Chapter II.

Review of Literature

Food-borne or waterborne microbial pathogens are leading causes of illness and death in less developed countries, killing an estimated 1.9 million
people annually at the global level. Even in developed countries, it is estimated that up to one third of the population are affected by microbiological food-borne diseases each year (Schlundt et al., 2004). The factors involved in significant increase are generally agreed to include changes in animal production systems and in the food production chain. Both types of changes can cause corresponding changes in patterns of exposure to the pathogens and the susceptibility pattern of the human population (Schlundt et al., 2004).

Mastitis, on account of its causing serious wastage and undesirable milk quality, is emerging as a major challenge among the others (like breeding improvement, nutrition management, control of infectious, tick-borne, blood and internal parasitic diseases) in dairy development of tropics. Subclinical mastitis was found more important in India (varying from 10-50% in cows and 5-20% in buffaloes) than clinical mastitis (1-10%) (Joshi and Gokhale., 2006).

2.1 Microbiological quality of milk

The total count of bacteria in milk has a decisive effect on the quality and safety of dairy products (Szteyn et al., 2005). Contamination of milk with high levels of spoilage bacteria is usually unsuitable for further processing since it does not meet the consumer's expectations in terms of health (nutritional value), safety (hygienic quality) and satisfaction (sensory attributes) (Nanu et al., 2007). As a result, total viable bacterial counting has become one of the accepted criteria for grading milk intended for consumption and processing for dairy products. The importance of various etiological agents in milk-borne diseases has changed dramatically over time. However, more than 90% of all reported cases of dairy related illness continued to be of
bacterial origin, with at least 21 milkborne or potentially milkborne diseases currently being recognized (Bean et al., 1996). Pathogens that have been frequently involved in foodborne outbreaks associated with the consumption of milk include *Listeria monocytogenes*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Clostridium botulinum*. The presence of these pathogenic bacteria in milk emerged as major public health concerns, especially for those individuals who still drink raw milk (Ryser, 1998). Keeping fresh milk at an elevated temperature together with unhygienic practices in the milking process may result in microbiologically inferior quality (Chye et al., 1994).

Fresh milk drawn from a healthy cow normally contains a low microbial load (less than 1000 CFU/ml), but the loads may increase up to 100 fold or more once it is stored for some time at normal temperatures (Richter et al., 1992). Contamination of mastitis milk with fresh clean milk may be one of the reasons for the high microbial load of bulk milk (Jeffery and Wilson, 1987). The detection of coliform bacteria and pathogens in milk indicates a possible contamination of bacteria either from the udder, milk utensils or water supply used (Olson and Mocquot, 1980; Bonfoh et al., 2003).

Of the 507 milk samples collected from 16 milk collection centres in Trinidad, 454 (89.5%) were California mastitis test (CMT)-positive. The total aerobic plate count per ml was generally high for all samples ranging from \(5.8 \times 10^5 \pm 3.1 \times 10^5\) to \(5.7 \times 10^8 \pm 1.5 \times 10^9\) (Adesiyun, 1994).

Evaluation of bacteriological quality of raw cow's milk taken from udder, bucket, storage container before and after cooling and upon arrival at the processing plant from four dairy farms and a milk collection centre in and
around Addis Ababa (Godefay and Molla, 2000) indicated a high increase in the mean total aerobic plate count in milk samples taken from the bucket (1.1 x $10^5$ CFU/ml), storage container before cooling (4 x $10^6$ CFU/ml) and upon arrival at the processing plant (1.9 x $10^8$ CFU/ml). The mean coliform counts ranged from 1.3 x $10^4$ CFU/ml (storage container before cooling) to 7.1 x $10^4$ CFU/ml (upon arrival at the processing plant). Lack of knowledge about clean milk production, use of unclean milking equipment and lack of potable water for cleaning purposes were some of the factors which contributed to the poor hygienic quality of raw milk in the study farms (Godefay and Molla, 2000).

Analysis of the microbiological quality of raw cow’s milk taken at different intervals from the udder to the selling point in Bamako, Mali revealed a strong increase in the total count (TC) of bacteria during transport from the farm to the market ($10^7$ CFU/ml). The milk containers of the farmer and the milk vendor played a major role in the increase in the milk flora that occurred during transport from the farm to the selling points (Bonfoh et al., 2003).

Quantification of viable cells is a critical step in almost all biological experiments. A Methylene Blue dye Reduction Test (MBRT) to quantify viable cells based on reduction of methylene blue dye in cell cultures has correlated well with colony forming units (cfu) up to an 800 live cells as established by plating. The utility of the developed assay to monitor cfu rapidly and accurately for *E. coli*, *Bacillus subtilis* and a mixed culture of *E. coli* and *B. subtilis* has been demonstrated (Bapat et al., 2006).

In a study to improve the microbiological quality of the milk, from cow’s udder to the selling point by container washing and disinfecting, a
significant decrease of the total counts and *Enterobacteriaceae* counts in milk at the selling point as compared to the cow’s udder was reported (Bonfoh *et al*., 2006). The study suggested that in milk production area, besides udder infection and water quality, hygiene behaviour with respect to hand washing, containers cleaning and disinfection were the key areas of relevance to milk hygiene intervention (Bonfoh *et al*., 2006).

The bacterial composition of bulk tank milk from 13 farms was examined over a 2-week period to characterize sudden elevations in the total bacterial count referred to as "spikes." Twenty standard plate count spikes were observed: 12 associated with *streptococci*, 4 associated with gram-negative organisms, 2 associated with *streptococci* and gram-negative organisms, and 2 that were not definitively characterized. Spikes ranged from 14,000 to 600,000 CFU/ml (Hayes *et al*., 2001). Microbiological enumeration of 112 samples of raw buffalo milk collected at four locations in China revealed total mesophilic aerobic bacteria counts of 5.59 log CFU/ml (Beizhong *et al*., 2007).

Analysis of bulk-tank milk samples in Estonia for lactic acid bacteria count (LABC), psychrotrophic bacteria count (PBC), aerobic spore-forming bacteria count (ASFBC) and total bacterial counts revealed LABC below $10^4$ CFU/ml in most samples, while psychrotrophic micro-organisms dominated in 60% of farms. PBC ranged from $4.2 \times 10^2$ to $6.4 \times 10^4$ CFU/ml, and ASFBC varied from 5 to 836 CFU/ml. The microbiological quality of the farm bulk-tank milk was good - more than 91% of samples contained <50,000 CFU/ml, and SCC in the majority of samples did not exceed the internationally recommended limits (Stulova *et al*., 2010).
During an evaluation of on-farm pasteurization systems, milk samples were examined for standard plate count (SPC), coagulase-negative *Staphylococcus* count, environmental *Streptococci* count, coliform count, gram-negative non-coliform count, and *Staphylococcus aureus* count. Bacteria counts were significantly reduced by pasteurization, and pasteurized milk contained acceptable numbers of bacteria in >90% of samples indicating pasteurization to be effective in lowering bacterial contamination of milk. However, bacteria numbers significantly increased after pasteurization and, in some cases, bacteria counts in milk fed to calves were similar to pre-pasteurization levels. Milk handling after pasteurization was identified as an important issue on the farms studied (Elizondo-Salazar *et al.*, 2010). While determining the total plate counts and total coliform counts in 250 samples of kraals and indigenous milk products in the coastal savannah zone of Ghana, total plate counts exceeded $10^5$ CFU/ml in 45.2% of the samples while coliforms exceeded $10^3$ CFU/ml in 66.0%. *E. coli* was detected in 11.2% samples (Addo *et al.*, 2011).

In an investigation, the effects of season, cow cleanliness and milking routine on bacterial and somatic cell counts of bulk tank milk was studied on a total of 22 dairy farms in Lombardy, (Italy) (Zucali *et al.*, 2011). Season had effect on cow cleanliness with a significantly higher percentage of non-clean (NC) cows during cold compared with mild season. Standard plate count (SPC), laboratory pasteurization count (LPC), coliform count (CC) in milk significantly increased in hot compared with cold season. The effect of cow cleanliness was significant for SPC, PBC, CC and *Escherichia coli* in bulk tank milk. Milking operation routine strongly affected bacterial counts: farms
that accomplished a comprehensive milking scheme including two or more operations among fore stripping, pre-dipping and post-dipping had lower teat contamination and lower milk SPC, PBC, LPC, CC and LS than farms that did not carry out any operation (Zucali et al., 2011).

