006). Four different manifestations of listeriosis were observed during an outbreak of Listeriosis which occurred in a flock of 55 sheep which were fed from the same batch of silage. Nine ewes aborted, 4 were septicemic and 1 was encephalitic. An isolate of *L. monocytogenes* indistinguishable from the outbreak strain was also isolated from a farm worker, however the cheese produced on the farm was free from the pathogen (Wagner *et al*., 2005). The above case proves that if adequate food safety rules are followed than even if the pathogen is present in the farm environment it may not contaminate the farm produce. In India sporadic cases as well as outbreaks of listeriosis have been reported from animal populations.

Different clinical manifestations have been reported including mastitis, meningoencephalitis and endometriosis. The first case of listeriosis in a domesticated animal was of an abortion in a ewe (Dhanda *et al*., 1959), subsequently a lot of work has been carried out on listeriosis occurring in different animal species. Studies carried out in migratory flocks of sheep and goats in the sub Himalayan region with a history of abortion storms revealed the presence of *L. monocytogenes* as well as *L. ivanovii* (Nigam *et al*., 1999). Mastitis caused by *Listeria* spp. is most economically damaging to the farmer as it is difficult to treat and results in culling of the infected animal. Pathogenic *L. ivanovii* has been isolated from the faces of a mastitic buffalo (Rawool *et al*., 2007); Bhilegaonkar and others (1997) and D'Costa and others (2012) isolated *L. monocytogenes* from milk and milk products in India. 4.4% of *L. monocytogenes* and one isolate of *L. ivanovii* have been isolated from buffaloes and ewes with reproductive disorders (Shakuntala *et al.,* 2006; Kaur *et al*. 2010). Elezebeth and others (2007) isolated *L. ivanovii* from aborted, mastitic and apparently healthy goats with the highest rate of isolation from the apparently healthy goats, and the highest seropositivity was shown by goats exhibiting abortion when tested for anti-LLO antibodies. Large outbreaks of listeriosis in the animal population have not been frequently reported in India, however, an outbreak of listeriosis with neurological symptoms was reported in a flock of broiler chickens (Vijaykrishna *et al*., 2000). Although, listerial meningoencephalitis in swine is infrequent a couple of outbreaks have been reported in India with circling movements and meningoencephalitis (Rahman *et al*., 1985; Dash *et al*., 1998). A recent study reports isolation of *L. monocytogenes* (5.11%) and *L. ivanovii* (0.93%) from clinical cases of sheep, goat, pig and pig rearing environment from the Konkan region (Raorane *et al*., 2014). Detection of listerial antibodies in serum samples of cattle from different

regions of Gujarat showed 31.13% of seroprevalence in case of tube agglutination test and 28.49% seroprevalence in case of indirect haemagglutination test (Rathod *et al*., 2010).

Animal production units may act as focal points for cross contamination of pathogenic *Listeria* into food and to humans and total eradication of the pathogen from the farm environment is impossible because of its ubiquitous nature, however intervention strategies should be planned and implemented in order to reduce animal infection and also to avoid its introduction into the human food chain.

2.6 Virulence genes

Though the traditional culturing techniques and immuno-based virulence testing methods are helpful to a certain extent, they are time-consuming and sometimes gives false positive results (Zhang *et al*., 2009). Hence, a need of more reliable methods as compared to phenotypic methods was obvious. Methods based on DNA are relativelt faster, accurate, reproducible and sensitive (Frece *et al*., 2010).

DNA microarrays use species probes that are designed considering the genetic structure of particular bacteria. Based on species specific gene polymorphism, particular bacteria are identified. Microarray techniques using probes for virulence genes, serotype specific sequences as well as lineage specific markers (Volokhov *et al*., 2002; Borucki and Call, 2003; Zhang *et al*., 2003; Doumith *et al*., 2004) have been developed for *Listeria*. However, DNA microarrays may be difficult to perform and may seldom show cross hybridization with DNA of organisms which show relatedness.

Though the *in vitro* tests give an idea about the presence of virulence characters, the actual virulence ability has to be confirmed by the *in vivo* test. Suspected strains can be tested by chick embryo. The mouse inoculation test is a highly preferred and essential method to correlate the isolates with the advent of infection. However, these *in vivo* methods are ethically demanding and can be performed in experimental situations only having facilities for animal house. Therefore, the pathogenicity and virulence mostly determined based on virulence markers (Notermans *et al*., 1991).

Identification of members of *Listeria* species from samples contaminated with other organisms by using conventional methods is time consuming and laborious. *In vitro* amplification by polymerase chain reaction of specific DNA markers makes it possible to detect as well to identify *L. monocytogenes* (Herman *et al.,* 1995). The detection of virulence causing factors *hlyA, plcA, actA* and *iap* are key aspects in case of *L. monocytogenes* (Kaur *et al*., 2010; Rawool *et al*., 2007). As several virulence genes have been identified from *L. monocytogenes* genome, detection of a single gene is not sufficient. Therefore, several genes are targeted in order to predict the pathogenicity (Nishibori *et al., 1*995). Absence of one or two genes due to spontaneous mutation may be observed, which leads to cause decreased virulence (Shakuntala *et al*., 2006). Therefore, detection of multiple virulent genes simultaneously in a single protocol is recommended.

Detection of virulence genes is an imperative factor in determining the pathogenicity of an isolate of *Listeria* especially from clinical cases. The virulence associated genes that are most frequently targeted for the purpose of amplification are *inlA, inlC, plcA, prfA, actA, hlyA* and *iap* genes (Vazquez-Boland *et al*., 2001). *L.*

monocytogenes isolated from water, human clinical and milk samples were observed to possess *inlA, inlC, plcA, prfA, actA, hlyA* and *iap* genes (Soni *et al*., 2013). *L. monocytogenes* and *L. ivanovii* isolated from bovine mastitis cases were seen to possess *plcA, prfA, actA, hlyA* and *iap* genes (Rawool *et al.*, 2007). A study carried out with clinical samples obtained from patients with spontaneous abortions yielded 3 *L. monocytogenes* isolates and were found to possess *plcA, prfA, actA, hlyA* and *iap* genes (Kaur *et al*., 2007). PCR fror the same five genes was performed for *L. monocytogenes* isolates obtained from cattles with a history of reproductive disorders , in which one isolate was seen to possess all the genes and the remaining isolates which lacked the *plcA* gene were seen to be non pathogenic in case of mice and chick embryo inoculation tests (Shakuntala *et al*., 2006). 25 *L. monocytogenes* isolates obtained from cases of human listeriosis in Italy were studied for PCR amplification of *inlA, inlJ* and *inlC* genes and all the isolates were positive for the genes (Mammina *et al*., 2009). A rapid microarray based assay has been developed for the reliable and simultaneous detection and discrimination of 6 species of *Listeria* based on the sequence differences in the *iap* gene and presence of *hlyA* indicates occurrence of *L. monocytogenes* and *L.ivanovii* (Volokhov *et al*., 2002).

Routine *in vitro* assays such as PI-PLC when clubbed with multiplex PCR assays of virulence genes demonstrate good potential to use as quick and reliable *in vitro* alternatives to *in vivo* pathogenicity tests (Aurora *et al*., 2008; Kaur *et al*., 2010).

2.7 Immunobased techniques

Antigen-antibody reactions are highly specific which can identify whole cells and sub-cellular components as well. In ELISA, antilisteria antibodies are used in a

microtitre well and they react with *Listeria* antigens. Results are obtained quicker than culture methods (Barbuddhe *et al*., 2002) but not competitive to molecular methods (Jadhav *et al*., 2012). A commercial test VIDA LMO2 assay (Bio Merieuse) based on an enzyme-linked fluorescent assay specific for *L. monocytogenes* (Ueda and Kuroabara, 2010) is used to test food samples.

Immunomagnetic separation uses paramagnetic polystyrene beads to which specific antibodies are linked covalently. Antigen antibody reaction takes place in a magnetic field (Wadud *et al*., 2010). Magnetic capture hybridization using nanoparticles has also been used an immunobased techniques (Amagliani *et al*., 2006). Immunomagnetic nanoparticles coated with rabbit anti-*Listeria monocytogenes* antibodies in combination with RT-PCR are used for quick detection of *L. monocytogenes* in milk (Yang e*t al*., 2007).

2.8 Molecular methods of detection

Conventional culture methods as well as immunobased methods are time consuming and can result in false positives (Zhang *et al*., 2009) and hence genetic methods are more reliable (Freece *et al*., 2010).

DNA microarrays use bacteria or species/strains specific probes in order to detect particular bacteria or to detect variations in species specific genes. This technique has been used to identify *Listeria* spp. by using species specific probes for virulence genes (Volokhov *et al.*, 2002), serotype specific differentiation as well as lineage specific markers (Borucki and Call, 2003; Doumith *et al*., 2004a).

PCR based methods are carried out by performing conventional PCR technique and targeting various virulence genes, intergenic spacer regions, rRNA genes, *hly*A, *actA, iap, plcA, fbpA,* for detection of *L. monocytogenes* using PCR (Aznar and Alarcon, 2002). As PCR detects the DNA irrespective of dead or live cells, the disadvantage is that it is unable to distinguish between DNA from dead or viable cell, (Lei *et al*., 2008). To distinguish the live cells, mRNA can be used in place of DNA by reverse transcriptase PCR (RT-PCR) (Jadhav *et al*., 2012). As mRNA degenerates faster as compared to DNA, detection through mRNA is more valid for live cells.

Several multiplex PCR have been designed in order to detect different foodborne pathogens in single reaction. Therefore is economical, reliable and time saving than conventional PCR (Kaur *et al*., 2007). Though, the problem comes when the resulting amplicons are of same size. However, with the increading trends of genome and genomic tools, it is easy nowadatys to design the primers quickly and of the size of your choice. Therefor, it gives an advanatges that one will be able to see the target amplicons size on agarose gels (Mustapha and Li, 2006).

Taking the advantages of polymerase chain reaction and double strand binding dyes, real-time PCR have been designed. In this technique DNA binding dyes have been employed. SYBR-Green dye is most commonly used dye which binds to double stranded DNA only is cost effective and easily available. During the PCR the amplicon constructed in run get illuminated due to binding of these dyes. The illuminated fluorescence then gets detected by the CCD camera attached to machine. The level of fluorescence indirectly accesses the level of double strand DNA or

amplicons. The other approach to SYBR green is the TaqMan real-time PCR probes. Attachments of probes quench the fluorescence molecule attached, which get measured by camera. In addition, the real-time PCR, one can monitor the live coverage of the polymerase reactions (Norton, 2002). These approaches have been standardized to enumerate the bacterial cells in particular food samples (Gasanov *et al*., 2005, Churchill *et al*., 2005, Liu, 2006; Jadhav *et al*., 2012).

2.9 Other techniques for detection of *Listeria.*

With the advances in technologies, the boundaries to detect the pathogens have also been increasing. Various methods have been developed to detect *L. monocytogenes* from foods and clinical samples.

The denaturing gradient gel electrophoresis along with temperature gradient can distinguish the different nucleotide sequence. Taking the advantage of this method, *Listeria* species can be distinguished. Despite the lengthy procedure involved in this technique, several researchers used DGGE and TGGE to detect the *Listeria* species from food samples (Cocolin *et al*., 2002; Tominaga, 2006).

Biosensors also have been developed to detect *L. monocytogenes.* Biosensor generally contains a signal induing molecule which then get amplified and represented in digital manner. The BIA3000 biosensors were used to detect *L. monocytogenes* Leonard *et al.,* (2004); however the use is limited to liquid solutions. The sensitivity of BIA3000 is high and can detect as low as 10 cells/ ml. The other approach is Surface Plasmon Resonance (SPR) which is based on optical signals and their refractive index. These biosensors can detect the pathogens in water as well as in

environmental samples (Poltronieri *et al*., 2009). Banarjee and Bhunia (2010), have developed the biosensor was where lymphocytes embedded matrix acts as a biosensor which differentiate the pathogens and non-pathogens based on cytotoxicity to lymphocytes. The sensitivity of these sensors is relatively poor and only 10^2–10^4 cfu/g could be detected.

LAMP is a method in which uses Bst DNA polymerase ((large fragment of *Bacillus stearothermophilus* DNA displacement activity) to amplify the target DNA. This amplification does not require a thermal cycler and can be carried out in a water bath (Prasad and Vidyarthi, 2009). The hlyA gene based LAMP assay developed by Tang *et al*. (2011) exhibited 100 fold more sensitivity thatn conventional PCR (Tang *et al*., 2011). Compared to PCR, this assay is simple, quicker, low in cost and very sensitive.

Due to simplicity and economical feasibility, methods based on spectroscopy are prefered across several laboratories (Holt *et al*., 1995). Spectroscopy based methods generally targets either single or combined basic elements such as DNA, RNA, lipids, proteins and carbohydrates (Al-Qadiri *et al*., 2008). Based on profile of standard bacteria, the profile of test bacteria is compared and identified (Janbu *et al*., 2008). The Fourier transforms infrared spectroscopy (FTIR) could detect all the *Listeria* even at species level (Gupta *et al*., 2006; Jadhav *et al*., 2012).

Colloidal silver surface enhanced Raman spectroscopy (SERS) technique was used to differentiate between important foodboene pathogenic species (Liu *et al*., 2009). An exclusive species specific fingerprint of ribosomal proteins has been obtained by using MALDI-TOF MS to whole cells to identify bacterial species from

different sources (Barbuddhe *et al*., 2008). The same technique has also been used for differentiation of various genera including *Staphylococcus, Streptococcus, Neisseria, Salmonella, Aeromonas, Campylobacter* and *Helicobacter* (Jadhav *et al*., 2012) and also to detect bacteria isolated from urine and blood samples (Ferreira *et al*., 2010; Drancourt, 2010). However, this technology needs standardization as the several growth and environmental factors can significantly affect the mass spectra obtained (Murray, 2010).

2.10 Typing methods

Bacterial typing and subtyping differentiate the particular bacterial strain. Typing/sub-typing of the pathogens is very important from taxonomy, epidemiology and phylogeny; mainly for characterization, food borne illness surveillance, investigation of outbreaks and tracking of sources throughout the food production chain (Wiedmann, 2002). Criteria for choosing a typing method include ability to distinguish pathogens, cost, standardization to suit laboratory conditions and ability to reproduce the results, ease of automation and use and its suitability for discrimination of different bacterial species.

Bacterial subtyping methods can be subdivided as conventional or phenotypic and genetic or nucleic acid based methods. Serotyping, phagetyping, plasmid profiling and multilocus enzyme electrophoresis are the commonly used conventional typing methods.

Serotyping has been proved to be an important tool in epidemiological investigations. The typing method targets antigens present on cell surface including

somatic (O), and flagellar (H) antigens. These surface antigens that are presented on the cell wall can be detected by antibodies and antisera reactions (Wiedmann, 2002). Thirteen serotypes of *L. monocytogenes* have been identified based on the combination of agglutination reactions of O and H antigens (McLauchlin and Jones, 1998). However, serotyping has the disadvantage of being expensive, tedious, and much time consuming. At times, there may be probability of cross reaction of the antigens from different strains with different antibodies. Also antigens that do not get expressed on the cell surface cannot be detected by this method (Riley, 2004).

However, *L. monocytogenes* serotyping has relevance in epidemiology. It has been used to authenticate the epidemiological concordance of other molecular subtyping methods used in conjunction. Also the molecular methods that give data matching with serotyping are believed to be epidemiologically and phylogenetically pertinent (Nightingale *et al*., 2005; Chen and Knabel, 2007; Nadon *et al*., 2001).

Phage typing is based on susceptibility of the *Listeria* isolates to lysis by a standard set of phages (Loessner and Busse, 1990). However, not all *L. monocytogenes* isolates were susceptible to the phages and results were not reproducible because of unstable phages, the technique also requires specialized skills (Graves *et al*., 1999).

Bacterial tying using plasmid profiling in comination with antibiotic resistance typing can be used for subtyping *L. monocytogenes* (McLauchlin *et al*., 1997).

Multilocus enzyme electrophoresis (MEE) uses the electrophoretic mobility patterns of different constitutive enzymes to distinguish different bacterial strains. Soluble enzymes from cell extracts are electrophoretically separated in starch gels and the enzyme activities are detected in the gel by using colour generating substrates. MEE has increased typing ability , ability to reproduce results and discriminatory power as compared to other phenotypic methods (Bibb *et al*., 1990) but is less discriminatory then DNA subtyping (Graves *et al*., 1999).

Serotyping is also carried out by using ELISA and multiplex PCR assays. An ELISA based assay which is similar to the slide agglutination technique has been used for serotyping but it is time consuming and requires high quality antisera (Palumbo *et al*., 2003).

Listeria isolates have been subtyped on the basis of MAMA PCR and the results obtained were similar to those obtained by slide agglutination (Jinneman and Hill, 2001). The four major strains which were serotyped were 1/2a, 1/2b, 1/2c & 4b.

A microarray using probes specific for regions of genetic dissimilarity between different *Listeria* spp. based comparative genomics approach was employed to define 3 genetic lineages I,II and III of *L. monocytogenes.* (Doumith *et al*., 2004a). These lineages were again subdivided into five serovar specific groups namely I.1(1/2a, 3a), I.2(1/2c,3c), II.I (4b,4d,4e), II.2 (1/2b,3b,7) and III (4a,4c). However, the assay failed to distinguish between 1/2a and 3a,1/2c and 3c, 1/2b, 3b and 7, 4a and 4c and between 4b, 4d and 4e . A similar multiplex PCR assay was also proposed

by Kerouanton *et al*., (2010) wherein all serotypes were subgrouped molecularly except serovars 4a, 4d and 7.

Chen and Knabel (2007) designed a PCR assay to recognize the genus *Listeria*, discriminate the *L. monocytogenes* strains and characterize them as epidemic clones.

PCR based methods of serogrouping have been developed and validated as alternatives to serological based methods which employ antigen and antibody reactions as conventional serotyping method. The techniques used can not only be used for serogrouping but also for identifying presence of variants within the serogroups.