Relationships of cleaning procedures for milking equipment applied in intensive dairy farms in Lombardy, (Italy) with bacterial count of bulk milk and hygienic condition of milking machine components was studied on a group of 22 dairy farms. The results showed that farms classified as high and low milk total bacteria count significantly differed both in terms of liners and receiver bacterial contamination and in terms of water temperature reached during the detergent phase of cleaning milking equipment. Significant positive correlations were found among total bacterial counts in milk and bacterial contamination of the liners. Routine check and regulation of water temperature during the washing phase of the milking machine can be a simple and effective way to control one of the main risk factors for bacteriological quality of bulk tank milk (Bava et al., 2011).

In a nationwide survey on the microbial etiology of cases of subclinical mastitis in dairy cows on dairy farms in Sweden among 583 quarter milk samples collected from 583 dairy cows, the most common bacteria isolated were S. aureus - 31%, CNS - 27%, Str. Dysgalactiae - 15%, Str. Uberis - 14%, E. coli - 4.8%, and Streptococcus spp. - 3.1% (Persson et al., 2011).

2.2 Coliforms in Milk

The presence of total coliforms in foods of animal origin indicates environmental sources of contamination (Mhone et al., 2011). Amongst the coliforms, Escherichia coli is the most common contaminant of raw and
processed milk (Quinn et al., 2002). It is a reliable indicator of faecal contamination of water and food such as milk and dairy products (Todar, 2008). Coliforms were detected in 62.3% of 131 bulk tank milk samples in eastern South Dakota and western Minnesota (Jayarao and Wang., 1999). Counts ranged from 0 to 4.7 log10 CFU/ml. The mean count was 3.4 log10 CFU/ml. Gram-negative non-coliform bacteria were observed in 76.3% of bulk tank milk. Counts ranged from 0 to 6.2 log10 CFU/ml. The mean count was 4.8 log10 CFU/ml. Coliforms and gram-negative non-coliform bacteria accounted for 32.9 and 67.1% of the total isolates, respectively. Examination of bulk tank milk for coliforms and non-coliform bacteria could provide an indication of current and potential problems associated with bacterial counts and milk quality (Jayarao and Wang., 1999). Investigation of the rate of contamination with coliforms and incidence of E. coli in raw milk supplied by farmers to dairy cooperative societies for marketing revealed about forty two (42.2%) percent of the milk samples from farmers’ cans and 10.3% of samples from cooperative cans to be free of coliforms (Ombui et al., 1994), while 89.5% of the samples from farmers cans and 50% samples from cooperative cans could be considered to be of good quality with no more than 50,000 coliforms/ml of milk. A good number of farmers were drawing milk under satisfactory conditions, but awareness campaigns on clean milking, milk handling and storage practices should be stepped up in order to reach farmers who may not be informed (Ombui et al., 1994).

As part of the NAHMS Dairy 2002 survey, 861 bulk tank milk samples from farms in 21 states, coliforms were detected in 95% (818 of 860) of the samples, and the average SCC was 295,000 cells/ml (Van kessel et al., 2004).
Total plate counts, total coliform counts and the presence of *Escherichia coli* and *E. coli* O157:H7 were determined in 250 samples of kraals and indigenous milk products in the coastal savannah zone of Ghana. Total plate counts exceeded $10^5$ CFU/ml in 45.2% of the samples while coliforms exceeded $10^3$ CFU/ml in 66.0% and *E. coli* was detected in 11.2%. Antibiotic residues were detected in 3.1% of raw cow milk samples (Addo et al., 2011).

2.3 *Escherichia coli*

Shiga toxigenic *Escherichia coli*, including *E. coli* O157:H7, produce a family of toxins known as Shiga toxins, or verotoxins, related to the toxin produced by *Shigella dysenteriae*. This bacterium is one of the major bacterial pathogens causing food-borne illnesses, ranging from mild diarrhea to a life threatening complication known as hemolytic uremic syndrome (Friedrich et al., 2002). The large number of cases of human illness caused by Shiga toxin-producing *Escherichia coli* (STEC) worldwide has raised safety concerns for foods of bovine origin. These human illnesses include diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. Severe cases end with chronic renal failure, chronic nervous system deficiencies, and death (Hussein and Sakuma, 2005a). The cattle have been shown to be a major reservoir of STEC and raw foods such as ground beef and milk are the most common vehicles of infection (Gomez et al., 2002). A large number of STEC strains (e.g., members of the serogroups O26, O91, O103, O111, O118, O145, and O166) have caused major outbreaks and sporadic cases of human illnesses that have ranged from mild diarrhea to the life-threatening hemolytic uremic syndrome. The global nature of food supply
suggests that safety concerns with beef and dairy foods will continue and the challenges facing the dairy industry will increase at the production and processing levels (Hussein and Sakuma, 2005a).

Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains have been linked to outbreaks and sporadic cases of illness worldwide. Illnesses linked to STEC serotypes other than O157:H7 appear to be on the rise in the United States and worldwide, indicating that some of these organisms may be emerging pathogens (Mathusa *et al.*, 2010). Various virulence factors are involved in non-O157 STEC pathogenicity; the combined presence of both *eae* and *stx* genes has been associated with enhanced virulence. Worldwide, foods associated with non-O157 STEC illness include sausage, ice cream, milk, and lettuce, among others. Subtilase cytotoxin SubAB is an additional STEC virulence factor which contributes to HUS. SubAB has a strong preference for a sialic acid that humans are unable to synthesize but which is derived from meat and dairy products. Thus, a two-hit process is seen in the pathogenesis of milk-borne SubAB-producing STEC strains, thereby causing HUS (Lofling *et al.*, 2009). Results from several studies suggest that control measures for O157 may be effective for non-O157 STEC (Mathusa *et al.*, 2010). Non-O157 STEC contributes to the burden of illness but has been under recognized as a result of diagnostic limitations and inadequate surveillance (Brooks *et al.*, 2005). Of the 940 human non-O157 STEC isolates from persons with sporadic illnesses submitted between 1983 and 2002, by 43 state public health laboratories to the Centers for Diseases Control and Prevention reference laboratory for confirmation and serotyping, the most common serogroups were O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145.
Non-O157 STEC infections were most frequent during the summer and among young persons. Virulence gene profiling revealed 61% $stx_1$ but not $stx_2$; 22% $stx_2$ but not $stx_1$; 17% both $stx_1$ and $stx_2$. STEC O111 accounted for most cases of HUS and was also the cause of 3 of 7 non-O157 STEC outbreaks reported in the United States. Strains that produce Shiga toxin 2 are much more likely to cause HUS than are those that produce Shiga toxin 1 alone. Improving surveillance will more fully elucidate the incidence and pathological spectrum of these emerging agents (Brooks et al., 2005).

In US, 27 milkborne general outbreaks of infectious intestinal disease characterized by significant morbidity were reported to the Public Health Laboratory Service (PHLS) Communicable Disease Surveillance Centre (CDSC). Unpasteurized milk (52%) was the most commonly reported vehicle of infection in milkborne outbreaks, with milk sold as pasteurized accounting for the majority of the rest (37%) and most outbreaks were linked to farms (67%) (Gillespie et al., 2003).

Ruminants are an important source of serologically and genetically diverse intimin-harboring $E. coli$ strains. Moreover, cattle have not only to be considered as important asymptomatic carriers of O157 STEC but can also be a reservoir of EPEC and $eae$ positive non-O157 STEC, which are described in association with human diseases (Blanco et al., 2005).

### 2.3.1 Isolation of $E. coli$

Universal pre enrichment broth (UPB) can be effectively used for the isolation of $E. coli$ from dairy farm environmental samples (Nam et al., 2004). A sensitive procedure based on ISO/DIS 16654:1999 (later ISO 16654:2001),
which includes an immunomagnetic separation step can be used for the isolation of *E. coli* (Conedera *et al.*, 2004).