A detailed analysis of five 4b variant strains which showed the presence of an additional 1/2a-3a specific amplicons in addition to the standard 4b, 4d, 4e specific amplicons revealed predisposition of some 4b strains to accept DNA from related organisms (Laksanalamai *et al*., 2014). A study carried out on 211 samples from Sardinian swine slaughter houses yielded 50 *L. monocytogenes* isolates in which only 2 serotypes were detected 1/2c (78%) and 1/2a (22%) (Meloni *et al*., 2013). Subtyping of *L. monocytogenes* isolates obtained from RTE food processing plants and listeriosis patients in Sweden in 2010 was carried out and it was observed that 18% of human isolates and only 1.4% of food isolates belonged to serotype 4b (Lambertz *et al*., 2013). A study carried out in India also showed a predominance of the 4b serotype in *L. monocytogenes* isolates of human origin (Soni *et al*., 2013). Somewhat similar results were also obtained in a study on clinical isolates carried out in Andalusia in the

period 2005-2009 wherein it was observed that 61% of the isolates belonged to serotype 4b, 19% to serotype 1/2b, 18% to serotype 1/2a and 2% to serotype 1/2c (Lepe *et al*., 2012). Seven *L. monocytogenes* isolates obtained from healthy sheep of Gujarat were seen to belong to serotype 4b (Yadav and Roy, 2009). In a study carried out in Poland 75 *L. monocytogenes* strains were first serotyped by conventional serotyping method by using liquid antisera (Mast Diagnostics UK) and later on multiplex PCR according to Doumith *et al*., 2004b was performed. 34 isolates belonged to serogroup 1/2a-3a, 2 to serogroup 1/2c-3c, 11 to serogroup 4b, 4d, 4e, 21 to serogroup 1/2b, 3b, 7 and 9 to serogroup 4a, 4c (Madaiczk and Majczyna, 2009).

Various other subtyping methods such as biotyping, antibiotic resistance typing and monocine typing (Harvey and Gilmour, 2001; Wilhelms and Sandow, 1989) have been used to a limited extent.

Genetic subtyping methods which use restriction enzymes are described. Ribotyping is basically a RFLP method which employs polymorphism detected in the rRNA gene. It has been used for lineage specific discrimination of *L. monocytogenes* (Wiedmann *et al*., 1997; Jaradat *et al*., 2002). It is able to discriminate the strains on the basis of their source of origin, and the system has also been automated (Schukken *et al*., 2003). Although this technique has a discriminatory power which is comparable to MLEE and it is reproducible, it cannot efficiently distinguish between strains belonging to 1/2b and 4b serotypes (Graves *et al*., 2007).

Pulsed field gel electrophoresis (PFGE) technique involves macrorestriction of DNA wherein restriction endonucleases are used which produce several fragments of

chromosomal DNA ranging in size from 40 to 600 kb. The genomic DNA is immobilized in an agarose plug and generally a combination of *Asc*I and *Apa*I is used for digestion of *L. monocytogenes* genomic DNA (Wiedmann, 2002). The plugs with the restriction-digested DNA are placed into an agarose gel and electrophoresed for longer time (10-52 h) with alternating currents. PFGE generates distinct band patterns and the isolates can be chracterised to belonging to a particular pulsotypes and this subtype differentiation is considered as the reference for comparison (Graves and Swaminathan, 2001) or "gold standard" for epidemiological investigations of foodborne illnesses. Considering the usefulness, the CDC has developed a network programme, Pulsenet (www.cdc. gov/pulsenet/) for health and food regulatory laboratories that regularly practice PFGE for subtyping of foodborne pathogens including *L. monocytogenes* (Graves and Swaminathan, 2001). PFGE is a highly sensitive method which can detect changes at a genomic level such as point mutations, insertions, deletions and transpositions and it is particularly used for subtyping 4b serotype involved in outbreaks. Some of its disadvantages are that PFGE needs skilled labour, particular equipment, costly restriction endonucleases and is a laborious and lengthy process (Graves and Swaminathan, 2001).

PFGE is generally used to elucidate the difference between isolates belonging to the same serogroup at a molecular level. In a study carried out on 54 *L. monocytogenes* isolates from human clinical cases it was seen that on macrorestriction with *Asc*I, 3 pulsotypes were recognized and 11 molecular subtype clusters could be identified including some unique pulsotype patterns (Mammina *et al*., 2009). PFGE analysis of five 4b variant strains which showed presence of an additional *lmo*0737 amplicon showed 3 distinct banding patterns, however the PulseNet Database of *L.*

monocytogenes did not reveal any matches (Laksanlamai *et al*., 2014). 20 different pulsotypes were revealed among clinical isolates from Spain and when compared to the PFGE profiles of foodborne isolates it was seen that the same pattern was shared by a clinical strain and 7 isolates obtained from food (Garrido *et al*., 2009).

Amplified fragment length polymorphism (AFLP) is a sensitive technique having high reproducibility which can be used for strain level differentiation of *L. monocytogenes* (Lomonaco *et al*., 2011). AFLP makes use of two restriction enzymes and sequences complementary to the recognition site of restriction enzymes linked to double stranded adaptors . Denaturing polyacrylamide gels are used and PCR is used to amplify the fragments and subsequently subjected to capillary gel electrophoresis with an automated sequencer for accurately determining the size (Lomonaco *et al*., 2011; Mikasova *et al*., 2005). This technique has been used in tracking the source of contamination in the food processing environment.

Restriction fragment length polymorphism (RFLP) aims polymorphisms present within and between restriction sites, purified genomic DNA is cut with restriction endonucleases and the fragments obtained are separated by electrophoresis. The location of recognition sequence for the restriction endonucleases will be different in different strains and so different gel patterns will be obtained. PCR- RFLP is carried out by amplifying a housekeeping or virulence gene of *L. monocytogenes* followed by digestion with restriction endonucleases. Comparison of the banding patterns is used for subtyping of the *L. monocytogenes* isolates (Tamburro *et al*., 2010; Paillard *et al*., 2003).

2.11 Antibiotic sensitivity

Listeriosis is a severe deadly disease among the immunocompromised perosnnels (Farber and Peterkin, 1991; Goulet and Marchetti, 1996; Mylonakis *et al*., 1998). The recovery of the patient is dependent to a large extent on the early antibiotics treatement with rapid bactericidal activity against *L. monocytogenes* (Goulet and Marchetti, 1996; Hof, 2004; Mylonakis *et al*., 1998). Generally, *L. monocytogenes* strains are susceptible to a wide range of antibiotics, except for the expanded spectrum cephalosporins, fluoroquinolones and fosfomycin (Hof, 1991). Currerntly, combination of high doses of aminopenicillin and gentamicin is preferred (Lorber, 1997; Hof, 2004; Temple and Nahata, 2000). While, Rifampin, vancomycin, linezolid and carbapenems are also used in treatment (Benes *et al*., 2002; Goulet and Marchetti, 1996; Hof, 2003; 2004; Temple and Nahata, 2000). In situations, where beta-lactam antibiotics treatement remains unresponsive combination of trimethoprim with a sulfonamide, such as in co-trimoxazole is preferred (Hof, 2004; Temple and Nahata, 2000). Comparative to other bacterial pathogen, less acquired resistance has been observed in case of *L. monocytogenes*. However, since the first report of *L. monocytogenes*, increasing reports of antibiotic resistance have been reported. (Aureli *et al*., 2003; Charpentier and Courvalin, 1999; Conter *et al*., 2009; Li *et al*., 2007; Srinivasan *et al*., 2005; Walsh *et al*., 2001; Poyart-Salmeron *et al*., 1992; Safdar and Armstrong, 2003; Godreuil *et al*., 2003). The first strains of *L. monocytogenes* resistant to antibiotics were reported in 1988 (Poyart-Salmeron *et al*., 1990). In a study carried out in France to check the prevalence of resistance among *L. monocytogenes* strains isolated from humans 4,668 clinical strains of *L. monocytogenes* were subjected to a panel of 23 antibiotics. 2 isolates showed multidrug resistance to chloramphenicol, erythromycin, streptomycin and tetracycline,

34 isolates showed resistance to tetracycline, 20 were found to be resistant to flouroquinolones, 4 strains were resistant to streptomycin, 3 strains each were resistant to chloramphenicol and erythromycin, 1 strain was seen to be resistant to trimethoprim which is the first report of a strain of human origin showing resistance to the antibiotic (Morvan *et al*., 2010). Multidrug resistant strains of *Listeria* due to acquisition of plasmids have been sporadically detected (Quentin *et al*., 1990; Hadorn *et al*., 1993; Tsakris *et al*., 1997). In a study carried out in Spain resistance towards tetracycline and doxycycline has been observed (Vitas *et al*., 2007). A major study on antibiotic resistance carried out with 1100 isolates from all over the world reported tetracycline, minocycline, streptomycin as well as trimethoprim resistance in *Listeria* species (Charpentier *et al*., 1995).

In order to assess the antibiotic resistance profiles of bacteria, several methods have been standardised. In case of pathogens, these resistance pofiles provide valuable guidance and revealed to be valuable in final treatements. Also, contineous monitoring allows understanding the emerance of the antibiotic resistance. Standard regulatory authorities such as CLSI, EUCAST, OIE, BSAC, SFM, SRG and CDS have provided several guidelines and recommendations to study the antiobiotics resistance of *L. monocytogenes.* The organizations which specify methods as well as interpretive criteria for veterinary pathogens are CLSI in USA, OIE in European Union and CDS-AST in Australia (Turnidge and Peterson, 2007; Watts and Lindemann, 2006).

Antibiotic susceptibility can be tested using various methods such as quantitative, qualitative, automated susceptibility, newer non automated susceptibility and molecular techniques.

2.11.1 Quantitative methods.

Broth dilution test is also called as macrobroth or tube dilution method (Ericsson and Sherris, 1971). In this test serial twofold are made in test tubes from 0 to maximum concentration that can be given in vivo without resulting in toxicity to the patient dilutions (Ericsson and Sherris, 1971, Turnidge and Peterson, 2007). 1 ml of bacterial suspension is added to the dilutions, incubated overnight and checked for lowest concentration of antibiotic that shows visual inhibition of growth (Balows, 1972). The main disadvantages of this method are the antibiotic solutions are supposed to be prepared manually and therefore large amounts of reagents and space is required.

Microbroth dilutions are small, disposable trays containing 96 wells with small volumes of serial twofold dilutions of different antibiotics. Inoculation of panels with standard amount of inoculum is carried out by using a disposable device and after incubation MIC is determined by using an automated viewing device (Jorgensen and Turnidge, 2007). This method has the advantage of having readymade panels, economy of reagents and space.

Compared to the above two methods, commercially available antibiotic concentration gradient strips gives several advantages. This test uses a plastic strip with gradients of antimicrobial agent. To test the particular bacterial strain, the growth is generally spread plated onto the agar media and this strip is placed on it. The

antiobiotic present on the strip get diffused according to their concentration and inhibit the growth. The main advantages of this strips are that they come prelabeled and in dry conditions, therefore easy to use. Also, one can test whole range of MIC in a sigle experiment. Several strips can be placed on a single agar plates (Gómez *et al*., 2014; Moreno *et al*., 2014; Hansen *et al*., 2005; Okada *et al*., 2011). These tests have been show to exibit good correlation with results obtained from other methods.

Agar dilution method uses twofold dilution of antibiotics incorporated into Mueller-Hintons agar. Bacterial inoculum is standardized and inoculated on the surface of the agar by using calibrated loops. After overnight incubation the lowest concentration of antibiotic that inhibits visible growth is taken as MIC (Baker *et al*., 1991).

2.11.2 Qualitative methods.

If the antibiotic resistance profiles for particular pathogens are known, qualitative methods help to determine the overall antibiotic sensitivity. Disk diffusion methods are generally used for the qualitative analysis of the antibiotic resistance. In this method bacterial inoculum is spreaded onto the Muller-Hinton agar and paper-disc containing specific concentration of antibiotics are placed on it and incubated overnight. The no-growth zone is then measured and recorded. Clinical and Laboratory Standards Institite CLSI have provided the guidelines (CLSI, 2009). The disc diffusion method is universally preferred.

2.11.3 Automated susceptibility methods.

Detection of culture in well plates with antimicrobial agent is carried out by using computer aided instruments. Several commercial systems are available that use readymade and arranged microdilution trays that carry out automated reading of plates. These tests are rapid, convenient and provide automated reading and interpretation based on turbidimetric, fluorimetric detection and video image processing e.g. Vitek system (bioMerieux, France), Walkaway system (Dade International, California), Sensititre ARIS (Trek Diagnostic Systems, UK), Avantage Test System (Abbot laboraties , Texas), Micronaut (Merlin, Germany), Phoenix, BD Biosciences , Maryland) etc.

2.11.4 Non Automated susceptibility methods.

Mechanism specific tests can directly detect the occurrence of a particular resistance mechanism. β–lactamase finding is carried out by using the chromogenic cephalosporinase test and chloramphenicol resistance is detected by CAT assay which identifies the enzyme chlromamphenicol acyl transferase.

2.11.5 Molecular techniques

Genotypic methods detect specific genes that confer the property of antibiotic resistance. Some of the methods used for this purpose are PCR using specific primers to amplify specific DNA sequences. Use of labeled probes, DNA arrays, DNA chips, molecular beacons etc.

2.12 Glutamate decarboxylase system

The glutamate decarboxylase system (GAD) has been related to acid resistance in many bacteria and it has been found in bacteria that are foodborne pathogens and need to transit the stomach in order to invade and colonize the gut epithelium (Bank *et al*., 1989; Cozzani *et al*., 1970; Smith *et al*., 1992).

Listeria monocytogenes, an intracellular facultatively anaerobic pathogen implicated in many foodborne outbreaks (Schlech *et al*., 1983). The *L. monocytogenes* abililty to resist low pH and bile gives advantages to cells to survive in the stomach and intestine. These two abilities are responsible for survival and asymptomatic carriage. *L. monocytogenes* is not a part of normal intestinal flora of human and animal (Karatzas *et al*., 2012; Feehily *et al*., 2014), however recent symptomless infections in healthy individuals with *L. monocytogenes* may play an important role in transmission of the pathogen.

The main mechanism lying behind the tolerance of pH is ability of *L. monocytogenes* cells to exclude the excessive proton ions from the cytolplasm. This exclusion is carried out by the F_0F_1-ATPase (Cotter *et al*., 2000). While, glutamate decarboxylase (GAD) system is also has been shown to involve partially in removal of protons (Cotter *et al*., 2001). The GAD system converts glutamate to γ-aminobutyrate (GABA) which involves a utilization of protons reducing the final concentration. Thefore, overall cytoplasmic final proton concentration gets reduced, allowing the cells to survive in the acidic conditions (Small and Waterman, 1998). Glutamates are generaly added in different forms to enhance the flavor and also to enhance the acidity. *L. monocytogenes* contamination in such food therefore selectively enriches the growth (Cotter *et al*., 2001).

The tolerance limit of *L. monocytogenes* strains can be studied *in vitro* by carrying out Glutamate decarboxylase activity assay (Cotter *et al*., 2001a, b). Cells are allowed to grow in presence of pH indicator bromocresol green. The change in colour of the test solution after respective incubation exhibits the ability to neutralize pH. The test is useful for primary screening of *L. monocytogenes.*

Porcine bile salt tolerance assay is performed by spot inoculating the isolates onto the BHI agar plates supplemented with 1% porcine bile salts and incubating at 37^{o}C for 48 h. The extent of bile salt degradation is based on the diameter of the zone around the colony.

The genetic determinants of the GAD system has been traced to the *gad*F and *gad*G genes (Sanders *et al*., 1998; Cole *et al*., 1998). The homolgous genes to the *gad*F and *gad*B genes from *E. coli*, *L. lactis* and *M. tuberculosis* were knocked out and showed that absence of GAD system decreases the acidic tolerance limits (Smith *et al*., 1992).

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CHAPTER 3

ISOLATION OF *Listeria* FROM HUMANS AND ANIMALS

3.1 Introduction

Listeria monocytogenes is an important pathogenic bacteria which is transmitted through food and therefore food samples, clinical samples as well as environmental samples are screened for its presence. The traditional methods of identification include growing the bacteria by using selective enrichment media and subsequent identification and characterization by observing primarily the morphology of the colonies followed by sugar fermentation tests as well as tests to observe haemolytic activity (Gasanov *et al*., 2005). Numerous traditional and quick protocols are presently accessible for the identification and characterization of *L. monocytogenes* in human and animal clinical as well as food samples. Most of the conventional methods are considered to be the 'gold standards' and the results obtained by the newer methods are compared and confirmed with respect to them. They usually yield pure culture of the organism and do not need expensive and sophisticated equipment. However, they have a few disadvantages such as lengthy protocols, experimental errors, the need of various chemicals, supplements and basal or selective growth medium, the probability of overgrowth of contaminants as compared to the target organism, identification of atypical variants and the difficulty in interpretation of colonies on the selective agar or differential agar mediums (Liu, 2006; Gasanov *et al*., 2005). The methods used for isolation and identification of *Listeria* make use of selective agents and enrichment procedures in order to exclude contaminants and permit multiplication of *Listeria* population enough for detection of the organism. One of the first methods used to isolate *Listeria* was the cold enrichment method (Gray *et al*., 1948, Lorber, 2007) and it was based on the fact that *Listeria* can multiply at refrigeration temperatures, whereas the contaminants could not. However, the cold enrichment method required long incubation periods,

frequently extending to months, thus, it was not practical for study of epidemic cases, as well as for the implementation of efficient control programmes like Hazard Analysis at Critical Control Points in food proceesing and manufacturing plants and factories where fast results are required. To solve this problem, nowadays *L. monocytogenes* is grown at normal incubation temperatures on selective culture media, containing compounds inhibitory to fungus, other Gram positive and Gram negative bacteria such as cycloheximide, acriflavine, $LiCl_3$, nalidixic acid, phenylethanol, ceftazidime, polymixin B and moxalactam which prevent growth of other contaminants (AFNOR, 1997; AFNOR, 2000; AOAC, 2000; Hitchins, 1998; ISO, 1996; USDA-FSIS, 2002).

The United States FDA protocol (Hitchins, 1998), the Association of Official Analytical Chemists (AOAC) (AOAC, 2000), the ISO-11290 method (ISO, 2004), the USDA-Food Safety and Inspection Service (FSIS) method (USDA-FSIS, 2002) and the French Standards (AFNOR, 1997; AFNOR, 2000) are some of the conventional isolation methods which are used worldwide. Some of the above methods give better results with certain type of samples. ISO standard 11290 method can be used for isolation and detection of *Listeria* from food and animal feed. Milk and dairy products are screened by using FDA and AOAC methods and the USDA-FSIS method is best suited for meat and meat products, poultry products and environmental samples. In order direct plating on sheep blood agar, and a modified AOAC method is recommended to isolate *Listeria* from animal and human clinical samples (Alles *et al*., 2009; Feldsine *et al*., 2009).

Most of the methods include selective incubations step using selective liquid media. After enrichment, the cultures are plated on selective/differential media such as PALCAM and MOX agar for isolation of presumptive colonies of *Listeria*. Chromogenic media like ALOA are also used on which typical colonies of *Listeria* appear blue–green in colour and colonies of *L. monocytogenes* are blue-green with an opaque halo around them (ISO, 2004). Typical colonies of *Listeria* obtained on ALOA agar should be carefully stored for characterization and further studies.