A rapid fluorogenic medium (4-methylumbelliferyl-beta-D-glucuronide (MUG-7) capable of detecting *E. coli* after 7.5 h incubation at 41.5°C was evaluated for the detection of *E. coli* in dairy products in comparison with Violet red bile agar. There were no significant differences between the numbers of *E. coli* detected on the two media (Sarhan *et al.*, 1991). Immunomagnetic separation (IMS) has been found to be a sensitive method for the recovery of *E. coli* O157:H7 in milk creams and recommended for isolation of the pathogen, the enrichment in tryptone soya broth with vancomycin, cefixime and tellurite, application of IMS, and plating of immunobeads onto nonselective agar (sorbitol MacConkey agar) and selective agar (sorbitol MacConkey agar with cefixime and tellurite or chromogenic agar with tellurite and cefixime) (Rojas *et al.*, 2006).

Alternative media such as LES Endo agar medium (LES Endo), Colilert-18 with 51-well Quanti-tray (Colilert), Chromocult Coliform agar (CC), Harlequin *E. coli* Coliform medium (HECM) and Chromogenic *E. coli* Coliform medium (CECM) for detection and enumeration of *E. coli* and coliform bacteria were compared to the reference method ISO 9308-1 (LTTC) using non-disinfected water samples with background flora. Results suggested that Colilert, CC and CECM are potential alternative media for detection of coliform bacteria and *E. coli* from non-disinfected water (Pitkanen *et al.*, 2007).

Enrichment in TSB added with yeast extract (YETSB) resulted in higher detection rates of *E. coli* O157 and O26 than in TSB. When YETSB
was used as enrichment broth, the analysis of the confluent growth from the media gave more positive results than that from *E. coli* O157:H7-ID medium (Caro *et al.*, 2011).

Chromogenic agars have been developed to recognize frequently occurring microorganisms directly on primary cultures, thus reducing the daily workload in a clinical microbiology laboratory. A new chromogenic medium CCEA was compared with a classical medium of violet red bile agar (VRBA), and other two Chromogenic media Agar I and Agar II. The accordant rates were 90%, 71.88%, 86.25% and 81.25% respectively, showing CCEA > Agar I > Agar II > VRBA. The CCEA might be more advantageous than the VRBA, having the same efficacy as with Agar I and Agar II (Lu *et al.*, 2007). Two chromogenic agars, CHROM agar Orientation (CO) and CPS ID 3 (CPS3) compared with routine media (biplate technique using trypticase soy blood agar and eosin methylene blue agar) for the isolation, enumeration and identification of organisms in urinary tract infection (UTI). Approximately 91.9% of *E. coli* could be identified directly on CO media, while 97.5% of *E. coli* could be identified on CPS3 media. The use of CO and CPS3 as single media is promising for clinical urine culture (Chang *et al.*, 2008).

The Sanita-kun *E. coli* and coliform sheet medium, a chromogenic medium containing X-Gal, consisting of a transparent cover film, an adhesive sheet, a layer of nonwoven fabric, and a water-soluble compound film, including a culture medium formula has been devised for the enumeration of total coliforms and differentiation of *E. coli* (Ushiyama and Iwasaki., 2010). The X-Gal is hydrolyzed by beta-galactosidase from coliforms to produce a visible blue dye and Salmon-glucuronic acid, which is
hydrolyzed by beta-glucuronidase from *E. coli* to produce a red-purple dye. It is easy to distinguish the difference between *E. coli* and coliform (other than *E. coli*) colonies. The Sanita-kun *E. coli* and Coliform sheet medium has been granted performance tested method status (Ushiyama and Iwasaki., 2010).

### 2.3.2 Prevalence of *E. coli* in foods

Shiga toxin-producing *E. coli* can potentially enter the human food chain from a number of animal sources, most commonly by contamination of meat with feces or intestinal contents after slaughter or cross-contamination of unpasteurized milk products (Alexandre and Prado., 2003). *E. coli* particularly O157:H7 have emerged as significant foodborne pathogens that pose major public health concern in the world, with milk and milk products constituting the high-risk foods category (Manoj *et al.*, 2004). In cheese, *E. coli* is used as an indicator to assess post-pasteurisation contamination and its presence may indicate inadequate pasteurisation, poor hygiene conditions during processing or post-processing contamination (Martina *et al.*, 2009). Bulk tank milk samples from the 248 dairy herds were examined for foodborne pathogens and Shiga toxin-producing *Escherichia coli* were detected in 2.4% samples (Jayarao *et al.*, 2006). The examination of 2005 raw bovine (*n* = 950), caprine (*n* = 460) and ovine (*n* = 595) bulk milk samples collected throughout several regions in Greece for the presence of *Escherichia coli* serogroup O157 resulted in the isolation of 29 strains (1.4%) of which 21 were isolated from bovine (2.2%), 3 from caprine (0.7%) and 5 from ovine (0.8%) milk (Solomakos *et al.*, 2009).
All STEC strains from frozen hamburgers and soft cheeses were characterized as eaeA-/EHEC-hlyA+. The stx2 genotype was highly prevalent (77.8%) (Gomez et al., 2002). Raw poultry samples were highly contaminated with *E. coli* (45%) and classified as high-risk food in Vietnam. *E. coli* was also detected in raw meat, fish, and vegetables with the rate of 21.3%, 6.6%, and 18.5%, respectively (Ha and Pham., 2006).

STEC were detected in 12% of the 785 minced beef samples collected from 30 local stores in Lugo city, Spain. PCR showed that 28 (29%) isolates carried *stx*1 genes, 49 (51%) possessed *stx*2 genes, and 19 (20%) both *stx*1 and *stx*2. The highly virulent seropathotypes O26:H11 *stx*1 eae-beta1, O157:H7 *stx*1 *stx*2 eae-gamma1 and O157:H7 *stx*2 eae-gamma1, which are the most frequently observed among STEC causing human infections in Spain, were detected in 10 of the 96 STEC isolates (Mora et al., 2007).

*Escherichia coli* is a common contaminant of seafood in the tropics and is often encountered in high numbers. A survey of *E. coli* was conducted in 644 molluscan shellfish samples marketed in the Apulia region of southern Italy. Levels of *E. coli* and fecal coliforms were above the Italian legal limit in 27 and 34 samples (4 and 5%), respectively (Parisi et al., 2004). Shellfish collected in coastal environments can serve as a vehicle for STEC transmission (Gourmelon et al., 2006). In a study carried out to evaluate the presence of Shiga toxin-producing *Escherichia coli* (STEC) and *E. coli* O157:H7 in shellfish from French coastal environments, *stx* genes were detected in 40 of 144 (27.8%) sample enrichments from mussels, oysters or cockles. Five strains carrying *stx*1 or *stx*(1d) genes and one *stx* negative, *eae* and *ehxA* positive *E. coli* O157:H7 were isolated from six of 40 *stx*-positive enrichments.
In another study, all the fish (*Rastrineobola argentea*) samples (60) analysed from in Kisumu town, Kenya were found to be contaminated with *E. coli*. The occurrence of multiple drug resistant (MDR) *E. coli* was identified as some of the possible health risks that may be associated with *R. argentea* (Sifuna et al., 2008). Pao et al. (2008) evaluated the microbial quality of raw fillets (n=272) of aquacultured catfish, salmon, tilapia, and trout. *Escherichia coli* was detected in 1.4, 1.5, and 5.9% of trout, salmon, and tilapia, respectively. *E. coli* was also found in 13.2% of catfish, with an average of 1.7 log MPN/g. Sushi is a traditional Japanese food, mostly consisting of rice and raw fish. Sushi samples (250) were analyzed for their microbiological status and the prevalence of pathogenic bacteria. The prevalence of *Escherichia coli* was higher in the fresh samples (Atanassova et al., 2008).