Characterization of *Listeria* using conventional methods is carried out by using several frequently used tests such as the Gram-staining, catalase test and motility test by microscopy or by motility in agar detection, hemolysis and sugar utilization tests (Robinson *et al*., 2000). The CAMP test forms the part of the ISO-11290 and AOAC procedures and the test is also mentioned in the FDA and USDA-FSIS techniques. It can aid in differentiation of *Listeria* spp. isolates. In case of positive reactions, a prominent zone of ß-hemolysis appears at the junction of the test/control and indicator strains. *L. monocytogenes* shows enhanced haemolysis with *S. aureus* and none with *R. equi*, whereas the reverse is observed in case of *L. ivanovii* (Quinn *et al*., 1999; McKellar, 1994).

In India, *L. monocytogenes* infections have been considered to occur as sporadic cases. It is not a notifiable disease in India. Consequently, no systematic data is available on occurrence of listeric infection. The first objective of this thesis focuses on isolation of *Listeria* spp. from suspected cases of humans and animals and characterization by conventional biochemical methods. Clinical samples were

collected from Goa and parts of Maharashtra and processed for isolating *Listeria* spp. using recommended standard methods

3.2 Material and Methods

3.2.1 Bacteria

Listeria monocytogenes (MTCC 657 / NCTC 7973 /ATCC 19111), *L. monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135) strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The reference strain of *Listeria ivanovii* (NCTC 11846) was procured form VPH Division, Indian Veterinary Research Institute, Izatnagar.

The strains were verified for their morphology, biochemical and cultural characteristics. Subsequently, the strains of *L. monocytogenes* were tested for their pathogenicity by *in vitro* tests. Finally, the strains of *L. monocytogenes* were tested for PI-PLC activity by ALOA assay (Ottavaini *et al*., 1997).

3.2.2 Samples

The details of samples processed are given in Table 3.1. A total of 596 clinical samples were analysed. Of these 481 samples were of human origin and comprised of urine, faeces**,** blood, serum, placental bits, vaginal and cervical swabs and cerebrospinal fluid. The samples were obtained from different hospitals of Goa and parts of Maharashtra. One hundred fifteen samples were of animal origin and comprised of blood and milk samples obtained from buffaloes and cows from various farms in Goa. The samples were processed for detection of *Listeria*.

3.2.3 Isolation

Isolation of *Listeria* was attempted from the clinical samples employing the USDA method as described by McClain and Lee (1988) with necessary modifications.

3.2.3.1 Enrichment

Tissue samples were first cut into pieces and then macerated in a stomacher. 25 gm of macerated sample was inoculated in 250 ml of University of Vermont Medium-1 (UVM-1) and incubated overnight at 30^0C. Blood and milk samples (10 ml each) were inoculated into 90 ml of UVM-1 directly and incubated overnight at 30^0C. An aliquot from the first enrichment broth, UVM-1 (0.1 ml) was then transferred to 9 ml UVM-2 medium and again incubated overnight at 30^0C. Cerebrospinal fluid samples (0.1 ml) were directly streaked on sheep blood agar.

Table 3.1. Details of samples collected from humans and animals.

Place	Samples (Human Origin)	Number Of Samples
Panjim (GMC)	Placental bits	114
Panjim (GMC)	Urine	318
Panjim (GMC)	Stool	13
Panjim Private Hospital	Cervical and vaginal swab	5
Margao Private Hospital	Vaginal swab	1
Porvorim	Paediatric stool sample	1
Primary Health Centre	Paediatric stool sample	8
Nagpur	Cerebrospinal fluid	10
Pune	Blood and serum	2
Kolhapur	Placental bits	4
Kolhapur	Vaginal swabs	5
	Samples (animal origin)	
Goa	Buffaloes (blood)	4
Goa	Buffaloes (milk)	69
Goa	Cows (milk)	42

3.2.3.2 Plating on Selective media

The enriched inoculum from UVM-2 was plated on PALCAM agar (Himedia Labs). The plates were incubated at 37^0C for 24-48 h. Typical grey green colonies with black sunken centers or bulls eye appearance on PALCAM were presumed to be of *Listeria*. The presumptive colonies (at least 3/plate) were subcultured for further processing.

3.2.4 Confirmation of Isolates

3.2.4.1. Biochemical characterization

From the selective medium, presumptive colonies of *Listeria* were streaked on 5% SBA. Colonies showing characteristic morphology were confirmed by morphological, biochemical tests, motility at 25^{O}C and fermentation of carbohydrates (xylose, rhamnose, mannitol and α-methyl- D-mannopyranoside).

3.2.4.2 Hemolysis on Sheep Blood Agar (SBA)

The presumptive *Listeria* isolates that were confirmed using biochemical tests were analyzed for the type of hemolysis on SBA as per the method described by Seeliger and Jones (1986). The isolates were plated on 5% SBA and incubated at 37^{o}C overnight. The plates were observed for hemolytic activity surrounding the bacterial growth. The presence of *L. ivanovii* is characterized by the typical wide and clear zone of β-haemolysis while, *L. monocytogenes* shows a narrow zone of haemolysis.

3.2.4.3 Christie-Atkins-Munch-Peterson (CAMP) test

All the presumptive *Listeria* isolates were subjected to CAMP test (BIS, 1994). The standard strains of *Staphylococcus aureus* and *Rhodococcus equi* grown in BHI broth for 18 h were plated on 7% SBA distantly and analogous to each other. The cultures to be tested were streaked in parallel at right angles between the *S. aureus* and *R. equi.* After incubation at 37^{0}C, the plates were observed for enhanced hemolysis after 24-48 h. *L. monocytogenes* is confirmed by observing the enhancd haemolysis towards *S. aureus* .

3.2.4.4 ALOA (Agar *Listeria* according to Ottaviani and Agosti) assay

The ALOA assay is an *in vitro* pathogenicity assay and an alternative way to assess PI-PLC activity. It was carried out using Chromogenic media, ALOA which helped to differentiate pathogenic *Listeria* spp (Ottaviani *et al.,* 1997). The isolates (72) characterized biochemically were subjected to PI-PLC assay on ALOA (Hi-media, Mumbai, India) media. The *Listeria* strains were grown overnight at 37^0C on SBA. A loopfull of *Listeria* isolates collected from SBA were placed as spots on ALOA plates. The *Listeria* species exhibit bluish green colonies on ALOA due to detection of β-glucosidase activity by utilization of a chromogenic compound X-glucoside present in the medium. *L. monocytogenes* colonies show formation of greenish-blue colour surrounded by an opaque halo on ALOA agar. The larger the zone size of the halo the greater is the enzymatic activity, if the zone size is >8-9mm than the intensity of the enzymatic activity was considered to be high.

3.2.5 Antibiotic sensitivity

Listeria monocytogenes isolates were examined for the susceptibility to antibiotics by the standard Kirby- Bauer disc diffusion method (Baeur *et al*.,1966) employing National Committee for Clinical Laboratory Standards (NCCLS) guidelines, 1997. *L. monocytogenes* MTCC 1143 and *L. monocytogenes* ATCC 19114 were used as the reference strains.

All the isolates (72) confirmed as *L. monocytogenes* were grown in BHI broth at 37^0C with overnight incubation. The culture suspension was adjusted to 0.5 McFarland Standard (approximately 1.5 x 10^8 cells) tube. After adjusting the turbidity of the bacterial suspension, a presterilized cotton swab was immersed in the

suspension and agitated several times to drain out the excess inoculum. The dipped swab was was spread on Mueller-Hinton Agar (Hi-media) supplemented with 5% defibrinated sheep blood within 15 minutes of preparation of inoculums. Later impregnated antibiotics discs were placed on the agar. Commercially available disks (Hi-Media) with the following antibiotics were used: ciprofloxacin (30µg/disc), vancomycin (30µg/disc), chloramphenicol (30µg/disc), meropenem (10µg/disc), penicillin (10µg/disc), gentamycin (10µg/disc), trimethoprim (25µg/disc), kanamycin (30µg/disc), erythromycin (15µg/disc) and ampicillin (25µg/disc). The discs were placed onto the Mueller-Hinton Agar inoculated with the test strains of *L. monocytogenes* were incubated at 37^0C overnight. Zones of inhibition were observed after overnight incubation and interpreted as per manufacturer's instructions.

3.2.6 Glutamate decarboxylase (GAD) activity assay

The *L. monocytogenes* isolates (72) were analysed for GAD activity using a quick colorimetric method employing indicator, bromocresol green (Cotter *et al*., 2001a, b). Cultures (1 ml) grown overnight in BHI were pelleted. After washing in normal saline solution, the pellets were resuspended in 0.5 ml of test solution. The test solution consisted of L-glutamic acid, Triton X-100, NaCl and bromocresol green as indicator. The pH was adjusted at 4. The change in colour from yellow to green or blue was observed. If there was no change in coloration from yellow after 4 h of incubation at 37^0C, the assay was regarded as negative for GAD activity. Change in colour from yellow to green colour was indicative of low activity and development of blue colour indicated the high activity. The experiments were repeated thrice for each of the strain.

3.2.7 Survivability of *L. monocytogenes* in synthetic gastric fluid

A study was carried out to detect the glutamate decarboxylase activity of *L. monocytogenes* and to examine the protective effect of glutamate (Cotter *et al*., 2001a, b). 4 selected strains of *L. monocytogenes* were analysed. *Listeria* strains were grown overnight in BHI broth. The cultures were pelleted by centrifugation. The pellets were reconstituted in equal quantity of synthetic gastric fluid, pH of 3.5 and prewarmed at 37^0C. In order to assess the protecting effect of glutamate 10 mM monosodium glutamate was added to synthetic gastric fluid before pH adjustment. Synthetic gastric fluid, with or without monosodium glutamate, was inoculated with the strains . The sensitivity of the strains was determined by sampling at 15 min intervals over a period of 1 hour. The colony-forming units (CFU) were determined for viable bacteria by plating the inoculated samples onto BHI agar.

3.3 Results and Discussion

Listeriosis is caused due to infection by *L. monocytogenes* and usually affects individuals with immune system weakened by underlying conditions such as cancer, HIV infection, cirrhosis and also other immunocompromised individuals like organ transplant patients, the elderly, pregnant women, newborn or unborn babies. The bacterium has ability to colonise and survive in food processing environments and to grow at refrigeration temperature. Foodborne transmission is the main route for infecting individuals. Listeriosis outbreaks similar to massive outbreaks observed in developed countries have not been reported in India. One of the reasons may be the difference in the lifestyle and food habits amongst the population, but with

urbanization and change in the lifestyle and the propensity to consume ready to eat and processed foods may prove to be hazardous unless adequate preventive measures are taken. In developed countries, due to its clinical severity and high fatality rate, invasive listeriosis is of great concern to public health despite its low incidence (Pontello *et al*., 2012). Significant increasing trends in listeriosis notification rates were noted in Austria, Denmark, Hungary, Italy, Spain and Sweden from 2005 to 2009 (Parihar *et al*., 2008; Pontello *et al*., 2012). However, there is lack of knowledge of listeriosis both of sporadic as well as of epidemic nature in India.

In the present study, 596 samples comprising of 481 from humans and 115 from animal origin were processed for isolation of *Listeria* (Table 3.1). The samples were attempted for the detection of *Listeria* as per USDA method. In case of blood, urine and cerebrospinal fluids, samples were directly processed while in case of tissue, samples were macerated and then used for isolation. A two step method of enrichment was used followed by streaking on selective media. The typical greyish black colonies with a bulls eye appearance surrounded by a black halo (Fig. 3.1) were presumed to be of *Listeria* spp. Suspected colonies were confirmed by subculturing on PALCAM agar and then grown in BHI broth for 18 h at 37^{o}C. These stored cultures were used for the further study. Gram-positive, coccobacillary forms with characteristic tumbling motility at 20 to 25^{0}C were further analysed biochemically and tested for their pathogenic potential.

Out of the 596 samples processed for isolation of *Listeria*, 10 (1.67%) samples were positive for *L. monocytogenes*. Of the 481 samples of human origin, 7 (1.45%)

samples found to be positive for *L. monocytogenes* (Table 3.2), while of the 115 samples of animal origin, 3 (2.6%) were positive for *L. monocytogenes* (Table 3.3).

Table 3.2. Isolation of *Listeria* from human clinical samples.

Place	Samples (Human Origin)	Number of Samples	Number of Isolates
Panjim (GMC)	Placental bits	114	-
Panjim (GMC)	Urine	318	2
Panjim (GMC)	Stool	13	1
Private Hospital	Cervical and vaginal swab	5	1
Private Hospital	Vaginal swab	1	-
Porvorim	Paediatric stool sample	1	-
Primary Hlth Cent.	Paediatric stool sample	8	2
Nagpur	Cerebrospinal fluid	10	-
Pune	Blood and serum	2	-
Kolhapur	Placental bits	4	-
Kolhapur	Vaginal swabs	5	1

Table 3.3. Isolation of *Listeria* from animal clinical samples**.**

Place	Samples (Animal Origin)	Number of Samples	Number of Isolates
Goa	Buffaloes (blood)	4	-
Goa	Buffaloes (milk)	69	2
Goa	Cows (milk)	42	1

In addition to the isolates of *L. monocytogenes* obtained in this study, clinical isolates (human=17 and animal =45) from different parts of India referred to our laboratory for characterization were also included for further study (Table 3.4).

Table 3.4. Details of isolates of *Listeria* referred from different places in India.

Place	Number of Isolates of Human Origin	Number of Isolates of Animal Origin
Pondicherry	5	-
Kolhapur	-	6
Bareilly	5	11
Himachal Pradesh	-	1
Kanpur	2	-
Nagpur	5	3
Mumbai	6	15
Ratnagiri	-	10
Bangalore	-	2

The purified suspected isolates were subjected to various tests for the purpose of characterization and identification. Catalase positivity and oxidase negativity were seen in case of all the isolates.

On carrying out sugar fermentation tests, the isolates exhibited production of acid from rhamnose, mannitol and α-D-methyl mannopyranoside but not from xylose and hence they were tentatively identified as *L. monocytogenes.*

Haemolysis, CAMP test, PI-PLC assay were performed for all the isolates. Moderate zones of haemolysis were observed on SBA (7%) (Table 3.5, Fig 3.2) which was typical for *L. monocytogenes* unlike the clear broad zones of β- haemolysis shown in case of *L. ivanovii* isolates.

On performing the CAMP test enhanced zone of haemolysis was seen close to the *Staphylococcus aureus* line of streak as compared to *Rhodococcusequi* (Table 3.5, Fig 3.3). The CAMP test, therefore, confirmed the results obtained by the biochemical sugar fermentation tests.

The PI-PLC assay was performed by spot inoculating the isolates on ALOA agar. The enzymatic activity expressed by *L. monocytogenes* isolates on the ALOA agar was estimated as high since the zone size was >8-9 mm in case of all the isolates except two which did not show presence of the halo (Table 3.5, Fig 3.4).

Based on the data obtained on performing the conventional biochemical tests 70 isolates were proved as *L. monocytogenes*, while two isolates were confirmed as *L. innocua*.

The isolates from clinical samples from humans were recovered from one placental bit sample, two urine samples, one vaginal swab and three from faecal samples. No isolates were obtained from CSF. It was observed that vaginal swabs and fecal samples showed a higher rate of isolation as compared to other clinical samples. In India, commonly reported clinical form is genital listeriosis. Detection of *L. monocytogenes* from cases having poor obstetrics history were reviewed by Barbuddhe *et al*., (2012). Krishna and co-workers (1966) isolated *L. monocytogenes*

from the cervix of 14 % of 150 patients of bad obstetric history i.e., past history of abortions, miscarriages, still births or neonatal deaths. The samples were collected from Mumbai. Later, *L. monocytogenes* was isolated from 3 % (Bhujwala *et al*., 1973) and 10% patients having a history of abortions (Stephen *et al*., 1978*)*. Isolation of pathogenic *L. monocytogenes* was reported from 3.3% cases of miscarriages and multiple abortions (Kaur *et al*., 2007). Meningitis and hydrocephalus in children has been caused by *L. monocytogenes* in utero and was reported to be transmitted from infected mothers (Gogate and Deodhar 1981). A perinephric abscess in a 5 yr old malnourished child caused by *L. monocytogenes* has also been reported (Gomber *et al*., 1998). A case of listerial meningitis in a 17-year-old immunocompetent patient was reported (Kalyani *et al*., 2006). In earlier studies from India, Dhanashree *et al*. (2003) isolated *L. monocytogenes* from 3.1% placental bit samples. In a recent study, *L. monocytogenes* was recovered from 5.3% of placental bits and 1.3% of vaginal swabs (Soni *et al*., 2013). Abdel Malek *et al*. (2010) reported an isolation rate of 7.14% for *Listeria* spp. from faecal samples of children in a paediatric hospital in Egypt. Gupta *et al*. (1997) isolated *L. monocytogenes* from blood samples. Bhujwala *et al*. (1973) could not isolate *Listeria* from CSF samples. Rate of isolation of *L. monocytogenes* was low from urine samples. Following delivery, *L. monocytogenes* may be shed in vaginal secretions or urine of mothers of infected newborns for 7–10 days (Anon, 2002). The organism has been isolated from urine of an individual suffering from repeated abortions (Nicolosi *et al*., 1978).

In case of animals, *L. monocytogenes* was isolated three (2.7%) from mastitic milk (111) samples. Only a few cases of bovine listerial mastitis have been reported in the literature (Bourry *et al*., 1995; Wagner *et al*., 2000). In an earlier study, *L.*

monocytogenes and *L. ivanovii* were isolated from 4 (0.55%) and 1 (0.14%) samples from mastitic cows and buffaloes (Rawool *et al*., 2007). Kalorey *et al*. (2008) reported 5.1% milk samples to be positive for *L. monocytogenes*. D'Costa *et al*. (2012) reported a prevalence rate of 4.82% for *L. monocytogenes* from milk samples. *L. monocytogenes* was isolated from 7.6% samples of bovine colostrum in Japan, comprising 55% isolates of serotype 1/2b and 45% isolates belonging to serotype 4b (Hasegawa *et al*., 2013).

In the present study, the rate of isolation from animal samples is much more than that from human samples which may be because of contamination in the fodder and improper sanitation and lack of attention towards healthcare. However, the rate is less than that reported from other parts of India (D'Costa *et al.*, 2012).

FIG. 3.1. Colonies of *Listeria* spp. on PALCAM agar

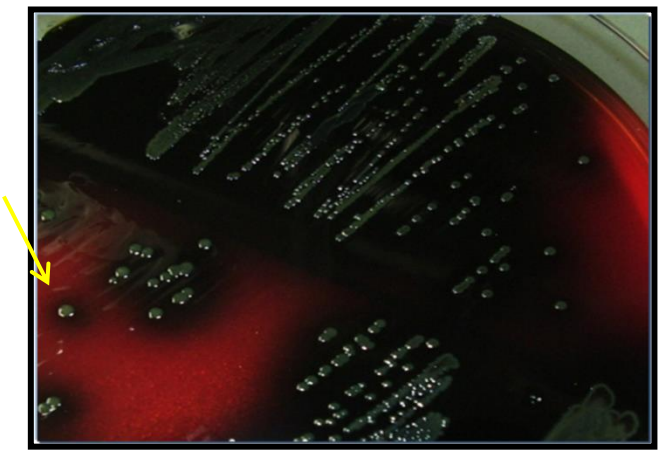


Fig. 3.2. Haemolysis on SBA

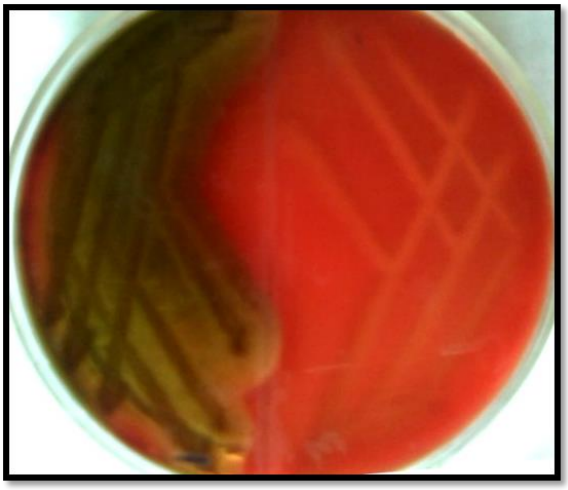


Fig. 3.3. CAMP test with *Staphylococcus aureus*

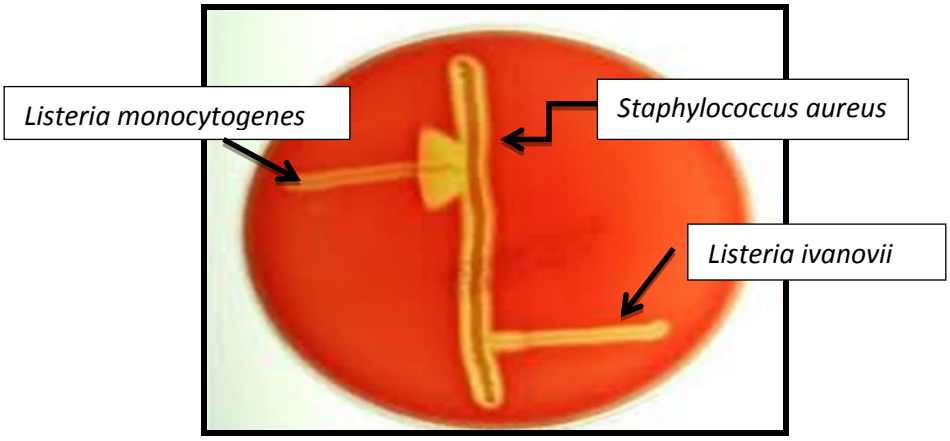


Fig. 3.4. *Listeria monocytogenes* colonies on ALOA (chromogenic media)

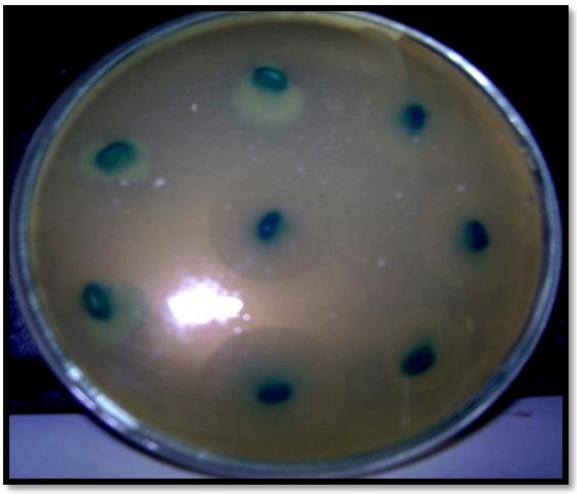


Table 3.5. Biochemical and pathogenicity profile of *Listeria* isolates.

Sr. No.	Isolate	Biochemical Profile				Species identified	CAMP with S/R	Haemolysis on SBA	PI-PLC assay
		Xylose	Rhamnose	Mannitol	α-Methyl D-mannopyranoside				
1	H1	_	+	_	+	LM	+S	+	++
2	H2	_	+	_	+	LM	+S	+	++
3	H3	_	+	_	+	LM	+S	+	++
4	H4	_	+	_	+	LM	+S	+	++
5	H5	_	+	_	+	LM	+S	+	++
6	H6	_	+	_	+	LM	+S	+	++
7	H7	_	+	_	+	LM	+S	+	++
8	H8	_	+	_	+	LM	+S	+	++
9	H9	_	+	_	+	LM	+S	+	++
10	H10	_	+	_	+	LM	+S	+	++
11	H11	_	+	_	+	LM	+S	+	++
12	H12	_	+	_	+	LM	+S	+	++
13	H13	_	+	_	+	LM	+S	+	++
14	H14	_	+	_	+	LM	+S	+	++
15	H15	_	_	_	+	LI	_	-	-
16	H16	_	+	_	+	LM	+S	+	++
17	H17	_	+	_	+	LM	+S	+	++
18	H18	_	+	_	+	LM	+S	+	++
19	H19	_	+	_	+	LM	+S	+	++

20	H20	_	+	_	+	LM	+S	+	++
21	H21	_	+	_	+	LM	+S	+	++
22	H22	_	+	_	+	LM	+S	+	++
23	H23	_	+	_	+	LM	+S	+	++
24	H24	_	+	_	+	LM	+S	+	++
25	A1	_	+	_	+	LM	+S	+	++
26	A2	_	+	_	+	LM	+S	+	++
27	A3	_	+	_	+	LM	+S	+	++
28	A4	_	+	_	+	LM	+S	+	++
29	A5	_	+	_	+	LM	+S	+	++
30	A6	_	+	_	+	LM	+S	+	++
31	A7	_	+	_	+	LM	+S	+	++
32	A8	_	+	_	+	LM	+S	+	++
33	A9	_	+	_	+	LM	+S	+	++
34	A10	_	+	_	+	LM	+S	+	++
35	A11	_	+	_	+	LM	+S	+	++
36	A12	_	+	_	+	LM	+S	+	++
37	A13	_	+	_	+	LM	+S	+	++
38	A14	_	+	_	+	LM	+S	+	++
39	A15	_	+	_	+	LM	+S	+	++
40	A16	_	+	_	+	LM	+S	+	++
41	A17	_	+	_	+	LM	+S	+	++
42	A18	_	+	_	+	LM	+S	+	++
43	A19	_	+	_	+	LM	+S	+	++

44	A20	_	+	_	+	LM	+S	+	++
45	A21	_	+	_	+	LM	+S	+	++
46	A22	_	-	-	+	LI	_	-	-
47	A23	_	+	_	+	LM	+S	+	++
48	A24	_	+	_	+	LM	+S	+	++
49	A25	_	+	_	+	LM	+S	+	++
50	A26	_	+	_	+	LM	+S	+	++
51	A27	_	+	_	+	LM	+S	+	++
52	A28	_	+	_	+	LM	+S	+	++
53	A29	_	+	_	+	LM	+S	+	++
54	A30	_	+	_	+	LM	+S	+	++
55	A31	_	+	_	+	LM	+S	+	++
56	A32	_	+	_	+	LM	+S	+	++
57	A33	_	+	_	+	LM	+S	+	++
58	A34	_	+	_	+	LM	+S	+	++
59	A35	_	+	_	+	LM	+S	+	++
60	A36	_	+	_	+	LM	+S	+	++
61	A37	_	+	_	+	LM	+S	+	++
62	A38	_	+	_	+	LM	+S	+	++
63	A39	_	+	_	+	LM	+S	+	++
64	A40	_	+	_	+	LM	+S	+	++
65	A41	_	+	_	+	LM	+S	+	++
66	A42	_	+	_	+	LM	+S	+	++
67	A43	_	+	_	+	LM	+S	+	++

68	A44	_	+	_	+	LM	+S	+	++
69	A45	_	+	_	+	LM	+S	+	++
70	A46	_	+	_	+	LM	+S	+	++
71	A47	_	+	_	+	LM	+S	+	++
72	A48	_	+	_	+	LM	+S	+	++

CAMP : Christie, Atkins, Munch- Petersen test

+S : Enhanced zone of haemolysis with *Staphylococcus aureus*

LM : *Listeria monocytogenes*, LI*: Listeria innocua*

SBA : Sheep blood agar

PI-PLC: Phosphotidylinositol specific phospholipase C

H : Human source

A : Animal source

Administration of antibiotics having quick antibacterial activity is essential for the successful treatment of listeriosis (Goulet and Marchetti, 1996; Hof, 2004; Mylonakis *et al*., 1998). The treatment currently advocated is a combination of high doses of ampicillin or amoxicillin and gentamicin (Hof, 2004; Temple and Nahata, 2000). Other possible alternatives recommended include rifampin, vancomycin, linezolid and carbapenems (Benes *et al*., 2002; Goulet and Marchetti, 1996; Hof, 2003; Hof, 2004; Temple and Nahata, 2000). Trimethoprim along with a sulfonamide such as cotrimoxazole has been used in case of intolerance to the beta-lactams (Hof, 2004; Temple and Nahata, 2000). Erythromycin and vancomycin are also used to combat listeriosis. It was thought that *L. monocytogenes* rarely develops acquired resistance to antibiotics, however, an increased resistance rate to one or several

clinically important antibiotics in environmental and clinical isolates have recently been reported (Aureli *et al*., 2003; Charpentier *et al*., 1999; Conter *et al*., 2009; Li *et al*., 2007; Srinivasan *et al*., 2005; Walsh *et al*., 2001, Charpentier *et al*., 1995; Godreuil *et al*., 2003; Poyart-Salmeron *et al*., 1990; Safdar and Armstrong, 2003).

In the present study, the isolates were tested against a panel of ten antibiotics namely gentamicin, kanamycin, chloramphenicol, ciprofloxacin, erythromycin, meropenem, trimethoprim, ampicillin, vancomycin and penicillin. It was seen that the isolates showed highest resistance to meropenem (77.58%), penicillin (62%), erythromycin (55.17%), vancomycin (56.89%) intermediate resistance to ampicillin (46.55%), kanamycin (31%), ciprofloxacin (22.41%) whereas the least resistance was shown by trimethoprim (5.17%) (Table 3.6, Fig. 3.5).

The results of antibiotic sensitivity of animal isolates were compared to those of human isolates and it was seen that animal isolates showed higher resistance to meropenem, penicillin and vancomycin. In case of human isolates higher resistance was observed in case of meropenem, ampicillin, erythromycin, vancomycin and penicillin (Table 3.7, Fig. 3.6). The resistance shown by human as well as animal isolates to penicillin, erythromycin, ampicillin and vancomycin is significant because these antibiotics are routinely used in therapy and hence can lead to complications in the treatment of listeriosis. In India, antibiotic resistant *L. monocytogenes* strains were reported sporadically from food and clinical sources (Dhanashree *et al*., 2003; Soni *et al*., 2013; Tirumalai, 2013; Sharma *et al*., 2008; Gupta and Sharma, 2013). Nigam *et al*. (1998) reported that isolates of *Listeria spp.* were 100% sensitive to ampicillin, chloramphenicol, penicillin and 88% sensitive to ciprofloxacin and gentamicin. Increase in resistance to antibiotics like erythromycin, ampicillin and

penicillin which are widely used in the treatment could pose a problem in treatment of listeriosis.

Fig 3.5. Antibiotic resistance profile of *L.monocytogenes* clinical isolates.

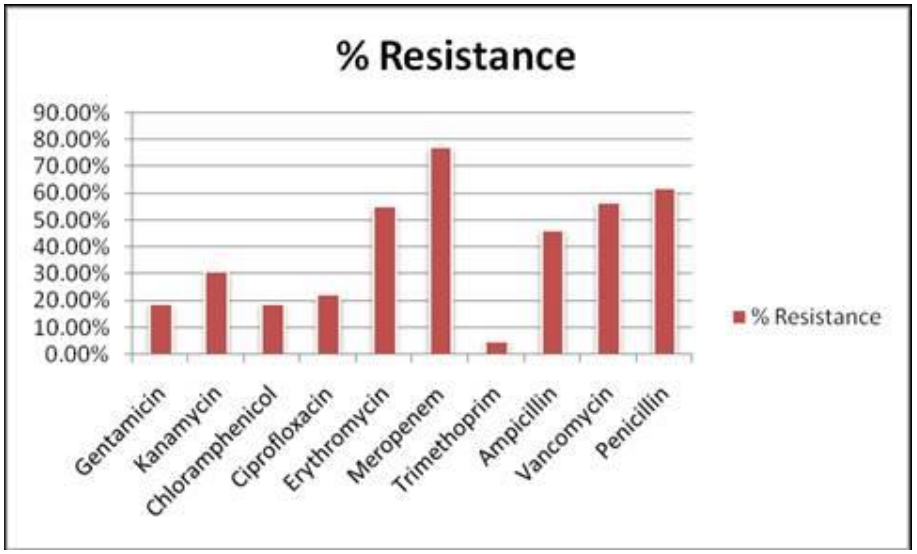


Table 3.6. Resistance of *L. monocytogenes* isolates to antibiotics

Antibiotic	% Resistance
Gentamicin	18.97%
Kanamycin	31.03%
Chloramphenicol	18.96%
Ciprofloxacin	22.41%
Erythromycin	55.17%
Meropenem	77.58%
Trimethoprim	5.17%
Ampicillin	46.55%
Vancomycin	56.89%
Penicillin	62%

Fig 3.6. Antibiogram of human and animal *L. monocytogenes* isolates

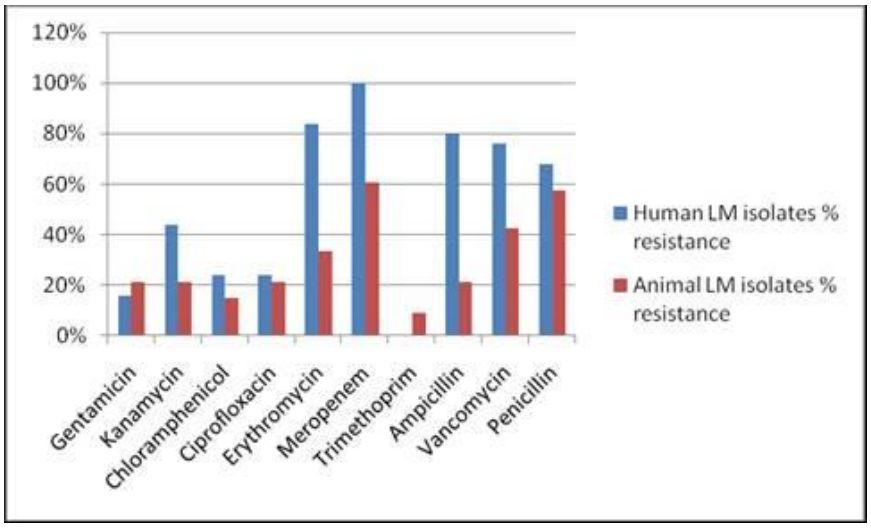


Table 3.7. Resistance of *L.monocytogenes* isolates of human and animal origin.

Antibiotic	Human LM isolates % resistance	Animal LM isolates % resistance
Gentamicin	16%	21.21%
Kanamycin	44%	21.21%
Chloramphenicol	24%	15.15%
Ciprofloxacin	24%	21.21%
Erythromycin	84%	33.33%
Meropenem	100%	60.61%
Trimethoprim	0	9.09%
Ampicillin	80%	21.21%
Vancomycin	76%	42.42%
Penicillin	68%	57.58%

The results obtained are similar to those obtained in a study carried on clinical and environmental isolates in which high resistance was observed to gentamicin, penicillin G, cephadroxil, cephtriazome, cephotaxim and oxytetracycline (Sarangi and Panda, 2011) . A study was carried out to determine antimicrobial susceptibility of *L. monocytogenes* strains recovered from human clinical cases in France against a panel of 23 antibiotics, 1.27% strains showed resistance to at least one antibiotic, two isolates were seen to exhibit acquired multiple drug resistance to chloramphenicol, erythromycin, streptomycin and tetracycline (Morvan *et al*., 2010). Resistance to tetracyclines and flouroquinolones was seen to be more common (Morvan *et al*., 2010). *L. monocytogenes* strains showing multidrug resistant against nalidixic acid, amoxicillin+sulbactam, cloxacillin, erythromycin, kanamycin and vancomycin have been reported (Sharma *et al*., 2012). In a study carried out in France on *Listeria* isolates phenotypic resistance was detectable against clindamycin, tetracycline and trimethoprim. The intermediate susceptibility was seen to be highest in isolates obtained from clinical sources. It was also deduced that resistance to tetracycline was attributed to the *tet*M gene which in some isolates was present on a transposon of Tn916 family (Bertsch *et al*., 2014).

This proved that indiscriminate use of antibiotics as well as discharge of contaminated effluent could lead to antibiotic resistance emergence (Hansen *et al*., 2005) and such multidrug resistant pathogens can cause widespread public health complications (Sugiri *et al*., 2014; Moreno *et al*., 2014).

The main route of transmission of *L. monocytogenes* is through food. When the pathogen passes along with the food into the stomach it encounters stressful

conditions in the form of low pH and its ability to survive in such conditions would influence its ability to survive in such conditions would influence its ability to establish infection. There is increasing evidence which showed that *L. monocytogenes* isolates differed in their ability to tolerate natural acids (Dykes and Moorhead, 2000). In the presence of low pH of gastric fluids, the F_0F_1-ATPase system of *L. monocytogenes* pumps out excess protons from the cytoplasm. The GAD has an important role in acid resistance in the host (Cotter *et al*., 2001a, b). Strains of *L. monocytogenes* may vary in the expression of GAD activity and their resistance to to gastric fluids in the presence of glutamate. It suggests that the appearance of GAD acitivity is an important condition for survival and colonization of the gut and stomach (Cotter *et al*., 2001a, b).

Screening of all the isolates, using rapid colorimetric assay revealed the presence of high GAD activity in 86% of the isolates , intermediate and low activities in 7% each (Table 3.8). A high proportion of the isolates showed increased GAD activity suggesting that it may be an important factor in enhancing infection. Similar results were observed in a study involving clinical, food and environmental *L. monocytogenes* isolates in which most of the clinical strains exhibited high GAD activity as compared to the others (Olier *et al*., 2004).