In India, four shiga toxin-producing *Escherichia coli* (STEC) strains were isolated from seafood, six from beef and one from a clinical case of bloody diarrhoea in Mangalore, the isolates were positive for Shiga toxins *stx*1 and *stx*2 and also for *stx*1 and *stx*2 genes (Kumar et al., 2004). While, in a study in Cochin a total of 484 presumptive *E. coli* were isolated from 414 finfish samples composed of 23 species of fresh fish from retail markets and frozen fish from cold storage outlets. Results indicated 81.4% of the *E. coli* isolates to be sorbitol positive (Thampuran et al., 2005). Microbiological quality of fish and shellfish from Kolkata with special emphasis on *E. coli* was determined and indicated poor hygiene and sanitary conditions. Although *E. coli* O157 could not be detected, a few samples were contaminated with non-O157 serotypes of enterohaemolysin- and Shiga toxin-producing *E. coli*, raising
public health concern (Manna et al., 2008). In another study, screening of fish and shrimp samples obtained from different retail fish markets in Cochin, India, by direct PCR assays targeting the eaeA gene, hlyA gene and stx gene revealed one shrimp sample to be positive for all these virulence markers, and recovery of seven typical E. coli O157:H7 isolates from the marker-positive shrimp sample indicated the need for strict adherence to hygienic handling methods and proper cooking or processing before consumption of these products (Surendraraj et al., 2010).

E. coli O157:H7 is capable of survival but not growth on the surface of fresh strawberries throughout the expected shelf life of the fruit and can survive in frozen strawberries for periods of greater than 1 month (Knudsen et al., 2001).

2.3.4 Milkborne E. coli outbreaks

Haemolytic uremic syndrome (HUS) is characterized by thrombotic microangiopathy with acute renal failure, haemolytic anaemia with schizocytes and thrombocytopenia. It is caused by gastrointestinal infection with Escherichia coli species producing verotoxins (or Shiga toxins, STEC) (Bertholet-Thomas et al., 2011). It is estimated that 5-8 % of infected individuals will develop HUS following STEC infection. Vehicles of STEC transmission are contaminated food (ground beef, unpasteurised dairy products, unwashed and uncooked fruit and vegetables), person-to-person transmission and contact with farm animals with STEC. After an average incubation period of 3 to 8 days, patients develop painful bloody diarrhoea followed by systemic toxemia. This may lead to thrombotic microangiopathy
with endothelial damage and activation of local thrombosis (Bertholet-Thomas et al., 2011).

Raw milk has been implicated as source of foodborne outbreaks. In Connecticut, two children experienced *Escherichia coli* O157-associated hemolytic uremic syndrome (HUS) after consuming raw milk purchased at a retail market and a farm (Guh et al., 2010). *E. coli* O157: NM outbreak strains were isolated from stool specimens of 6 case patients and 1 milking cow. The total estimated outbreak cost was $413,402. The outbreak resulted in substantial costs and proposed legislation to prohibit non-farm retail sale, strengthen advisory labels, and increase raw milk testing for pathogens (Guh et al., 2010). Annual Listings of Disease Outbreaks and the Foodborne Outbreak Database (FOOD) to establish epidemiologic baseline characteristics for disease outbreaks associated with fluid milk during 1990-2006 data by the Centers for Disease Control and Prevention reported eighty-three fluid milkborne outbreaks between 1990 and 2006, resulting in 3621 illnesses (Newkirk et al., 2011). The mean number of illnesses per outbreak was 43.6 (illness range: 2-1644). Consumption of unpasteurized milk was associated with 55.4% of reported outbreaks and *Escherichia coli* in 10.8% of reported outbreaks. Private homes accounted for 41.0% of outbreak locations (Newkirk et al., 2011).

A study was carried out on hospitalized patients with hemorrhagic colitis in Georgia who have consumed not washed raw fruits or vegetables, non pasteurized dairy products, food from street vendors, soft cheeses made from raw milk and untreated water in areas lacking adequate chlorination. Increased rate of patients indicated circulation of shiga-toxin producing *E.
coli (Vashakidze et al., 2010). Another mixed-serotype outbreak of
verocytotoxin-producing *Escherichia coli* (VTEC) O145:H28 and O26:H11
was reported in the province of Antwerp, Belgium in September-October 2007
(Buvens et al., 2011). The epidemiological and laboratory investigations
revealed ice cream as the most likely source of the outbreak. The ice cream
was produced at a local dairy farm using pasteurized milk. VTEC of both
serotypes with indistinguishable pulsed-field gel electrophoresis patterns were
isolated from patients, ice cream, and environmental samples. The data
suggested that O145:H28 played the most important role in the outbreak
(Buvens et al., 2011).

2.3.5 Detection of virulence genes

The prevalence of Shiga toxigenic group of *E. coli* (STEC) in food
products of bovine origin was 16% in France (Madic et al., 2009). In USA,
samples of bulk tank milk from dairies suggested that 4.2% were positive for
one or both Shiga toxin genes (*stx*1 and *stx*2) (Karns et al., 2007). Baseline
data on the prevalence and characteristics of Vero cytotoxin-producing *E. coli*
(VTEC) organisms in lactating animals in Ireland suggested ~3% of milk
samples contained *E. coli* O157 (Murphy et al., 2007). In France, the
prevalence of STEC-positive samples in raw milk as determined by PCR-
ELISA was 21%, of these strains, ~72% were confirmed positive for *stx*
(Perelle et al., 2007).

Analysis of the *E. coli* O157:H7 strains from raw ewe's milk in Spain
that were positive for the rfbO157 and fliCH7 genes by multiplex PCR for the
presence of virulence genes revealed the predominance of *stx*2 type
demonstrating the raw ewe's milk used in cheese making as a source of *E. coli* O157:H7 strains that are potentially pathogenic for humans (Caro *et al.*, 2006). Thirty strains of *E. coli* isolated from raw milk cheeses including soft, hard, unripened and blue mould cheeses had the *stx*1 gene and one strain, the eae gene. Combinations of *stx*2 and *stx*1 genes were present in 17 (81%) of the STEC strains (Vernozy *et al.*, 2005).

While assessing the potential public health impact of STEC in Swiss raw milk cheese produced from cow's, goat's, and ewe's milk, 1,422 samples from semi hard or hard cheese and 80 samples from soft cheese were examined for STEC (Zweifel *et al.*, 2010). STEC was detected after enrichment in 5.7% of the 1,502 raw milk cheese samples collected at the producer level. The *stx*1 gene was only found in 2 strains, whereas 27 strains carried genes encoding for the *stx*2 group, mainly *stx*2 and *stx*2vh-a/b. Semi hard and hard raw milk cheese might be a potential source of STEC, and a notable proportion of the isolated non-O157 STEC strains belonged to serotypes or harboured Shiga toxin gene variants associated with human infections (Zweifel *et al.*, 2010).

A polyphasic approach was evaluated for the detection of *E. coli* genes most commonly associated with virulence factors (*eae, elt, ipaH, stx*) in traditional soft cheeses, manufactured artisanally from whole raw milk in the Lombardy region (northern Italy). Genes associated with enteroinvasive *E. coli, ipaH,* and Shiga toxin-producing *E. coli, stx,* were detected in two of the bulk samples analyzed constituting a potential hazard for consumer health (Bernini *et al.*, 2010).

The epidemiology of an extended spectrum beta-lactamase *E. coli* (CTX-M-15) was observed and described on a commercial dairy farm
located in the United Kingdom. The proportion of CTX-M-15 \textit{E. coli} positive samples was significantly (p<0.0.01) higher in milking cows (30.3%) than in the herd as a whole (17.0%). The increased prevalence of the CTX-M-15 \textit{E. coli} in certain cattle groups and farm environments including calving pens suggested that husbandry, antimicrobial usage and hygiene may play a significant role on a farm with regards to the epidemiology of CTX-M-15 (Watson \textit{et al.}, 2011).

The \textit{tccP2} gene was detected in a higher number of serotypes including the five most frequent EHEC serotypes (i.e. O26:H11, O103:H2, O111:H8, O145:H28 and O157:H7), and a few other serotypes that caused human infections (i.e. O4:H(-) , O45:H2 and O55:H7) (Madic \textit{et al.}, 2011a). Combinations of \textit{stx}, \textit{eae} variants, and O genetic markers, which are typical of the five targeted STEC serotypes, were detected by real-time PCR in 6.5% of the cheeses (26 samples) and included \textit{stx-wzx} (O26)-\textit{eae-β1} (4.8%; 19 samples), \textit{stx-wzx} (O103)-\textit{eae-ε} (1.3%; five samples), \textit{stx-ihp1} (O145)-\textit{eae-γ1} (0.8%; three samples), and \textit{stx-rfbE} (O157)-\textit{eae-γ1} (0.3%; one sample) (Madic \textit{et al.}, 2011b).

Dairy farmers perform various types of work and are in direct contact with dairy cattle nearly every day. Twenty (3.4%) of 589 stool samples from dairy farmers were \textit{stx}-positive. The distribution of \textit{stx}-positive stool samples revealed an increase in Shiga toxin-producing \textit{Escherichia coli} infection with age, duration of work, and herd size (Park \textit{et al.}, 2011).

A diversity of STEC strains in dairy cattle from Argentina, most of them carrying genes linked to human disease were detected (Fernandez \textit{et al.}, 2010). Of the 170 isolates analyzed by multiplex PCR and serotyping from 540
STEC positive samples, 11% carried \textit{stx}1, 52\% \textit{stx}2 and 37\% \textit{stx}1/\textit{stx}2. Thirty-five per cent of strains harboured the profile \textit{stx}1, \textit{stx}2, \textit{saa}, \textit{ehxA} and 29\% \textit{stx}2, \textit{saa}, \textit{ehxA} (Fernandez \textit{et al.}, 2010).

No virulence genes namely, \textit{irp}2, \textit{iucD}, \textit{papC iss}, \textit{svg}, \textit{stx}1, \textit{stx}2, \textit{cnf1} and \textit{hlyA} were detected in persistent \textit{E. coli} mammary infections (Suojala \textit{et al.}, 2011). However, PCR analysis revealed 37\% of 200 \textit{E. coli} isolates from food in Casablanca having one or more of virulence genes associated with strains causing intestinal and extra-intestinal infections. The virulence genes included \textit{stx}1, \textit{stx}2, \textit{lt}, \textit{st}, \textit{hlyA}, \textit{aggA}, \textit{saa}, \textit{astA}, \textit{iucD}, \textit{cnf1}, \textit{eaeA}, \textit{bfpA}, \textit{ial}, \textit{ipaH}, \textit{afa}, \textit{pap} and \textit{sfA} (Samira \textit{et al.}, 2009).

Shiga toxin genes were detected in enrichments from 15.2\% of the bulk tank milk (BTM) samples (536) and from 51.0\% of the in-line milk filters (519) by real-time PCR from dairy farms across the United States during the National Animal Health Monitoring System's Dairy 2007 study (Van Kessel \textit{et al.}, 2011).

In Indian context, virulence gene profiling of Shiga toxin-producing \textit{E. coli} (STEC) non-O157 strains isolated from human stool samples, cow stool samples, and beef samples in Calcutta, India, revealed the dominant combinations of \textit{stx}1 and \textit{stx}2 (44.5\% of strains) and \textit{stx}1, \textit{stx}2, and \textit{hlyA} (enterohemorrhagic \textit{E. coli} hemolysin gene) (19\% of strains). Only 6.4\% of the STEC strains harbored the \textit{eae} gene (Khan \textit{et al.}, 2002).

Pasteurization is used to inhibit microbial growth in milk. However, the studies that show if pasteurization inactivates Shiga toxins produced by the bacteria are largely lacking. An outbreak in North Cumbria in 1999, showed that haemolytic uraemic syndrome (HUS) was linked to drinking pasteurized
milk, but no live bacteria was found in the milk samples (Goh et al., 2002). However, it has been demonstrated that the stx2 is heat-stable and conventional pasteurization of milk, at the various suggested temperatures and times by the U.S. Food and Drug Administration, (63 °C for 30 min, or 72 °C for 15 s or 89 °C for 1 s), did not reduce the biological activity of stx2. However, treatment at 100 °C for 5 min inactivated the toxin (Reuven and Paula., 2009).

2.3.6 Serotypes of E. coli

In Brazil, twelve different serogroups of E. coli were isolated from mastitic milk, and among them were O26, O55, O111 and O119, all of them classic enteropathogenic E. coli (EPEC) serogroups (Correa and Marin., 2002). The predominant serogroups detected from milk and milk products were O174, O175, O176, O109, O76, O162 and O22 serogroups in decreasing frequency (Vernozy et al., 2005) in France; O26, O55, O111, O114, O125, O127, O128, O158). (Carneiro et al., 2006) in Brazil and O2, O15, O22, O91, O109, O113, O174 in Swiss cheeses (Stephan et al., 2008). Over 100 different STEC serotypes isolated from healthy cattle in Thailand other than O157: H7, e.g. O26, O91, O103, O111 and O113 have now been shown to cause illness (Panutdaporn et al., 2004).

Shiga-toxin-producing Escherichia coli were isolated from frozen hamburgers and soft cheeses and four different O:H serotypes were found, comprising: O8:H19 (5 strains), O113:H21 (1), O8:H16 (1), and O39:H49 (1). (Gomez et al., 2002). E. coli serogroups O26, O55, O86, O111, O114, O119, O125, O126, O1127, O128, and O142 were isolated in Sao Paulo from children with diarrhea (Campos et al., 2004). Most frequent serotypes among
the 106 VTEC strains isolated from goats in Spain were O5:H-, O76:H19, O126:H8, O146:H21, ONT:H- and ONT:H21 (Cortes et al., 2005).

A large number of STEC strains (e.g., members of the serogroups O26, O91, O103, O111, O118, O145, and O166) have caused major outbreaks and sporadic cases of human illnesses that have ranged from mild diarrhea to the life-threatening hemolytic uremic syndrome. These illnesses were traced to O157 and non-O157 STEC (Hussein and Sakuma, 2005b). The occurrence of \textit{E. coli} O157, O111 and O26 in 159 raw ewe's milk samples was examined. Percentage occurrences of \textit{E. coli} O157, O111 and O26 were 18.2, 8.2 and 5.7, respectively. Mean \textit{E. coli} O157 and O111 levels were 0.22 and <0.04 MPN/mL, respectively. Enrichment in YETSB resulted in higher detection rates of \textit{E. coli} O157 and O26 than in N-mTSB (Caro et al., 2011).

One hundred and fifty-six strains were associated with 29 different O serogroups, and 19 H antigens were distributed among 157 strains isolated in Argentina (Fernandez et al., 2010). STEC O113:H21, O130:H11 and O178:H19 were the most frequently found serotypes. The non-O157 STEC serotypes described are associated worldwide with disease in humans and represent a risk for public health. Therefore, any microbiological control in dairy farms should be targeted not only to the search of O157:H7 serotype (Fernandez et al., 2010).

Bacteriological analysis of 713 samples of various types of foods and related articles and potable water samples from different places in Ludhiana, Punjab revealed presence of EPEC (55) and ETEC (3) (Ram et al., 1996)

An epidemiological survey of \textit{E. coli} O157 in different regions of India based on 17093 samples received during the 10-year period from humans, food
items, animals and the environment at the National Salmonella and Escherichia Centre, a national reference centre for *Salmonella* and *Escherichia* for India revealed 0.5% of 5678 human samples positive for *E. coli* O157 (Sehgal *et al.*, 2008). A significantly high percentage of *E. coli* O157 were isolated from meat (0.9%, 13/1376), milk and milk products (1.8%, 10/553), seafood (8.4%, 16/190) and water (1.6%, 8/486). The isolates were found to be distributed among domestic and wild animals, and the maximum number of isolates of *E. coli* O157 was detected in samples received from coastal belt areas. *E. coli* O157 is widely distributed among humans and animals, food and environment in different geographical regions of India (Sehgal *et al.*, 2008).