By using gastric fluid having pH 3.5 in the presence and absence of 10mM monosodium glutamate, it was seen that the survival rate was higher in presence of MSG for all four isolates tested. It was observed that in case of three isolates namely H4, H8 and H16 no colonies were obtained after 45 min in gastric fluid without MSG, however, in presence of MSG although the colony count decreased the isolates still

survived even for 60 min incubation in gastric fluid. The fourth isolate H6 showed survival at 45 min but not at 60 min in absence of MSG and survival even at 60 min in the presence of MSG (Table 3.9, 3.10, 3.11, 3.12, Fig 3.7, 3.8, 3.9, 3.10), thus indicating that glutamate has a protective effect on the *L. monocytogenes* and ensures a higher rate of survival even at an acidic pH.

Table 3.8. Percent Glutamate decarboxylase activities shown by *Listeria* isolates.

Glutamate decarboxylase assay	% of isolates showing positive GAD assay
High activity	86%
Intermediate activity	7%
Low activity	7%

Fig 3.7. Survival of *L.monocytogenes* isolate H4 in gastric fluid

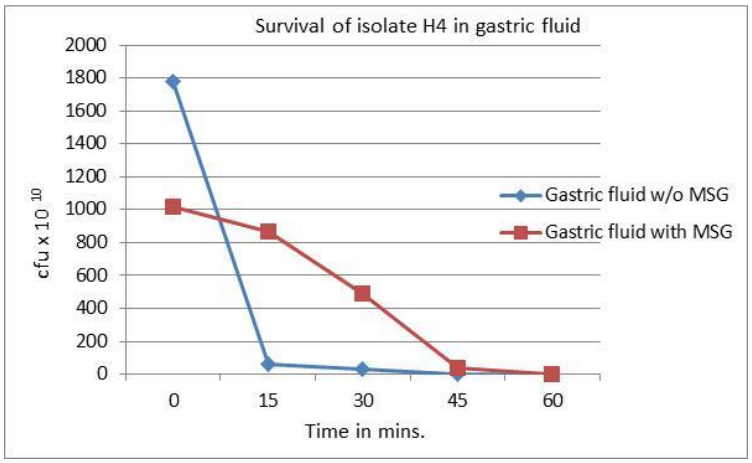


Table 3.9. Survival of *L.monocytogenes* isolate H4 in gastric fluid

Time in minutes	Viable count in gastric fluid without MSG cfu/ml	Viable count in gastric fluid with MSG cfu/ml
0	$1776X10^{10}$	$1016\ X10^{10}$
15	$64.2X10^{10}$	$865\ X10^{10}$
30	$27.5X10^{10}$	$494\ X10^{10}$
45	0	$41.5\ X10^{10}$
60	0	$0.0378\ X10^{10}$

Fig 3.8. Survival of *L.monocytogenes* isolate H6 in gastric fluid

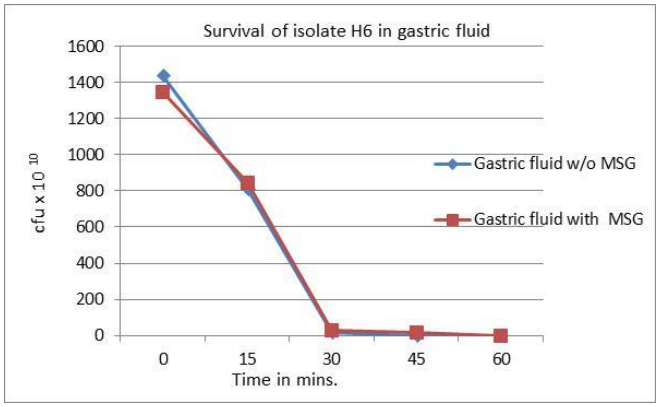


Table 3.10. Survival of *L.monocytogenes* isolate H6 in gastric fluid

Time in minutes	Viable count in gastric fluid without MSG cfu/ml	Viable count in gastric fluid with MSG cfu/ml
0	1440 $X10^{10}$	1350 $X10^{10}$
15	806 $X10^{10}$	845 $X10^{10}$
30	13.5 $X10^{10}$	27.1 $X10^{10}$
45	0.006 $X10^{10}$	18.4 $X10^{10}$
60	0	0.0377 $X10^{10}$

Fig 3.9. Survival of *L.monocytogenes* isolate H8 in gastric fluid

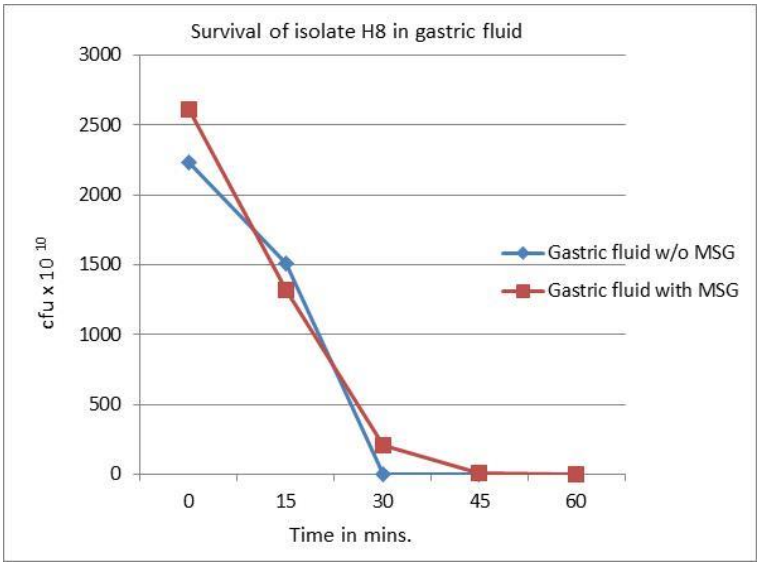


Table 3.11. Survival of *L.monocytogenes* isolate H8 in gastric fluid

Time in minutes	Viable count in gastric fluid without MSG cfu/ml	Viable count in gastric fluid with MSG cfu/ml
0	2230 $X10^{10}$	2610 $X10^{10}$
15	1507 $X10^{10}$	1320 $X10^{10}$
30	1.4 $X10^{10}$	209 $X10^{10}$
45	0	10.7 $X10^{10}$
60	0	0.2113 $X10^{10}$

Fig 3.10. Survival of *L.monocytogenes* isolate H16 in gastric fluid.

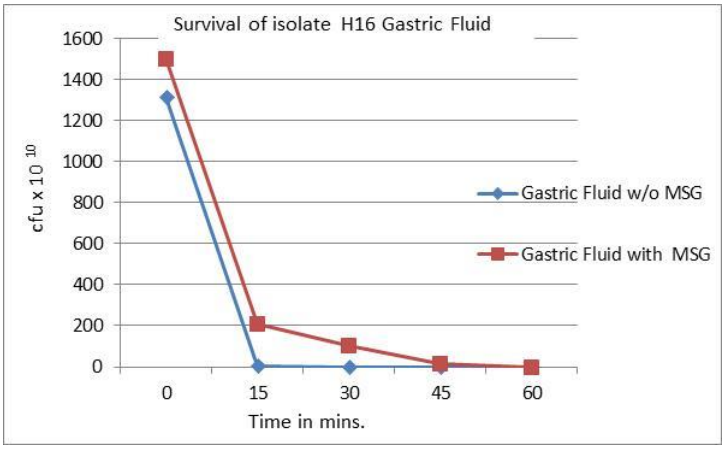


Table 3.12. Survival of *L.monocytogenes* isolate H16 in gastric fluid.

Time in minutes	Viable count in gastric fluid without MSG cfu/ml	Viable count in gastric fluid with MSG cfu/ml
0	1314 $X10^{10}$	1500 $X10^{10}$
15	1.16 $X10^{10}$	210 $X10^{10}$
30	0.008 $X10^{10}$	103.3 $X10^{10}$
45	0	12.88 $X10^{10}$
60	0	0.0181 $X10^{10}$

The results are in agreement with results obtained in a study carried out on survival kinetics of human asymptomatic carriage strains wherein most of the strains showed enhanced survival in presence of glutamate (Olier *et al*., 2004). Similar results were also obtained in a study involving a strain of *L. monocytogenes* which

stated that expression of GAD acitivity by *L. monocytogenes* was an essential requirement for endurance in the stomach milieu (Cotter *et al*., 2001b).

Reports describing cases of listeriosis from humans in India are rare, and this may be because of lack of awareness, failure in identification, improper isolation techniques and low rate of incidence (Malik *et al*., 2002; Barbuddhe *et al*., 2004). The current epidemiological scenario of listeric infections available in India is insufficient and unreliable to assess the extent of human and animal listertiosis, as it is based on conventional diagnostic tests showing considerable cross-reactivity. The disease mostly remains undiagnosed and underreported, mainly owing to the lack of awareness among physicians, veterinarians, food processors. Also, the non-availability of a reliable, quick and easy diagnostic tests hampers the detection of listeric infections (Malik *et al*., 2002). In view of the above, the data reported here is of considerable significance.

The study highlighted the occurrence of *L. monocytogenes* in humans and in animals. Considering the zoonotic potential of the pathogen and its public health significance, hygienic measures should be adopted at farms and public. Special care needs to be taken in case of pregnant women, the newborn and the unborn and also those having underlying conditions. Special advisories need to be developed for alerting the high risk groups. There is also an urgent need to study the occurrence of the pathogen among high risk foods, novel niches, and susceptible human groups. Nevertheless, development of diagnostics remains a priority for implementing the plans for detection of the pathogen in shortest possible time.

Chapter 4

GENOTYPIC CHARACTERIZATION OF *Listeria* ISOLATES

4.1 Introduction

Listeria monocytogenes is well known opportunistic pathogen affecting both humans and animals transmitted through food. Among the very few food borne pathogens, *L. monocytogenes* has special abilities by which it can infect the highly sequestered part of host system for example it can cross blood–brain and fetoplacental barriers to produce localized infection. Systemic infection of immunodeficient or pregnant individuals results in septicemia, nervous system infections, spontaneous abortions, and stillbirths (Latorre *et al*., 2009). With molecular methods, the rapid detection and typing of diseases causing pathogens is possible and which also complements the traditional epidemiological surveillance systems by providing suitable discriminatory analyses of pathogen associated with different outbreaks, and for detection and investigation origin and transmission in chains (Allerberger, 2013). *L. monocytogenes* strains can be discriminated on the basis of molecular typing methods using serotyping or geno-serotyping (Doumith *et al*., 2004) and by PFGE by using Pulse Net protocol (Graves and Swaminathan, 2001). In order to minimize listeriosis outbreaks, rapid, exact, and efficient surveillance network programmes are needed. Application of molecular subtyping surveillance of listeriosis may help in recognition of clusters of cases which otherwise may be considered as sporadic cases.

In addition to the conventional methods, a number of systems are available commercially for identification of *Listeria* taht include Vitek, API, MICRO-ID, ELISA and Nucleic acid assay Kits (OIE Terrestrial Manual, 2008). Conventional methods for detection of *L. monocytogenes* and immunodignostics are lengthy and give false positives results (Zhang *et al*., 2009), hence, molecular techniques are more reliable and sensitive as compared to others (Frece *et al*., 2010).

Some other techniques used for detection of *Listeria* spp. are biosensor based techniques in which a target organism can be detected by interaction of it with biomolecule which gives a typical signal when the organism is detected (Leonard *et al*., 2004; Poltronieri *et al*., 2009; Banerjee and Bhunia, 2010). *Listeria* from food sources have been detected by using DGGE (Cocolin *et al*., 2002) and TGGE (Tominaga, 2006) methods. Loop mediated isothermal amplification (LAMP) involves amplification of DNA was used to develop a more accurate and sensitive assay for targeting the *hly*A gene (Tang *et al*., 2011). Spectroscopy based techniques such as FT-IR, MALDI-TOF MS have been emplyed for detection of *Listeria*, as they give a typical fingerprint spectrum of *Listeria* at a species level (Holt *et al*., 1995; Jadhav *et al*., 2015; Hseuh *et al*., 2014; Barbuddhe *et al*., 2008; Ferreira *et al*., 2010; Drancourt *et al*., 2010).

Presence of numerous strains of *L. monocytogenes* necessitates an appropriate system for subtyping of bacterium which will provide effective measures for the control of disease outbreaks. Typing methods to discriminate *L. monocytogenes* strains have important applications in foodborne disease surveillance, outbreak detection and to track epidemiological links. To understand the ecology and nature of bacterial strains, their ability to cause disease, one should need the complete information related to the differentiation of the pathogenic strains, (Wiedmann, 2002). Subtyping methods can also be use as source tracking of *L. monocytogenes* which helps in the development of control strategies to avoid contamination in food processing environments (Palumbo *et al*., 2003). Two major approaches are commonly used in subtyping: phenotypic and genotyping methods. The phenotypic approaches such as phage typing, serotyping, MEE and esterase typing; however, in

genetic subtyping methods pulsed-field gel electrophoresis (PFGE) is considered as gold standard test for *L. monocytogenes* subtyping. Apart from these other methods such as ribotyping, random amplification of polymorphic DNA (RAPD), PCR-restriction fragment length polymorphism (PCR-RFLP),amplified fragment length polymorphism (AFLP), , repetitive element PCR (REP-PCR), and DNA sequencing-based subtyping techniques such as multilocus sequence typing (MLST) (Stessl *et al.,* 2014). The phenotypic typing protocols have low diffrentiation power and low reproducibility, whereas the genotypic pathogen typing methods are most accurate, reproducible and consistent (Shuckken *et al*., 2003). Combination of multiple subtyping techniques is frequently used to improve subtyping discrimination in epidemiologic investigation of listeriosis outbreaks (Liu, 2006).

Serotyping is a widely used method for strain differentiation and is based on antigen antibody reactions. However, the method has very less diffrentiation ability when compared with molecular typing methods, it is also costly and tedious and has to be performed by skilled technicians (Gorski *et al*., 2014; Doumith *et al*., 2004b). Sometimes ambiguous results may also be obtained due to shared antigens between strains.

Phage typing is based on susceptibility of the bacterial isolates to standard phages (Audurier *et al.,* 1984), however, since almost 10% of the *Listeria* strains are untypable the usefulness of this technique is limited (Liu, 2006).

Multi locus enzyme electrophoresis (MEE) is one of the typing methods to differentiate bacterial strains; the priniciple is based on the electrophoretic mobility of

different peptides. Different strain produces different type of enzymes and variations in the charges present on the variable aminoacids force the peptide to travel in specific direction (Shuckken *et al*., 2003, Gasanov *et al*., 2005). Esterase typing is a measure of the activity of esterase enzyme from the cell extrct. It is a variant of MEE method (Harvey and Gilmour, 1996).

DNA based methods provide powerful tools for differentiation of pathogenic strains and also useful in subtyping methods. These methods provide discriminatory typing results for *L. monocytogenes* (Liu, 2006). The most commonly used molecular methods are ribotyping, PFGE and PCR based subtyping techniques. Ribotyping method search the polymorphism stretches present in the rRNA gene. This technique was used for lineage specific differentiation of *L. monocytogenes* isolates (Matloob *et al*., 2014). Ribotyping has also been automated and the ribopatterns generated are compared with a ribotype library (Shuckken *et al*., 2003). PFGE gives high discrimination index with two endonucleases i.e *ApaI* and *Asc I.* These enzymes cut the whole bacterial DNA at rare sites and generate fingerprinting patterns of the bacterial DNA. Whole bacterial genome is purified and subsequently cut by using restriction enzymes into DNA fragments. Bacterial DNA fragments are separated electrophoretically after restriction digestion by size, using PFGE to generate DNA banding patterns. Endonucleases which cut the t DNA only at few sites are selected to get a range of 8- 25 large DNA bands of 40 to 600 kilobases (kb). DNA fingerprinting patterns of different strains are evaluated to distinguish different bacterial strains from those sharing very similar DNA banding patterns. *Asc*I and *Apa*I are the two rare cutting restriction endonucleases that are commonly used for PFGE of *L. monocytogenes* (Wiedmann, 2002). PFGE subtyping of strains is often performed

with types of restriction endonucleases in separate reactions to improve discriminatory index. For example, 2 isolates with similar PFGE banding pattern for *AscI* may have 2 different *ApaI* PFGE patterns. PFGE subtyping based national network (PulseNet) has been developed by the Centers for Disease Control and Prevention for sharing the DNA subtypes and information for isolates of foodborne pathogens (Lorber, 2007). *L. monocytogenes* strains can be classified into different subtypes based on distinct DNA fingerprinting patterns using PFGE, which provide ease to discriminate dominant subtype considered the reference standard (Graves and Swaminathan, 2001). PFGE is regarded as "gold standard" method for subtyping of *L. monocytogenes*, as it has high level of sensitivity for discrimination (Jiang *et al*., 2008).

Several PCR based techniques were used for subtyping of *L. monocytogenes.* Primers have been developed to serotype four major strains 4b, 1/2a, 1/2b, and 1/2c (Jinneman and Hill, 2001). A comparative genomic approach was used to describe the three different genetic lineages and to further subdivide them into 5 groups which are serovar specific: I.1 (1/2a, 3a), I.2 (1/2c, 3c), II.1 (4b, 4d, 4e), II.2 (1/2b, 3b, 7) and III (4a, 4c) (Doumith *et al*., 2004a). Other PCR based subtyping techniques such as RAPD, AFLP, REP-PCR,PCR-RFLP, and DNA sequencing based subtyping methods such as MLST are also used (Graves *et al*., 2007; Parisi *et al*., 2010; Jersek *et al*., 1999; den Bakker *et al*., 2010).

The second and third objectives of this thesis aimed to characterize *Listeria* spp. by using phenotypic and genotypic methods as well as to investigate and

elucidate the genetic diversity of the *L. monocytogenes* strains using various molecular tools.

4.2 Materials and Methods

4.2.1 Detection of *virulence* genes of *Listeria*

The multiplex PCR based assay for detection of the *hlyA, actA* and *iap* genes was standardized as per the method described (Notermans *et al.,* 1991) with necessary modifications. Oligonucleotides were synthesized by Sigma Aldrich, USA. The sequences of primers used are given in Table 4.1. Briefly, the genomic DNA of thebacterial cultures and standard strain of *L. monocytogenes* MTCC 1143 was extracted using bacterial genomic DNA extraction kit (Himedia). The obtained DNA was used as a template in PCR reaction mixture.

After optimization of the protocol, the PCR was carried out for 50 µl reaction mixture comparised of 10x PCR buffer (100 mM Tris–HCl buffer, 500 mM KCl (pH 8.3), , 0.2 mM dNTPs , 2 mM MgCl2 and 0.1µM of each primer,1 unit of Taq DNA polymerase, 2 µl of DNA template and lastaly the volume of reaction mixture was adjusted with sterilized milliQ water. Positive and negative controls were were run parllely. The DNA amplification was carried out in Mastercycler epGradient (Eppendorf, Germany) having a preheated lid. The optimum reaction conditions for PCR were set as, initial denaturation at 95^0C for 2 min followed by 35 cycles each for 15 s denaturation at 95^0C, 30 s annealing at 58^0C and 1 min 30 s extension at 72^0C, followed by a final extension of 10 min at 72^0C and hold at 4^0C. Amplified DNA were run on the agarose gel and observed under a gel documentation unit (Alphaimager, USA).

Table 4.1 Primer sequences for *L. monocytogenes* used in virulence gene-PCR.

Target gene	Primer sequence	Product size (bp)
hlyA	F5'-GCAGTTGCAAGCGCTTGGAGTGAA-3'	456
	R 5'-GCAACGTATCCTCCAGAGTGATCG-3'	
actA	F 5'-CGCCGCGGAAATTAAAAAAAGA-3'	839
	R 5'-ACGAAGGAACCGGGCTGCTAG-3'	
iap	F 5'-ACAAGCTGCACCTGTTGCAG-3'	131
	R 5'-TGACAGCGTGTGTAGTAGCA-3'	

4.2.2 Serotyping

Serotyping based on somatic (O) and flagellar (H) antigens was carried out using commercially available antisera (Denka Seiken Co., Tokyo, Japan) as described by the manufacturer. O-antigen was determined by slide agglutination method with heat inactivated bacteria and that of H-antigen was carried out with liquid bacterial cultures in a test tube.

4.2.3 Serotyping by multiplex PCR (mPCR)

The multiplex PCR based serotyping was carried out for all the isolates. The protocol was optimized as per the Doumith *et al.*, (2004b). Briefly, the reactions were set at 50 microliter volume comprising 10x PCR, 300 mM dNTPs, 2 mM MgCl2, 2 units *Taq* DNA Polymerase, 2 mM each of primer *lmo0737*, ORF2819, ORF2110 and *prs* and 10 mg/ml of DNA template. The sequences of primers used are given in Table 4.2. PCR reactions were performed in Gradient Thermocycler (Eppendorf) with a pre-heated lid. For the reaction, an initial denaturation DNA was performed at 94^0C for 5 min, followed by 35 cycles each of 94^0C for 30 s, 54^0C for 1 min 15 s, and 72^0C for 1 min 15 s, and final extention at 72^0C for 10 min. Agarose gel electrophoresis of

amplified products was carried out on 1.5% agarose gel. Gels were observed under gel documentation unit (Alphaimager, USA). All the *Listeria* show presence of *prs* amplicon(370bp), serotype 1/2a shows presence of *prs* amplicon(370bp) and *lmo0737* amplicon(691bp), serotype 1/2b shows presence of *prs* amplicon(370bp) and ORF2819 amplicon(471bp) and serotype 4b shows presence of *prs* amplicon(370bp), ORF2819 amplicon(471bp) and ORF2110 amplicon(597bp).

Table 4.2 Primer sequences for *L. monocytogenes* used in multiplex-PCR based

Target gene		Primer sequence	Product size (bp)
lmo0737	F	5'-AGGGCTTCAAGGACTTACCC-3'	691
	R	5'-ACGATTTCTGCTTGCCATTC-3'	
ORF2819	F	5'-AGCAAAATGCCAAAACTCGT -3'	471
	R	5'- CATCACTAAAGCCTCCCATTG-3'	
ORF2110	F	5'- AGTGGACAATTGATTGGTGAA -3'	597
	R	5- CATCCATCCCTTACTTTGGAC -3'	
prs	F	5'- GCTGAAGAGATTGCGAAAGAAG-3'	370
	R	5'- CAAAGAAACCTTGGATTTGCGG-3'	

4.2.4 Pulsed field gel electrophoresis (PFGE)

PFGE was performed as per standardized CDC PulseNet protocol (Graves and Swaminathan, 2001). A total of 70 *Listeria* strains from different sources were analysed by the CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, USA).

4.2.4.1 *Preparation of culture*

L. monocytogens strains were grown on BHI agar at 37^0C overnight. Growth on agar plates was harvested and suspended in to the tubes containing 3 ml of TE buffer. The cell density was adjusted in the range of OD 0.79 to 0.81. 240 µl adjusted cell suspension was then transferred to 1.5 ml tubes. Lysozyme solution (60 µl of 10 mg/ml) was added and mixed by pipetting. The mixture was then kept for incubation on shaking water bath at 37^0C for 10 min.

4.2.4.2 *Preparation of Plugs*

An equal amount of molten 1.2% PFGE grade agarose, 1% SDS, 0.2 mg/ml Proteinase K (Sigma, St. Louis, MO) were mixed in sterile distilled water and kept at 54–56^0C. The preparation was then added to the optimized bacterial cell suspension and mixed gently. The 600 µl of mixture was then distributed into the 2 lanes of (300 µl each) reusable plug molds (Bio-Rad) and allowed to solidify for 5 min.

4.2.4.3 *Lysis of Cells in Agarose Plugs*

The solidified plugs were kept in 50 ml conical tubes containing lysis buffer (0.15 mg/ml Proteinase K, 50 mM EDTA: pH 8.0, 50 mM Tris pH 8.0, 1% sodium lauryl sarcosine). Plugs were incubated with lysis buffer for 2 h at 54–5^0C in shaking water bath at 200 rpm. After proteolysis, the lysis buffer was removed and plugs were washed two times with 15 ml of warm sterile distilled water. Subsequently, plugs were washed four times with 15 ml of warm (50–54^0C) TE buffer for 15 min in the water bath shaker (50–54^0C).

After washing, the plugs were cut (2–2.5 mm slices) using a gel-cutting fixture and made ready for restriction digestion by endonucleases or plugs can be stored further at 4^0C in TE buffer until ready for restriction.

4.2.4.4 *Restriction digestion of agarose plugs*

Plugs were digested with two restriction enzymes separately: 25 U of *Asc*I (Fermentas, USA) at 37^0C for 2-3 h and 160-200 U of *Apa*I (New England Biolabs) at 30^0C for 3-5 h.

4.2.4.5 *Casting Agarose Gel*

The gel casting tray was assembled and the comb was fitted. 1% agarose in 1x TE buffer was prepared and poured when it was at 45^0C into the gel casting tray and allowed to solidify. Digested and sliced plugs of all isolates along with Lambda ladder (New England Biolabs) were loaded very carefully on the gel. Electrophoresis was performed using a 1% agarose gel in 0.5x Tris-borate EDTA buffer. The agarose gel containing plugs was removed from gasting platform and kept carefully into the electrophoresis tank containing 2 L of 0.5x TBE buffer. Electrophoresis was carried out using the parametets: 6 V; initial switch time, 4.0 s; final switch time 40 s; run time 22 h.

4.2.4.6 *Staining and Documentation of PFGE Agarose Gel*

Gel was stained with 25 ml ethidium bromide dye solution (10 mg/ml) suspended in 400 ml of 0.5x TBE. Gel was kept staining at RT for 20-30 min of and

destained by washing twice with 400 ml of milli Q water. After staining and destaining gel was observed under gel documentation unit (Alpha Imager). The gendograms were generated on the PFGE patterns by using the Gel Compare II (Applied Maths) software. The analysis was also done by visual assessment of the PFGE profiles. A similarity coefficient of 60% was used to describe the pulsotypes.

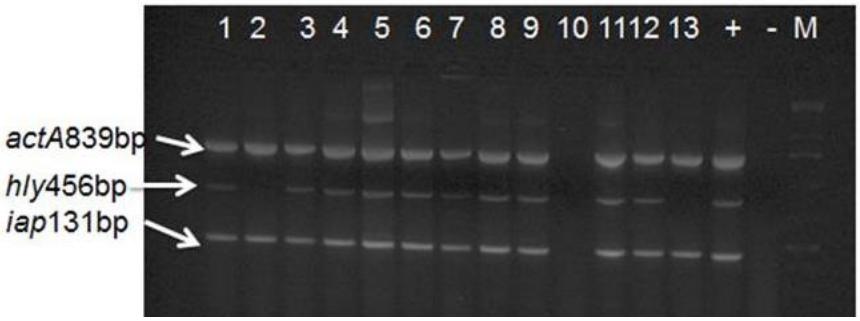
4.3 Results and Discussion

Listeria monocytogenes is a ubiquitously found in soil, faeces of animals, decaying vegetation. Listeriosis frequently affects the immuno-compromised, pregnant women, newborns, and the elderly. The incidence of the *L. monocytogenes* in the food production scene and its ability to form biofilm in food processing environments and growth in a wide range of food types at chilling temperatures allows its transmission.

Genomic methods were employed for the purpose of confirming the results obtained by the conventional methods. PCR was carried out for detection of virulence genes:*hlyA, actA* and *iap* (Rawool *et al*., 2007) which are significant in pathogenesis of *L. monocytogenes* (Li *et al*., 2015; Rawool *et al*., 2007). Detection of single virulence gene sometimes may not be sufficient to identify *L. monocytogenes*, so also some strains may be deficient in one or more virulence genes as a result of mutations (Nishibori *et al*., 1995; Cooray *et al*., 1994). Hence, simultaneous detection of more than one virulence gene of *L. monocytogenes* is desirable. PCR amplification of *hlyA*, *actA* and *iap* genes yielded amplification products of 456bp, 839bp and 131bp, respectively. Out of the 72 isolates, 70 isolates showed presence of all the three genes and only 2 isolates lacked the virulence genes tested for (Fig 4.1, Table 4.3).

Traditional culture based protocols are labor intensive as well as time consuming, hence, based on the results obtained it can be concluded that results of PI-PLC activity on ALOA medium coupled to PCR of virulence genes can be used for confirming the potential pathogenic nature of *L. monocytogenes.*

FIG. 4.1. Detection of virulence associated genes by PCR.



Lanes 1-13: *L. monocytogenes* isolates, + : *L. monocytogenes* MTCC 1143, -: Negative control, M: DNA ladder

A study carried out with eight virulence genes showed good correlation with mouse inoculation test (Liu *et al*., 2003). However, strains of *L. innocua* were lacking the virulence genes (Nishibori, 2013) and were not lethal to mice. *L. monocytogenes* strains recovered from RTE foods showed the presence of the *hlyA* and *prf* genes (Ahmed *et al*., 2013). Similarly, the *hlyA* and *iap* genes were observed in *L. monocytogenes* isolated from fish samples (Swetha *et al*., 2012). The *hlyA, iap* and *actA* genes were assessed for the virulence potential of *L. monocytogenes* strains recoverd from RTE meat-based food industries (Henriques and Normak, 2010). RT-PCR for *hly*A gene was used in diagnosis of CNS listeriosis and confirmed the results obtained by conventional culture methods (Monnier *et al*., 2011).

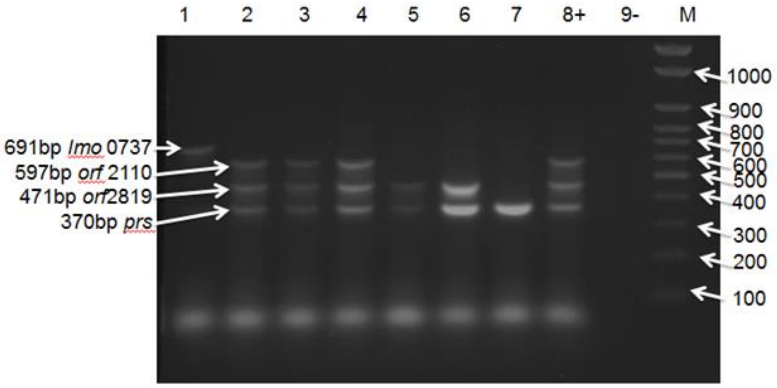
For epidemiological investigations of foodborne outbreaks and also for comparison of clinical and foodborne isolates, typing of *L. monocytogenes* is very important. Typing of strains is also important in the food-processing environment to track the sources of contamination and routes of spread of the pathogenic strains. This can be achived by using molecular methods as they are rapid and reliable. Conventional serotyping was carried out by using commercially available kits. The data indicated that in case of the strains isolated from human clinical cases serotypes 4b, 4d, 4e and 1/2b were identified with the major serotype being 4b (73.91%) followed by 4d, 4e and 1/2b (8.69% each). *L. innocua* was identified in one case (Table 4.5). Characterization of the isolates from animal clinical cases revealed serotypes 4b (65.95%), 4d (14.89%), 1/2a (8.51%), 1/2b (10.63%). *L. innocua* was confirmed from one case (Table 4.6). Serotype 4b was observed to be the major serotype in isolates from human as well as animal origin, however, isolates of serotype 1/2a were detected in animal isolates and 4e serotype was detected in human but not in animal isolates. Conventional serotyping is difficult to perform and also expensive, moreover, since antigenic sharing happens regularly among *L. monocytogenes* serotypes, it poses challenges to define the serotypes of few *L. monocytogenes* and variants (Schonberg *et al*., 1996, Liu *et al*., 2006).

A multiplex-PCR serotyping assay has been established which divided the four main *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c and 4b) into different groups (Doumith *et al*., 2004). This method was used in the present study to serotype *L. monocytogenes* strains recovered from human clinical cases. It is recommended that conventional and molecular methods should be used in conjunction to obtain correct results. Serogroups conforming to serotypes 4b, 4d and 4e was noticed in 21 strains

(86.6%) and 1/2b, 3b in 2 strains (8.69%) in case of human isolates. In case of isolates of animal origin, 4b, 4d and 4e group was found in 38 strains (80.85%), 1/2a, 3a serogroup in 4 strains (8.51%) and 1/2b, 3b serogroup in 5 strains (10.63%) (Fig. 4.2) . The results indicated that isolates were predominanatly of the serogroup 4b, 4d and 4e (Table 4.4, Fig 4.3) with the highest number belonging to serotype 4b which was documented from most of the listeriosis outbreaks (Ericsson *et al*., 1995: Kathariou *et al*., 2005). A clinical strain obtained from CSF samples of a newborn was serotyped as an atypical 4b serotype (Vasconcelos *et al*., 2008) by using molecular techniques along with conventional methods, a highly virulent veterinary outbreak strain of *L. monocytogenes* was also characterized as an atypical 4b serotype (Bundrant *et al*., 2011). In a study carried out on 251 stains of human origin collected in the DPVSA laboratory in Italy from 2000-2010 by using the multiplex serotyping PCR method, it was observed that the predominant serotype was 1/2a (46.6%) followed by 4b (30.7%) and 1/2 b (14.7) (Pontello *et al*., 2012).

In Sweden, 18% of the human isolates in 2010 were reported to be of serotype 4b (Lambertz et al., 2013). In another study, 154 isolates of *L. monocytogenes* isolated in Spain were grouped into four serotypes, 4b (61%), 1/2b (19%), 1/2a (18%) and 1/2c (2%), with 100% susceptibility to ampicillin and cotrimoxazole (Lepe *et al*., 2012). In a study in Greece, serogroup 4b, 4d, 4e was detected in 39.4%, in strains isolated from food and clinical while, only serogroup 1/2a, 3a (23.0%) was noticed among food isolates (Houhoula *et al.,* 2012). All three strains of *L. monocytogenes* from animal samples belonged to serotype 4b. The high incidence of the 4b serotype indicated the threat of epidemics if adequate preventive measures are not employed.

FIG. 4.2: Serotyping of *Listeria monocytogenes* using multiplex PCR.



Lane 1- *L. monocytogenes* serotype 1/2a, lanes 2, 3,4 and 8 - *L. monocytogenes* serotype 4b, lanes 5 and 6 - *L. monocytogenes* serotype 1/2b, lane 7 – *Listeria* spp., lane 8 - *L. monocytogenes* MTCC 1143 serotype 4b, lane 9 – Negative control, M - DNA ladder

Table 4.3: Sources , **v**irulence gene profiling and serotypes of *Listeria* isolates from human & animal clinical cases.

Sr. No.	Isolate	Source/syndrome	Sample type	Virulence genes			Genomic serogroup	Serotype
				hlyA	*actA*	*iap*		
1	H1	Kidney disease	Urine	+	+	+	4b,4d,4e	4b
2	H2	Septicaemia	Blood	+	+	+	4b,4d,4e	4d
3	H3	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
4	H4	Abortion	Placental tissue	+	+	+	1/2b,3b	1/2b
5	H5	Septicaemia	Blood	+	+	+	1/2b,3b	1/2b
6	H6	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
7	H7	Septicaemia	Blood	+	+	+	4b,4d,4e	4e
8	H8	Kidney disease	Urine	+	+	+	4b,4d,4e	4b
9	H9	Diarrhoea	Stool	+	+	+	4b,4d,4e	4e
10	H10	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
11	H11	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
12	H12	Abortion	Placental tissue	+	+	+	4b,4d,4e	4d
13	H13	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
14	H14	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
15	H15	Abortion	Placental tissue	_	_	_	_	_
16	H16	Kidney disease	Urine	+	+	+	4b,4d,4e	4b
17	H17	Diarrohea	Stool	+	+	+	4b,4d,4e	4b
18	H18	Diarrohea	Stool	+	+	+	4b,4d,4e	4b
19	H19	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
20	H20	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
21	H21	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
22	H22	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
23	H23	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b

24	H24	Abortion	Placental tissue	+	+	+	4b,4d,4e	4b
25	A1	Mastitis	Faeces	+	+	+	4b,4d,4e	4d
26	A2	Mastitis	Faeces	+	+	+	4b,4d,4e	4b
27	A3	Mastitis	Milk	+	+	+	4b,4d,4e	4d
28	A4	Mastitis	Faeces	+	+	+	1/2b,3b	1/2b
29	A5	Abortion	Vaginal swab	+	+	+	1/2b,3b	1/2b
30	A6	Mastitis	Milk	+	+	+	4b,4d,4e	4b
31	A7	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
32	A8	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
33	A9	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
34	A10	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
35	A11	Mastitis	Faeces	+	+	+	4b,4d,4e	4b
36	A12	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
37	A13	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
38	A14	Mastitis	Milk	+	+	+	4b,4d,4e	4b
39	A15	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4d
40	A16	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
41	A17	Abortion	Faeces	+	+	+	4b,4d,4e	4b
42	A18	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
43	A19	Abortion	Urine	+	+	+	4b,4d,4e	4d
44	A20	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4d
45	A21	Abortion	Urine	+	+	+	4b,4d,4e	4d
46	A22	Abortion	Vaginal swab	_	_	_	_	_
47	A23	Abortion	Faeces	+	+	+	4b,4d,4e	4d
48	A24	Abortion	Urine	+	+	+	1/2a,3a	1/2a
49	A25	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
50	A26	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b

51	A27	Abortion	Urine	+	+	+	1/2a,3a	1/2a
52	A28	Abortion	Faeces	+	+	+	1/2a,3a	1/2a
53	A29	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
54	A30	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
55	A31	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
56	A32	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
57	A33	Abortion	Vaginal swab	+	+	+	1/2a,3a	1/2a
58	A34	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
59	A35	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
60	A36	Abortion	Vaginal swab	+	+	+	1/2b,3b	1/2b
61	A37	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
62	A38	Abortion	Vaginal swab	+	+	+	1/2b,3b	1/2b
63	A39	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
64	A40	Abortion	Vaginal swab	+	+	+	1/2b,3b	1/2b
65	A41	Abortion	Vaginal swab	+	+	+	4b,4d,4e	4b
66	A42	Mastitis	Milk	+	+	+	4b,4d,4e	4b
67	A43	Mastitis	Milk	+	+	+	4b,4d,4e	4b
68	A44	Mastitis	Milk	+	+	+	4b,4d,4e	4b
69	A45	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
70	A46	Mastitis	Faeces	+	+	+	4b,4d,4e	4b
71	A47	Septicaemia	Blood	+	+	+	4b,4d,4e	4b
72	A48	Septicaemia	Blood	+	+	+	4b,4d,4e	4b

Table 4.4: Overall Serotypes of Clinical isolates.