In Kolkata, India, one hundred and thirty Sor(-) *E. coli* were isolated from 556 food samples and 177 cattle stool samples using cefixime tellurite-supplemented SMAC (CT-SMAC) and chromogenic HiCrome MS.O157 agar respectively. Based on typing of somatic antigen, the isolates were classified into 38 serogroups. PCR results identified about 40% strains, belonging to O5, O8, O20, O28, O48, O60, O78, O82, O84, O101, O110, O123, O132, O156, O157, O-rough and OUT as Shiga toxigenic. Majority of O5, O84, O101, O105, O123, O157, O-rough and OUT strains were enterohaemolytic (Manna *et al.*, 2009). During an investigation to determine the distribution, virulence gene profile and phenotypes of Shiga toxin-producing *Escherichia coli* (STEC) strains within a dairy farm in Kolkata, primary screening by multiplex-PCR detected *stx*1 and *stx*2 in 18.9% of cow faeces, 32.4% of calf stool samples, 21.6% of farm floor swabs and 4.5% of raw milk samples and viable STEC from 4.5, 9.9, 8.1 and 1.8% of the corresponding PCR-positive samples. The
study demonstrated the potential of dairy farm for housing virulent STEC (Das et al., 2005).

2.3.7 Methods of Detection

While cultural isolation of *E. coli* from foods and faeces is time-consuming, labour-intensive and hence, rapid detection systems have been developed which significantly reduce the analysis time. Various detection techniques such as enzyme-linked immunosorbent assays (ELISAs) (De Boer et al., 2000), direct immunofluorescent filter techniques (Chen et al., 1998), PCR-ELISA (Fach et al., 2001), colony hybridization (Vernozy et al., 2005), latex agglutination and by multiplex PCR (Maher et al., 2001) have been reported.

A series of mPCR assays were developed using primer pairs that identify the sequences of Shiga toxins 1 and 2 (*stx*1 and *stx*2, including the *stx*2c, *stx*2d, *stx*2e and *stx*2f variants), intimin (*eae*A), and enterohaemorrhagic *E. coli* enterohaemolysin (*ehly*A) (Osek, 2003). Shiga toxin (*Stx*)-producing *Escherichia coli* (STEC) are amongst major causes of food-borne infectious diseases and outbreaks. The automated method for enumeration of *E. coli*, TEMPO EC, in foods uses a dehydrated culture medium and enumeration card containing 48 wells across three different dilutions for the automatic determination of the most probable number (MPN). The alternative method was compared in a multilaboratory collaborative study to AOAC Official Method 966.24. The study demonstrated that the TEMPO EC method is a reliable, automated assay for the enumeration of *E. coli* in foods (Crowley et al., 2010).
A new quantitative PCR (qPCR) assay was designed to detect all known \textit{stx} gene subtypes in a single reaction, including the most distant variant \textit{stx2f}. The qPCR assay was 100\% specific and showed analytical sensitivity of two STEC genome copies per reaction, represents a valuable tool for rapid detection and quantification of STEC in foods taking into account the genetic \textit{stx} variability observed in STEC population (Derzelle \textit{et al.}, 2011).

A duplex real-time PCR assay targeting enterohaemorrhagic \textit{E. coli} (EHEC) type III effector TccP/TccP2-encoding genes which are pivotal to EHEC-mediated actin cytoskeleton reorganization in human intestinal epithelial cells was carried out using a large collection of 526 \textit{E. coli} strains of human, animal, food and environmental origins (Madic \textit{et al.}, 2011a). Combination of multiple real-time PCR assays was used for the screening of 400 raw-milk cheeses for the five main pathogenic STEC serotypes (O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7) (Madic \textit{et al.}, 2011b).

TaqMan real time PCR assays were designed for each of the non-O157 STEC O serogroups most commonly associated with human illness: O26, O45, O91, O103, O111, O113, O121, O128, and O145. The assays could also detect each O serogroup in human stool specimens inoculated with STECs at 1000 CFU/0.5 g of stool following 24 h enrichment (Lin \textit{et al.}, 2011).

A novel method with high specificity and stability for detecting \textit{E. coli} O157:H7 by using a quartz crystal microbalance (QCM) immunosensor based on beacon immunomagnetic nanoparticles (BIMPs), streptavidin-gold, and growth solution was described by Shen \textit{et al.} (2011). The detection limit was 23 CFU/ml in phosphate-buffered saline and 53 CFU/ml in milk.
A multiplex PCR (m-PCR) assay based on the amplification of the \textit{wzx}, \textit{stx}1, and \textit{stx}2 genes was developed for the identification and characterization of \textit{E. coli} O26 VTEC and its detection in raw milk and ground beef (Lorusso \textit{et al.}, 2011). This M-PCR assay had a sensitivity of $10^8$ CFU/ml when applied to a bacterial suspension and of $10^6$ CFU/ml or g when applied to both inoculated milk and minced beef samples and could be used for the rapid detection of \textit{E. coli} O26 VTEC from foods and for the rapid identification and characterization of clinical and environmental isolates (Lorusso \textit{et al.}, 2011).

Matrix-assisted laser desorption-ionization mass spectrometry considerably accelerates the diagnosis of mastitis pathogens, especially in cases of subclinical mastitis. More immediate and efficient animal management strategies for mastitis and milk quality control in the dairy industry can therefore be applied (Barreiro \textit{et al.}, 2010).

In India, a number of studies have been carried out to develop rapid assays for the detection of foodborne pathogens. A PCR assay to detect the major pathogens including \textit{E. coli} of bubaline mastitis (BM) directly from the mastitic milk samples of the buffaloes was carried out. Out of the 60 mastitic milk samples tested 30 were positive for \textit{E. coli} (Anandkumar., 2009).

A duplex real-time polymerase chain reaction assay targeting the \textit{rfb} gene of \textit{E. coli} O157:H7 and the \textit{hly} gene of \textit{L. monocytogenes} tagged with different reporter dyes was developed for \textit{E. coli} O157:H7 and \textit{L. monocytogenes} in such a way that each pathogen could be detected simultaneously in a single tube and differentiated. The detection limit of the assay in reconstituted non-fat dried milk (11%) spiked with the two targeted pathogens at different levels was 1 and 3 log CFU colony forming units/mL of each with and without
enrichment (6 h) of the sample (Singh et al., 2009a). The application of the duplex real time PCR assay on 60 market samples, including kulfi and paneer, revealed three samples involving one each of raw milk, kulfi, and paneer found to be positive for *E. coli* O157:H7, while one sample of raw milk was positive for *L. monocytogenes* (Singh et al., 2009a).

A multiplex PCR (mPCR) assay using previously known genetic markers of *E. coli* and Shiga-toxic *E. coli* was standardized. *uidA* gene was targeted for the common detection of *E. coli*, and *stx1* gene was used as markers for the detection of shiga-toxin producing strains, respectively. The reactions, individually as well as in the mPCR, could detect approximately 1 cell per 20-µl PCR assay. In naturally contaminated raw milk samples (n=100), *E. coli* were detected in all samples and VTEC verotoxinogenic *E. coli* in 15 samples (Riyaz et al., 2009).

A rapid real-time PCR (RTi-PCR) method based on Scorpion probe technology targeting the *eae* gene of *E. coli* O157:H7 was developed and applied using spiked and naturally contaminated products. The assay was sensitive enough to detect 2 log CFU/mL of the target pathogen in pure broth culture and 3 log CFU/ml in spiked skim milk (Singh et al., 2009b).

### 2.3.8 Molecular subtyping of *E. coli*

Systems of serotyping, subtyping, and virulence typing of STEC are used to aid in epidemiology, diagnosis, and pathogenesis studies (Gyles., 2007). A number of genotyping methods to track VTEC infections and determine diversity and evolutionary relationships among microorganisms
have been developed which facilitated monitoring and surveillance of foodborne VTEC outbreaks and early identification of outbreaks or clusters of outbreaks. Pulsed-field gel electrophoresis (PFGE), because of its high discriminatory power, reproducibility and ease of standardization, has been used extensively to track and differentiate VTEC (Fremaux et al., 2006; Karama and Gyles, 2010). Multiple-locus variable-number tandem-repeats analysis (MLVA), based on the occurrence of variable numbers of tandem duplications of short stretches of DNA at specific loci in the chromosome and microarrays have been applied to discriminate VTEC. MLVA, a simpler and less expensive method, is proving to have a discriminatory power comparable to that of PFGE. MLVA has been used to subtype O157:H7 STEC (Hyytia-Trees et al., 2006). Novel methods such as the detection of single nucleotide polymorphisms and optical mapping are being evaluated for subtyping VTEC (Karama and Gyles, 2010).

Multilocus sequence typing is based on variations in nucleotide sequences of internal fragments of selected housekeeping genes, has not been effective. MLST involving 7 housekeeping genes and genes for the membrane proteins \textit{ompA} and \textit{espA} has been applied to 77 isolates of O157:H7 STEC that were diverse by PFGE (Noller \textit{et al.}, 2003). No diversity in the sequences of the housekeeping genes and \textit{espA} and little diversity in \textit{ompA} was found.

DNA-based subtyping methods such as pulsed field gel electrophoresis (PFGE) are generally required to increase discrimination of strains so that the spread of specific strains can be monitored (Willshaw \textit{et al.}, 2001). Seven distinguishable PFGE patterns in 3 homology groups were identified among patient and dairy herd \textit{E. coli} O157:H7 isolates (Keene \textit{et al.}, 1997).
STEC strains isolated from human stool samples, cow stool samples, and beef samples over a period of 2 years in Calcutta, India that gave identical or nearly similar DNA fingerprints in RAPD-PCR and had similar virulence genotypes were further characterized by PFGE (Khan et al., 2002). Identical RAPD and PFGE profiles were observed in four sets of strains, with each set comprising two strains. There was no match in the RAPD and PFGE profiles between strains of STEC isolated from cows and those isolated from humans (Khan et al., 2002).

The randomly amplified polymorphic DNA-PCR (RAPD-PCR) profile of the STEC strains isolated from the farm milieu revealed diverse banding patterns and clonal analysis demonstrated that the strains from different sources were not identical but showed some genetic relatedness (Das et al., 2005). PFGE analysis revealed an "outbreak" profile in 13 cases of hemolytic uremic syndrome after consumption of unpasteurized gouda cheese. The cheese isolates had indistinguishable PFGE profiles as compared with outbreak case isolates (Honish et al., 2005). A dendrogram derived from the PFGE patterns of 22 strains of three predominant serogroups isolated from aquatic environments in Bangladesh indicated two major clusters, one containing mainly serogroup O55 and the other O8. Three strains of identical PFGE profiles belonging to serogroup O55 were isolated from three distinct areas, which may be of epidemiological significance (Alam et al., 2006).

PFGE identified seven distinct XbaI macrorestriction patterns among E. coli from milk samples in Lombardy region, Italy at a similarity level of 41%. (Picozzi et al., 2005). PFGE profiles of STEC Shiga toxin-producing E. coli from French dairy farms indicated genetic diversity of the STEC strains and
some of these persisted in the farm environment for up to 12 months (Fremaux 
et al., 2006).

PFGE confirmed persistence of mammary infection with *E. coli* in 11.8% of 144 cows, confirmed by re-isolation of the same type from the affected quarter at 3 weeks post-treatment. The majority of isolates (82.6%) belonged to phylogeny group A, which mainly consisted of commensal strains (Suojala et al., 2011). PFGE analysis showed high genetic diversity among STEC isolated from raw-milk cheeses, however, none of them had patterns identical to those of human O26:H11 strains investigated (Madic et al., 2011).

### 2.3.9 Antibiotic resistance

Resistance to one or more antibiotics was observed in 49.2% of the STEC strains isolated from human stool samples, cow stool samples, and beef samples in Calcutta, India, with some of the strains exhibiting multidrug resistance (Khan et al., 2002).

Susceptibility of sorbitol-negative or slow-fermenting *E. coli* from milk samples to 11 antibiotics highlighted the high resistance to tetracycline (50%), sulfonamide and streptomycin (33%) in Lombardy region, Italy (Picozzi et al., 2005). Eighty-four percent of the *E. coli* isolates from foods commonly sold in the market place in Vietnam were resistant to one or more antibiotics, and multi-resistance, defined as resistance to at least 3 different classes of antibiotics (Van et al., 2008).

All *E. coli* O157 isolates from raw bovine, caprine and ovine raw bulk milk samples displayed resistance to a wide range of antimicrobials, with the stx-positive isolates being, on average, resistant to a higher number of
antibiotics than those which were stx-negative (Solomakos et al., 2009). Antibiotic resistance among the E. coli isolates from quarter milk samples in Estonia, demonstrated resistance to ampicillin, streptomycin and tetracycline in 24.3%, 15.6% and 13.5% cases, respectively (Kalmus et al., 2011).

2.4 *Listeria monocytogenes*

Listeriosis is a serious invasive bacterial zoonotic disease characterized by neural, visceral and reproductive disorders. It is usually manifested as septicemia, abortion, stillbirth, meningitis, and meningo-encephalitis in a variety of animals including humans especially in immunocompromised individuals and persons in contact with animals. It is an important foodborne bacterial disease and a nagging public health hazard caused by ingestion of contaminated food and water. To manage the problem of foodborne listeriosis, it requires an understanding of the burden of the disease on a worldwide scale as foods that are prone to contamination are eaten widely domestically and many are traded globally (Todd and Notermans, 2011). *Listeria* spp. including *L. monocytogenes* are isolated from diverse environmental sources including soil, water, sewage, vegetation (e.g., grass, meadows, forests, silage), wild animal faeces, as well as on the farm and in food processing facilities (Barbuddhe and Chakraborty, 2009; Sauders and Wiedmann, 2007; Doijad et al., 2011).

Listeriosis was first recognized as a disease in 1926 during a spontaneous outbreak of infection among laboratory rabbits and guinea pigs in Cambridge characterized by mononuclear leucocytosis (monocytosis) and the isolated organism was named as “*Bacterium monocytogenes*” (Murray et al.,
1926). Subsequently, in 1927, Pirie isolated a similar bacillus from the liver of infected gerbils and named it *Listerella hepatolytica* in honour of Lord Lister (Gray and Killinger, 1966). Finally, the genus was named as *Listeria* in 1940 for taxonomic reasons (McLauchlin, 1987).

The genus *Listeria* (Group 19, Bergey’s Manual, 9th ed.), includes eight species, viz., *Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshmeri*, *L. seeligeri*, *L. grayi*, *L. marthii* and *L. rocourtiae*. Of these, *L. marthii* (Graves et al., 2010) and *L. rocourtiae* (Leclercq et al., 2010) have been described recently. *L. monocytogenes* is an opportunistic pathogen in human beings and various animal species, whereas *L. ivanovii* mainly affects ruminants. *L. monocytogenes* is composed of at least 12 serovars i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Liu, 2006); All 12 serovars of the pathogen are known to cause human listeriosis, but serovars 1/2a, 1/2b and 4b are associated with most cases.

*Listeria spp.* are Gram-positive non-sporulating, facultatively anaerobic rods that measure 1-2 µm in length and 0.5 µm width. Growth occurs between 3 and 50°C but the optimum temperature is 30 - 37°C and it shows typical tumbling motility at 20-25°C (Topley and Wilson, 1990), which is characteristic for the organism.

Listeriosis was first recognized as a disease of animals and although the link between silage feeding and infection in farm animals has been known for decades, it was the recognition in the 1980s of listeriosis as a foodborne human disease that promised intense research activity (Low and Donachie, 1997).

**2.4.1 Symptoms of listeriosis**
Human listeriosis typically occurs sporadically, but occasional nosocomial clusters have been reported. *L. monocytogenes* causes two forms of listeriosis: non-invasive gastrointestinal listeriosis and invasive listeriosis (Allerberger and Wagner, 2010). *L. monocytogenes* infects normally sterile parts of the body such as liver, spleen, cerebral spinal fluid and blood, and most cases end up being hospitalized (Todd and Notermanns, 2011). Persons with a predisposing condition linked to decreased level of cell-mediated immunity such as individuals with cancer malignancies, organ transplant, liver disease, HIV/AIDS, and diabetes are more prone to infection and can develop sepsis, meningitis, and serious infections affecting the nervous system (Allerberger and Wagner, 2010).