PCR Serotyping	Conventional Serotyping	Number of isolates	Percentage
4b,4d,4e	4b	48	68.57%
4b,4d,4e	4d	9	12.85%
4b,4d,4e	4e	2	2.85%
1/2a,3a	1/2a	4	5.71%
1/2b,3b	1/2b	7	10.0%
Listeria spp.	*Listeria* spp.	2	

Table 4.5: Serotypes of Clinical isolates from humans.

PCR Serotyping	Conventional Serotyping	Number of isolates	Percentage
4b,4d,4e	4b	17	73.91%
4b,4d,4e	4d	2	8.69%
4b,4d,4e	4e	2	8.69%
1/2a,3a	1/2a	0	0
1/2b,3b	1/2b	2	8.69%
Listeria spp.	*Listeria* spp.	1	

Table 4.6: Serotypes of Clinical isolates from animals.

PCR Serotyping	Conventional Serotyping	Number of isolates	Percentage
4b,4d,4e	4b	31	65.95%
4b,4d,4e	4d	7	14.89%
4b,4d,4e	4e	0	0
1/2a,3a	1/2a	4	8.51%
1/2b,3b	1/2b	5	10.63%
Listeria spp.	*Listeria* spp.	1	

Genomic methods of subtyping have a improved discriminatory index, reproducibility and fidelity as associated to phenotypic methods and hence, the methods are used for epidemiological investigations especially in case of outbreaks. However, in some situations their discriminatory power may be insufficient especially if they target restriction polymorphism only in some genes. PFGE targets restriction polymorphism in the genome and thus shows higher discriminatory power and hence, has animal been recommended as the gold standard method of subtyping by CDC for foodborne pathogenes.

Several pulsotypes of *Asc*I as well as *Apa*I were observed among the isolates (Fig. 4.3, 4.4, 4.5, 4.6). PFGE analysis showed differentiation among isolates belonging to the same serogroup as well as from the same sources and areas. Ten *Apa*I pulsotypes were observed among the 23 human isolates. Eight isolates observed with unique *Asc*I pulsotypes, and the remaining *Asc*I pulsotypes were allotted to four clusters. Each cluster contained one to three isolates. Among isolates from animals, at 60% similarity, 6 *Asc*I and 8 *Apa*I pulsotypes were observed. Analysis uing UPGMA showed differentiation of the isolates based on the lineages I and II which are simillar to results obtained in previous studies (Gilberth *et al*., 2009). The PFGE types were also compared with pulsotypes for isolates from other countries (Yde and Genicot, 2004, Mammina *et al*., 2009). The banding pattern was compared as described by this study had different PEGE pattern when compared with the isolates described earlier (Yde and Genicot , 2004 ; Mammina *et al.*, 2009). In an earlier study, PFGE analysis of 495 *L.monocytogenes* strains from food and human clinical cases showed persistence of 7 pulsotypes in food and food processing environment as well as clinical samples (Fugett *et al*., 2007). A similar study was also carried out to compare

the macrorestriction pattern of animal isolates with human isolates and 29% of the pattern of animal isolates thus suggesting an epidemiological link in infections (Okwumabua *et al*., 2005). An investigation in Chile revealed the presence of similar pulsotype among the isolates from poultry and human clinical cases (Foerster *et al.,* 2003).

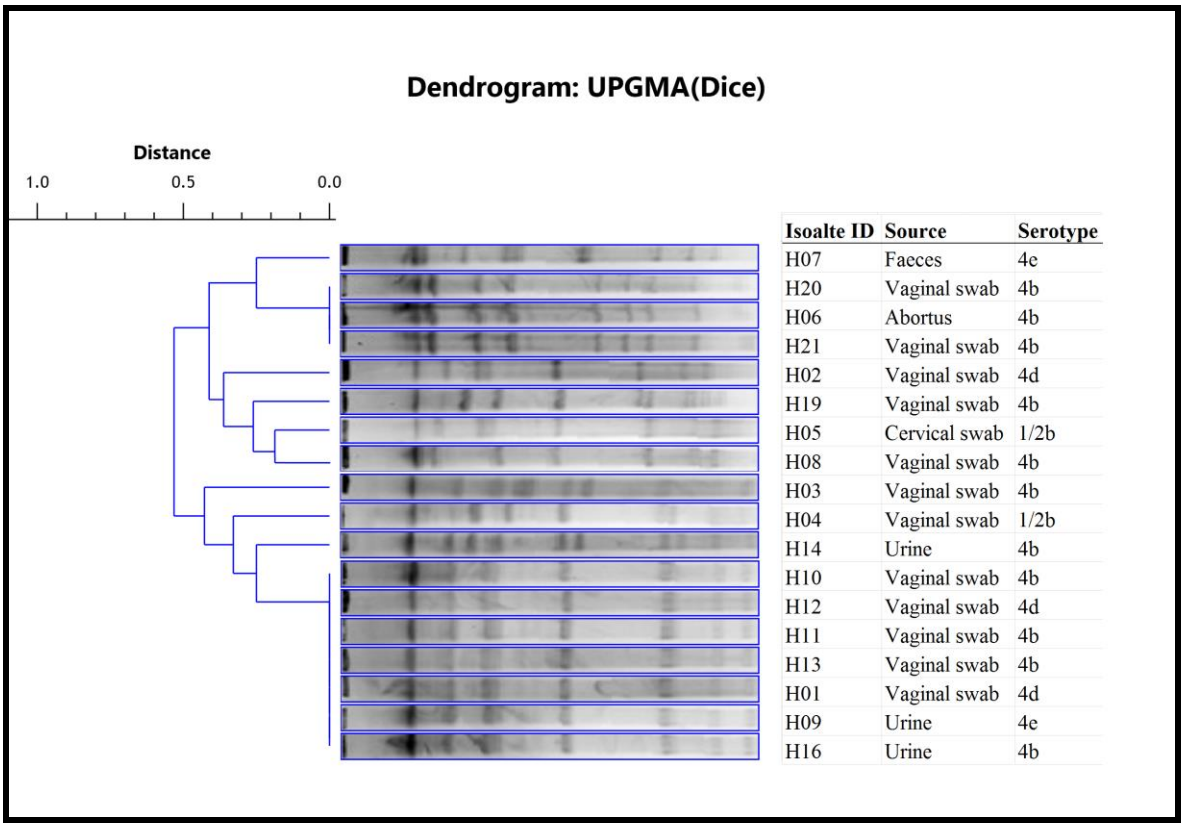
PFGE is also used by european Union Refence laboratory for typing of *L.monocytogenes* strains throughout the countries belonging to the European Union (Felix *et al*., 2010).

In India, according to earlier data, in the period between 1966 and 2005, a number of bacteriologically confirmed human listeriosis cases have been described (Malik *et al*., 2002 ; Dhanashree *et al*., 2003; Gupta and Sharma, 2013). The isolates from these studies were not available for analysis. Most industrialized countries have an yearly listeriosis occurrence of 2 to 10 reported cases per million people every year (Goulet *et al*., 2008). However, listeriosis has a 30% case fatality rate (Barbuddhe et al., 2012; CDC, 2013). Rapid and differentiative subtyping assays, such as various DNA-based methods, for example PFGE, PCR and restriction fragment length polymorphism analysis, or sequencing of some targeted sequences, are needed for the epidemiological investigation of *L. monocytogenes* and the tracing of precise clones along food-processing units (Wiedmann, 2002; Sauders *et al*., 2006). More recently, we have reported on the development of a 10-minute assay grounded on MALDI-TOF spectroscopy directly from colonies on agar plates. The method allows not only discrimination between pathogenic and nonpathogenic *Listeria* spp., but also permits resolution up to the level of the PCR serotype analysis described above (Barbuddhe *et*

al., 2008). Nevertheless, the high cost of the capital equipment involved, despite being offset by cheap running costs mof the assay, puts this method beyond the means of smaller diagnostic laboratories.

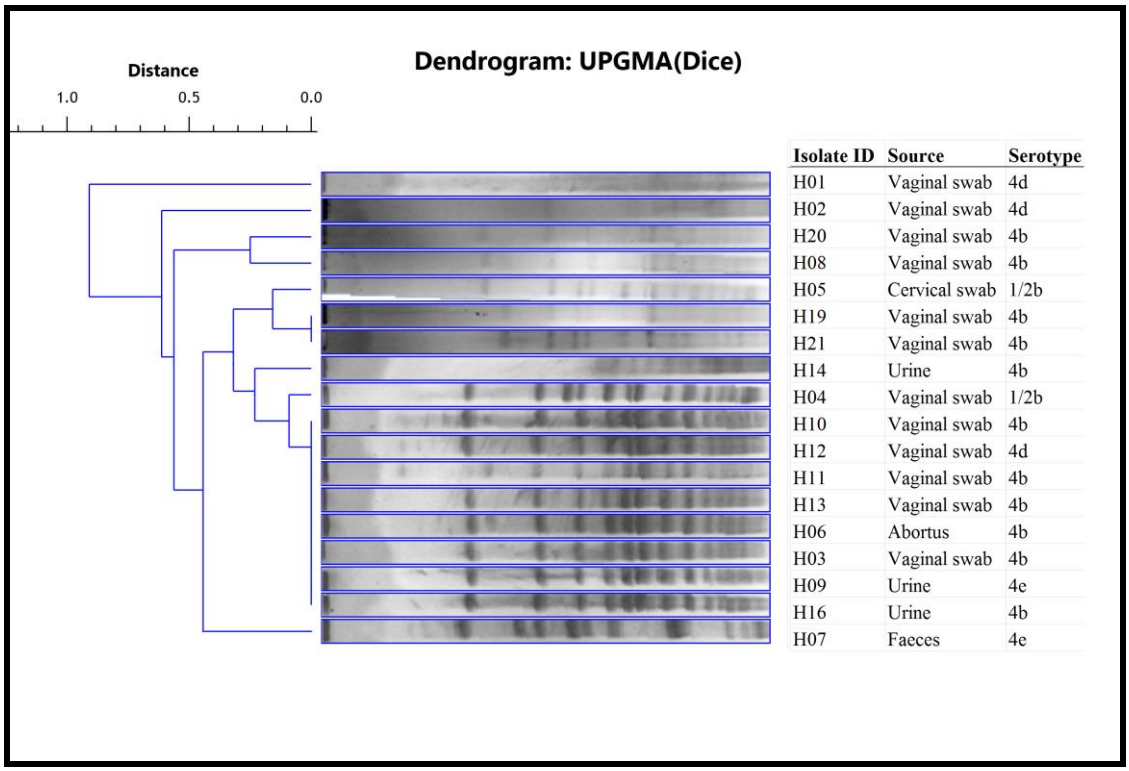
The application of PFGE in characterization of clinical isolates along with environmental and food samples can provide valuable information for epidemiological tracking in order to identify sites of cross contamination.

Fig 4.3 PFGE analysis of *L. monocytogenes* strains isolated from human clinical cases after restriction digestion by *Asc*I.



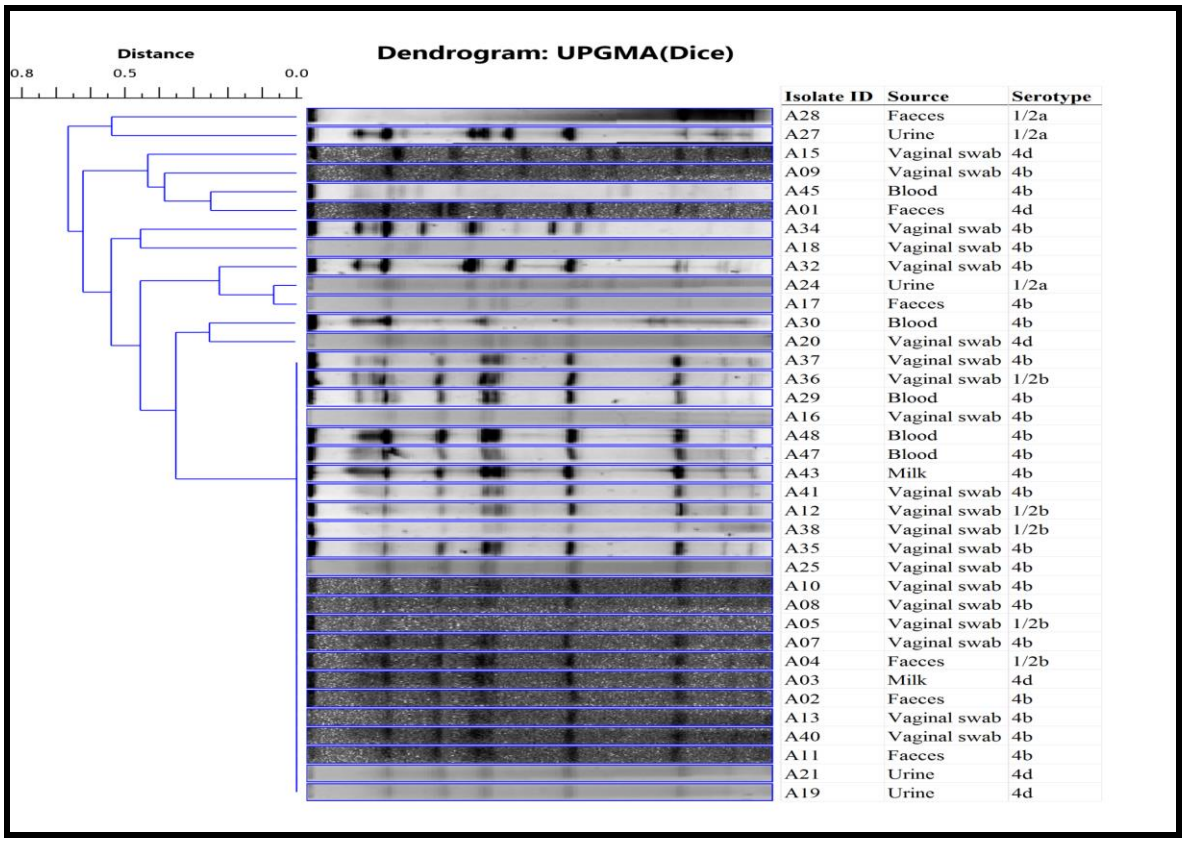
Isoalte ID	Source	Serotype
H07	Faeces	4e
H20	Vaginal swab	4b
H06	Abortus	4b
H21	Vaginal swab	4b
H02	Vaginal swab	4d
H19	Vaginal swab	4b
H05	Cervical swab	1/2b
H08	Vaginal swab	4b
H03	Vaginal swab	4b
H04	Vaginal swab	1/2b
H14	Urine	4b
H10	Vaginal swab	4b
H12	Vaginal swab	4d
H11	Vaginal swab	4b
H13	Vaginal swab	4b
H01	Vaginal swab	4d
H09	Urine	4e
H16	Urine	4b

Fig 4.4 PFGE analysis of *L. monocytogenes* strains isolated from human clinical cases restricted by *Apa*I**.**



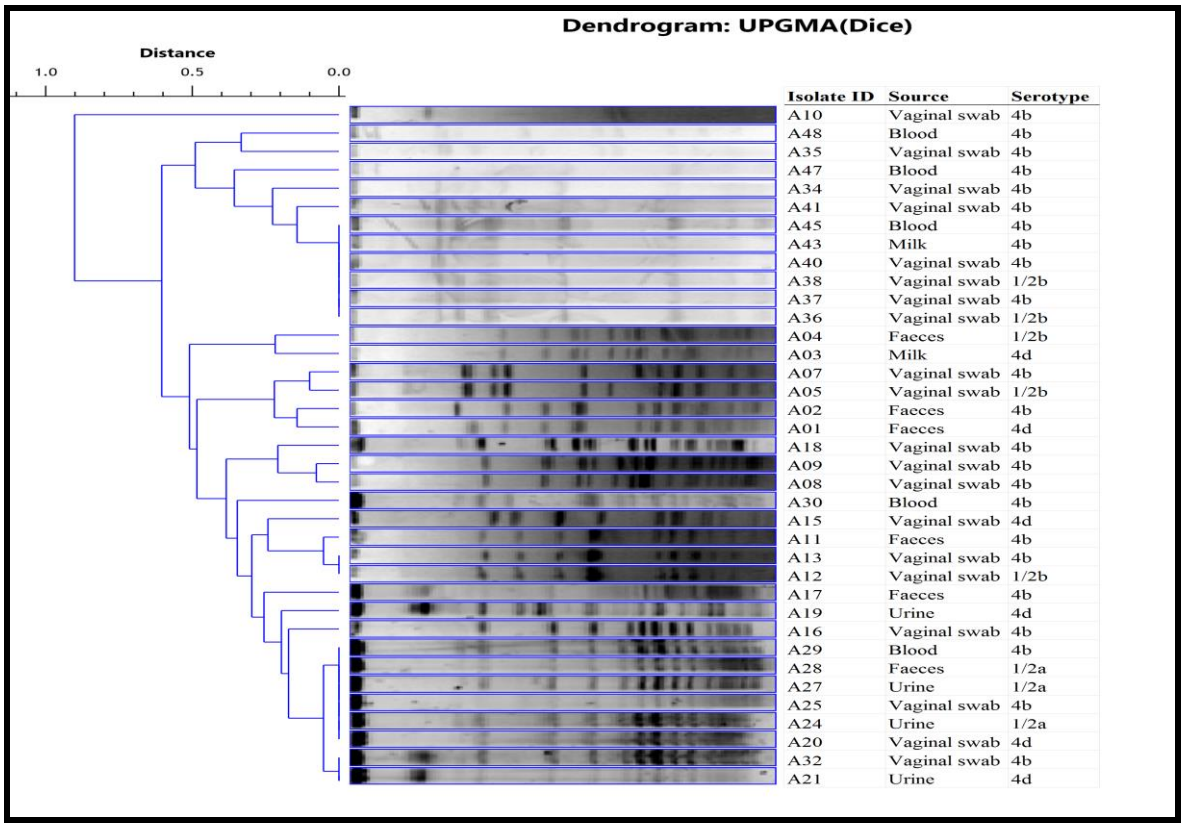
Isolate ID	Source	Serotype
H01	Vaginal swab	4d
H02	Vaginal swab	4d
H20	Vaginal swab	4b
H08	Vaginal swab	4b
H05	Cervical swab	1/2b
H19	Vaginal swab	4b
H21	Vaginal swab	4b
H14	Urine	4b
H04	Vaginal swab	1/2b
H10	Vaginal swab	4b
H12	Vaginal swab	4d
H11	Vaginal swab	4b
H13	Vaginal swab	4b
H06	Abortus	4b
H03	Vaginal swab	4b
H09	Urine	4e
H16	Urine	4b
H07	Faeces	4e

Fig 4.5 PFGE analysis of *L. monocytogenes* isolates of animal origin restricted by *Asc*I.



Isolate ID	Source	Serotype
A28	Faeces	1/2a
A27	Urine	1/2a
A15	Vaginal swab	4d
A09	Vaginal swab	4b
A45	Blood	4b
A01	Faeces	4d
A34	Vaginal swab	4b
A18	Vaginal swab	4b
A32	Vaginal swab	4b
A24	Urine	1/2a
A17	Faeces	4b
A30	Blood	4b
A20	Vaginal swab	4d
A37	Vaginal swab	4b
A36	Vaginal swab	1/2b
A29	Blood	4b
A16	Vaginal swab	4b
A48	Blood	4b
A47	Blood	4b
A43	Milk	4b
A41	Vaginal swab	4b
A12	Vaginal swab	1/2b
A38	Vaginal swab	1/2b
A35	Vaginal swab	4b
A25	Vaginal swab	4b
A10	Vaginal swab	4b
A08	Vaginal swab	4b
A05	Vaginal swab	1/2b
A07	Vaginal swab	4b
A04	Faeces	1/2b
A03	Milk	4d
A02	Faeces	4b
A13	Vaginal swab	4b
A40	Vaginal swab	4b
A11	Faeces	4b
A21	Urine	4d
A19	Urine	4d

Fig 4.6 PFGE analysis of *L. monocytogenes* strains isolated from animal clinical cases after restriction digestion by *Apa*I**.**



As the *L. monocytogenes* strains were obtained from limited geographical areas, generalization of the finding could not be made. Also, due to the pausity of information about the food sources or their exposure to consumers it was difficult to compare strain from different sources and to track their sources. Despite this, the results reported in this study might be helpful in interpretation of the epidemiology of listeriosis in India. Use of conventional and molecular techniques in comination in epidemiological investigations of food-borne episodes and surveillance is essential for management of human listeriosis. Proper communication of data on foodborne episodes between health and food authorities should improve the efficacy of control measures.

The use of PFGE in association with multiplex-PCR based serotyping allowed quick differentiation of *L. monocytogenes* strains**.** The detection of *L. monocytogenes* serotype 4b predominantly is a cause of concern owing to its association with human listeriosis outbreaks globally. The sharing of data on typing of strains fromhuman and food sources between health and food authorities should improve the efficacy of control measures.

CHAPTER 5

SUMMARY OF THE THESIS

Listeriosis, an important foodborne disease, has a high case fatality and hospitalization rates. Although outbreaks similar to the massive ones that were observed in Europe and North America have not yet occurred in India, with the changing lifestyles and food habits, the threat is increasing day by day. The global trade of foods produced from different countries is also increasing. Awareness and timely precautionary measures are very important to prevent the infection from vulnerable population.

The investigation was undertaken to assess the prevalence of *Listeria* in clinical samples collected from various places in Goa and Maharashtra and their characterization by phenotypic and genotypic methods.

Clinical samples (596) collected from humans and animals were analyzed by the USDA method. The presumptive *Listeria* isolates were characterized biochemically and using sugar fermentation tests. Further the isolates were characterized employing haemolysis on sheep blood agar, CAMP test and ALOA medium for phospholipase activity.

The percentage of isolation of *Listeria* from samples of human origin was 1.45% and that of animal origin was 2.6%. In humans, vaginal swabs and fecal samples showed a higher rate of isolation as compared to other clinical samples. *Listeria monocytogenes* isolates were also obtained from other parts of India and analyzed to know an overall picture of prevalence of the organism and its serotypes in India.

The isolates were serotyped by using multiplex PCR method as well as by conventional serotyping method. The data indicated that in case of the isolates obtained from human clinical cases serotypes 4b, 4d, 4e and 1/2b were identified with the predominant serotype being 4b (73.91%) followed by 4d, 4e and 1/2b (8.69% each). *L. innocua* was identified in one case. The strains isolated from clinical cases in animals, *L. monocytogenes* serotypes 4b (65.95%), 4d (14.89%), 1/2a (8.51%), 1/2b (10.63%) were confirmed. *L. innocua* was isolated from 2% cases. All the isolates except two showed the presence of the virulence genes, *hlyA*, *act*A and *iap* indicating that they are pathogenic in nature.

Molecular subtyping carried out by pulsed field gel electrophoresis (PFGE) revealed the presence of different pulsotypes. The isolates recovered from humans and animals were clonal indicating an epidemiological link between them. The organisms may get transmitted from animals to humans either through the foodborne route or through handling.

The results of antibiotic sensitivity showed a slight variation between results obtained with animal and human isolates. In case of human isolates trimethoprim (100% sensitive) was seen to be most effective whereas among the commonly used therapeutic antibiotics erythromycin (84% resistance), ampicillin (80% resistance) and vancomycin (76% resistance) were seen to be ineffective. While in case of animal isolates although trimethoprin (91% sensitive) was most effective, penicillin (57.58% resistance) was the most ineffective and this may be because of indiscriminate use of the antibiotic in animals which may have led to occurrence of resistance strains.

Most of the isolates (86%) showed high glutamate decarboxylase activity, which helps to enhance continued existence of *L. monocytogenes* in gastric juice in the stomach and in acidic foods such as yogurt, acidic juices, salad dressings etc.

Survivability studies of the isolates in gastric juice in the presence and absence of MSG was carried out and it was observed that survival was much greater in the presence of MSG than in its absence. Since many of the RTE foods contain MSG for flavor enhancement or adjustment of acidity, this may also be a contributory factor in the persistance of *L. monocytogenes* as a contaminant in such foods.

The study confirmed the presence of *Listeria* spp. in clinical samples. It is also indicative of the predominance of 4b serotype in the isolates of both animal as well as human origin. Since the study encompasses isolates from different parts of India it also suggests the dominance of 4b serotype in the country. The antibiotic profile points to emergence of resistance to some of the commonly used antibiotics like penicillin, ampicillin and vancomycin which could prove to be of significance in the future. Subtyping results showed the variation at a genomic level even between isolates of the same serotype.

The salient findings of the study included:

- The study confirmed the occurrence of *Listeria* in human as well as in animal clinical samples.
- The rate of isolation of *L. monocytogenes* from animal samples was higher than in human samples, probably due to improper housing and hygiene of animals.

- The predominant serotype of *L. monocytogenes* obtained was 4b. This serotype has been implicated in many outbreaks. Serotypes 1/2b, 1/2a, 4d and 4e were also detected.
- All the isolates except two possessed virulent genes reflecting on the pathogenicity of the isolates and the two isolates were later confirmed to be *L. innocua*.
- Various pulsotypes were seen on carrying out PFGE indicating the presence of variants even among the same serotypes. Common PFGE profiles were also observed among animal and human isolates suggesting an epidemiological link between them.
- *L. monocytogenes* isolates were highly resistant to meropenem, erythromycin, penicillin, vancomycin and ampicillin while majority of the isolates were sensitive to trimethoprim and gentamicin. Antibiotic sensitivity results showed variation with respect to human and animal isolates.
- A high rate of glutamate decarboxylase activity was seen in case of most of the isolates which may be an important factor in enhancing the rate of survival in the stomach and in acidic foods.
- A higher rate of survival in gastric juice was observed in the presence of MSG and since some RTE foods contain MSG as a flavouring agent this can prove to be an important factor in survivability of the pathogen in gastric juice.

In conclusion, awareness needs to be created among the public as well as among the medical practitioners about the etiology of this organism which is an opportunistic pathogen and an important foodborne zoonotic agent. Since the *Listeria* is a part of Food Safety and Standards Regulations by Govt. of India enhanced

surveillance of the pathogen need to be encouraged to break the chain of transmission to humans through foods of animal origin.

NEEDS FOR

FUTURE RESEARCH

Unplanned urbanization, globalization, climatic changes, fluctuations in microbial and ecological systems and poor sanitation have led to an enhancement in episodes of infectious diseases. As the situation persists, the occurrence of foodborne diseases may escalate particulalrly in the developing countries.

As reported by the World Health Organization microbes are responsible for 25% of the total 57 million deaths annually. *Listeria* is one of the leading lethal foodborne pathogen with a high case fatality rate. Therefore, *Listeria* has been the focus of research globally.

In India, the data on foodborne illnesses is largely lacking owing to poor networking and lack of foodborne disease surveillance. However, some reports on sporadic cases of both human as well as animal listeriosis are available. Due to the changing lifestyle and food habits as well as increasing awareness about the disease and availability of better diagnostic methods, an increased number of sporadic cases of listeriosis have been reported in India recently.

Epidemiological investigations has a major role in determining the sources of contaminations. Various techniques have been employed for tracking the source of contamination and study the epidemiological patterns. Novel habitats, and new epidemiological niches of the pathogen need to be explored.

Genotypic methods such as PFGE can be used to establish the prevalence of common pulsotypes in isolates obtained from various sources such as clinical, food, environmental samples, water and vegetation.

Further work has to be carried out to trace the epidemiological routes by comparing the PFGE patterns of isolates obtained from clinical sources, food, environmental samples, water, vegetation to establish prevalence of common pulsotypes from various sources. Other methods including MLST and MVLST can also be performed for further subtyping. Sharing of genomic data electronically may help effective surveillance of infections and rapid investigation of possible infection clusters as has been done in PulseNet programmes. *In vivo* pathogenicity studies of clinical isolates can be carried out to establish the degree of pathogenicity and hence deduce the propensity of the pathogens to cause infections.

Early treatment with proper antibiotics is essential for management of listeriosis. Indiscriminate and suboptimal use of antibiotics in animal farming and human treatment may lead to emergence of antibiotic resistance in bacteria including *Listeria* spp. There are reports of multidrug resistance *Listeria* strains. The antibiotic susceptibility testing of the isolates need to be advocated before treatment. The genomic nature of the strains can be determined by isolating the DNA and carrying out PCR of specific genes responsible for conferring resistance.

An effective surveillance system along with the laboratory facilities to isolate and maintain the cultures would also help in elucidating the incidence and distribution of *Listeria* in India. These cultures can then be genomically compared to different isolates from all over the world in order to establish genomic links or differences.

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APPENDICES

Appendix A. Media Composition

UVM 1 enrichment broth :

Proteose peptone	5.0 g
Tryptone	5.0g
Beef extract powder	5.0g
Yeast extract	5.0g
Potassium dihydrogen phosphate	1.35g
Sodium chloride	20.0g
Disodium hydrogen phosphate (anhyrous)	12.0g
Nalidixic acid (0.4% in 1N NaOH)	10 ml
Acriflavine (0.25 % in water)	5ml
Distilled water	1000ml
Autoclave at 15 lbs for 15 min.	
pH	7.4

UVM II enrichment broth

Composition is same as UVM I except instead of 5 ml, 10 ml of the acriflavine solution should be added.

PALCAM Agar

Ingredients	Grams/Litre
Peptone	23.0
Starch	1.0
Sodium chloride	5.0
D-Mannitol	10.0
Ammonium ferric citrate	0.5
Esculin	0.8
Glucose	0.5
Lithium chloride	15.0
Phenol red	0.08
Agar	13.0
Final pH	7.0 +/- 0.2 at 25°C.

Dissolve 68.8 g in 1 litre distilled water. Sterilize by autoclaving at 121°C for 15 minutes. Cool down to 50°C and add the dissolved contents of 2 vials PALCAM Listeria Selective Supplement.

Mueller- Hinton Agar

Ingredients	Grams/Litre
Beef Extract.	2.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g
pH	7.4

ALOA (Agar Listeria Ottavani & Agosti) Medium

Ingredients	**Grams/Litre**
Meat peptone	18.0
Tryptone	6.0
Yeast extract	10.0
Sodium pyruvate	2.0
Glucose	2.0
Magnesium glycerophosphate	1.0
Magnesium sulphate	0.5
Sodium chloride	5.0
Lithium chloride	10.0
Disodium hydrogen phosphate anhydrous	2.5
5-bromo-4-chloro-3-indolyl-ß-D-glucopyranoside	0.05
Agar, according to gelation-strength	12 to 18
Water, according to volume of fungistat supplement	925-930 ml

Dissolve the dehydrated components or dehydrated complete base in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is 7.2 ± 0.2.
Sterilize by autoclaving for 15 minutes at 121°C.

Supplements: Dissolve 2 g of L-α-Phosphatidylinositol (Sigma P 6636) in 50 ml of cold distilled water.

Stir for about 30 minutes until a homogeneous suspension is obtained.

Autoclave at 121°C for 15 minutes. Cool to 48-50°C.

Dissolve the following compounds individually in the specified solvents and filter-sterilize the solutions: 0.02 g nalidixic acid (sodium salt) in 5 ml of water; 0.02 g of ceftazidime in 5 ml of water; 76700 U of polymyxin B in 5 ml of water; 0.05 g of cycloheximide in 2.5 ml of ethanol and add 2.5 ml of water; 0.01 g of amphotericin B in a mixture of 2.5 ml HCl (1 M) and 7.5 ml dimethylformamide.

Complete medium: Agar base 925 ml (or 930 ml); Nalidixic Acid solution; 5 ml; Ceftazidime solution, 5 ml; Cycloheximide solution, 5 ml (or Amphotericin B solution 10 ml); Polymyxin B solution, 5 ml; L-α-Phosphatidylinositol solution, 50 ml.

Blood Agar

Ingredients	**Grams/Litre**
Proteose peptone	15.0
Liver extract	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0
Final pH (at 25°C)	7.4±0.2

70ml of defibrinated sheeps blood is added to 1 litre of the medium just before pouring the plates.

BHI broth

Ingredients	**Grams / Litre**
Calf brain, infusion from	200.0
Beef heart, infusion from	250.0
Proteose peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Final pH (at 25°C)	7.4±0.2

Synthetic Gastric Fluid

Ingredients	**Grams / Litre**
Proteose peptone	8.3
D-glucose	3.5
NaCl	2.05
KH_2PO_4	0.6
$CaCl_2$	0.4
KCl	0.37
Lysozyme	0.1
Porcine Bile Salts	0.05
Pepsin	0.13
Final pH (at 37°C)	3.5

To prepare synthetic gastric fluid with MSG, 10 mM monosodium glutamate (Sigma) was added to 1 litre of synthetic gastric fluid before pH adjustment.

Test Solution of GAD Assay

Ingredients	**Grams / Litre**
L-glutamic acid	1.0
NaCl	90
Bromocresol Green	0.05
Triton X	0.3ml
Final pH (at 25°C)	4.0

PFGE reagents

1. **TRIS stock (1M)**

TRIS	121.14 g
D/W	1000 ml

Adjust the pH to 8.0 if necessary

2. **EDTA stock (1M)**

EDTA	372.24 g
D/W	1000 ml

Adjust the pH to 8.0 if necessary

3. **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0):**

Tris (1M, pH 8.0)	10 ml
EDTA (1M, pH 8.0)	1 ml
D/W	89 ml

Adjust the pH to 8.0 if necessary

4. **1% PFGE grade agarose in TE Buffer:**

Agarose	1 gm
TE buffer (pH 8.0)	10 ml

5. **Phosphate-buffered saline (0.01 M; pH 7.2)**

Na_2HPO4 (anhydrous)	1.09 g
NaH_2PO4 (anhydrous)	0.32 g
NaCl	9.0 g
D/W	1000 ml

Mix to dissolve and adjust pH to 7.2 Store this solution at room temperature.

6. **Cell lysis buffer (50mM Tris:50mM EDTA, pH 8.0 + 1% Sarcosyl)**

Tris (1M, pH 8.0)	5 ml
EDTA (1M, pH 8.0)	5 ml
Sarcosyl	1 gm
D/W	90 ml

7. **Cell Lysis/Proteinase K Buffer:**

Cell lysis buffer	5 ml
Proteinase K (20 mg/ml)	25 µl

8. **SSP solution** (1.2% agarose:1% Sodium Dodecyl Sulfate: 0.2 mg/ml proteinase K)**:**

Agarose	0.12 gm
SDS	0.1 gm
Proteinase K (20 mg/ml)	100 µl
Distilled water	9.9 ml

Appendix B. List of primers used

Listeria primers

Table 1 : Primer sequences for *L. monocytogenes* used in Multiplex-PCR serotyping (Doumith et al., (2004b)

Target gene	Primer sequence	Product size (bp)
lmo0737	Forward 5'-AGGGCTTCAAGGACTTACCC-3' Reverse 5'-ACGATTTCTGCTTGCCATTC-3'	691
lmo1118	Forward 5'-AGGGGTCTTAAATCCTGGAA-3' Reverse 5'-CGGCTTGTTCGGCATACTTA-3'	906
ORF2819	Forward 5'-AGCAAAATGCCAAAACTCGT -3' Reverse 5'- CATCACTAAAGCCTCCCATTG-3'	471
ORF2110	Forward 5'- AGTGGACAATTGATTGGTGAA -3' Reverse 5- CATCCATCCCTTACTTTGGAC -3'	591
prs	Forward 5'- GCTGAAGAGATTGCGAAAGAAG-3' Reverse 5'- CAAAGAAACCTTGGATTTGCGG-3'	370

Table 2: Primer sequences for *hlyA*, *actA* and *iap* genes used in Multiplex-PCR of Virulence genes of *L. monocytogenes* (Rawool *et al*., 2007).		
Target gene	Primer sequence	Product size (bp)
hly A	Forward 5'- GCAGTTGCAAGCGCTTGGAGTGAA-3' Reverse 5'- GCAACGTATCCTCCAGAGTGATCG-3'	456
actA	Forward 5'-CGCCGCGGAAATTAAAAAAAGA-3' Reverse 5'-ACGAAGGAACCGGGCTGCTAG-3'	839
iap	Forward 5'-ACAAGCTGCACCTGTTGCAG-3' Reverse 5'-TGACAGCGTGTGTAGTAGCA-3'	131