In healthy adults, disease is mainly diarrhoea and fever. In pregnant women fever, diarrhoea, abortion or stillbirth are common; newborns get sepsis, pneumonia or meningitis (Todd and Notermanns, 2011). Most cases of confirmed listeriosis fall into the ageing category, especially over 65 years old. Case-fatality rates can be high (20-30%). The estimated number of cases in the United States in 1999 was 2500 with 500 deaths (Mead et al., 1999). It is estimated that 10% of the population in the developed world carry the bacterium in their gastro-intestinal tract (Swaminathan and Gerner-Smidt, 2007).

### 2.4.2 Isolation of *L. monocytogenes*

Meat, poultry and dairy products have been most frequently implicated as vehicles of transmission. Large outbreaks are usually linked to errors in food processing plants, such as contaminated slicing machines, followed by
opportunities for growth of the pathogen (Todd and Notermanns, 2011). Internationally significant efforts were initiated to isolate and identify *L. monocytogenes* and to limit its proliferation in foods (Farber and Peterkin, 1991; Dilon and Patel, 1992; Jemmi, 1993). A number of methods and media currently exist for the detection and enumeration of *L. monocytogenes*. For isolation of *L. monocytogenes* from foods, animals/human clinical samples and environmental samples, the use of enrichment cultures followed by selective plating is required (Curtis and Lee, 1995). The FDA bacteriological and analytical method (BAM), and the International Organization of Standards (ISO) 11290 method (Barbuddhe *et al.*, 2008) are the most widely used culture reference methods for detection of *Listeria* in all foods. Other methods, like the USDA and the Association of Analytical Chemists (AOAC/IDF) are also used.

### 2.4.2.1 Enrichment procedure/media

Isolation of *Listeria* from complex samples, such as food, environmental and stool samples, containing abundant background flora and a low number of *Listeria*, requires enrichment. The earliest method available was the cold enrichment technique (Gray *et al.*, 1948). This required inoculation of the sample into a nutrient broth lacking selective agents, followed by incubation at 4°C for long periods. However, the method was time consuming and has subsequently been replaced by methods involving selective enrichment and selective plating based on inhibition of the growth of background flora by adding such inhibitory agents as lithium chloride,
nalidixic acid, acriflavine, cefotetan, ceftazidime, colistin, cycloheximide, fosfomycin and polymyxin B (Gasanov et al., 2005).

Use of tryptose phosphate broth with polymixin (Bojsen-Moller, 1972) and Levinthal broth with trypaflavine and nalidixic acid (Ralovich et al., 1972) were the earliest attempts at isolation of *Listeria* within days.

Later, a number of media such as *L-PALCAMY* (Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk) (Van Netten et al., 1989), Lovett (FDA) (Lovett et al., 1987), University of Vermont medium (Donnelly and Baigent, 1986) and Fraser broth (Fraser and Sperber, 1988) were developed.

The ISO 11290 method employs a two-stage enrichment process: the first enrichment in half Fraser broth (Fraser and Sperber, 1988) for 24 h, then an aliquot is transferred to full-strength Fraser broth for further enrichment. In the FDA BAM method the sample (25 g) is enriched for 48 h at 30°C in *Listeria* enrichment broth (LEB) (Lovett et al., 1987) containing the selective agents acriflavin and nalidixic acid, and the antifungal agent cycloheximide. The USDA and the Association of Analytical Chemists (AOAC/IDF) methods use a modification of University of Vermont Medium (UVM) (Donnelly and Baigent, 1986) containing acriflavin and nalidixic acid for primary enrichment. The USDA method was designed and has been officially recommended primarily for meat and poultry products and the FDA method was designed for processing dairy products (Brackett and Beuchat, 1989).

### 2.4.2.2 Selective or differential plating media
Potassium tellurite was incorporated into a selective media to inhibit Gram-negative organisms (Gray et al., 1950). Subsequently, another selective agar was developed (McBride and Girard) by adding lithium chloride (LiCl), glycine and blood to phenyl ethanol agar. In modified MLA (MMLA), the blood was replaced with cycloheximide to inhibit growth of molds (Lovett et al., 1987). This was further modified as ARS-modified McBride agar (ARS-MMA) by adding Nalidixic acid, moxalactam and bacitracin (Buchanan et al., 1989).

Later, a number of media were developed which included Oxford agar (Curtis et al., 1989); LiCl-ceftazidine agar, modified (LCAM) (Lachica, 1990); polymixin- acriflavin- lithium chloride- ceftazidine- asculin- mannitol (PALCAM) (van Netten et al., 1989); Dominguez-Rodriguez isolation agar (DRIA) (Domínguez-Rodriguez et al., 1984); Dominguez Rodriguez Listeria selective agar medium, modified (LSAMm agar) (Blanco et al., 1989); modified Vogel Johnson agar (MVJ) (Buchanan et al., 1987) and MVJ modified further (MVJM) (Smith and Buchanan, 1990). *L. monocytogenes* Blood Agar (LMBA) proved to be a very useful tool and was able to detect *L. monocytogenes* from 94.1% of environemental sites compared to the 76.5% and 79.4% detection rate displayed by Oxford and Palcam agars, respectively (Kells and Gilmour, 2004).

### 2.4.2.3 Chromogenic media

The chromogenic media commercially available include Agar *Listeria* according to Ottoviani and Agosti, the BCM *L. monocytogenes* detection system, CHROM agar, and Rapid’ L.mono. Chromogenic media are simple, cost effective, and easy to interpret (Gasanov et al., 2005). Agar *Listeria*
according to Ottaviani and Agosti (ALOA) is both a selective and differential medium for the isolation of *Listeria* spp. and presumptive identification of *L. monocytogenes* (Ottaviani et al. 1997). ALOA was found to be superior to Oxford and PALCAM when samples containing both *L. monocytogenes* and *L. innocua* were examined (Vlaemynck et al., 2000).

### 2.4.3 Differentiation of *Listeria* species

Conventionally, the identification of *Listeria* spp. have relied on the results of fermentation of sugars and haemolytic reactions (Seeliger and Jones, 1986), and the commercially available API *Listeria* identification kit (Bille et al., 1992). The interpretation of haemolytic reaction, particularly CAMP test is difficult (McKeller, 1994; Fernandez-Grarayzabal et al., 1996), and the API *Listeria* kit has been reported to produce occasional discrepant results (Bille et al., 1992).

A rapid and inexpensive identification scheme relying on the detection of hemolysin and on two carbohydrate (L-rhamnose and D-xylose) fermentation tests has been proposed (Lachica, 1990). Similarly, use of agar-based media in place of conventional liquid media has been proposed (Evanson et al., 1991), however, haemolysis can be difficult to assess, and various atypical *L. monocytogenes* strains (non-haemolytic, catalase-negative, rhamnose-negative, non-motile) would not be identified (Bille et al., 1992). Genomic methods can firmly identify various isolates of *Listeria* (Liu, 2006), however, such methods require sophisticated and costly equipments.

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

### 2.4.4 Foodborne listeriosis

There have been many outbreaks linked to *L. monocytogenes* since it was first identified as a foodborne pathogen in 1981. Various types of RTE foods and different types of deli meat, pâté, soft cheeses, smoked fish/shellfish, coleslaw, raw milk and pasteurised milk were implicated (Norton and Braden, 2007). A non-invasive febrile gastroenteritis, or non-invasive gastroenteritis, has been linked to outbreaks of deli meat, cheese, chocolate milk, smoked fish, corn and rice salad. Most outbreaks have occurred in Europe, US, Canada, and to a lesser extent Australia and New Zealand (Todd and Notermans, 2011).

As per estimates each year approximately 76,000,000 cases of foodborne illness occur in USA, and of these about 2,500 (<1%) are attributed to *L. monocytogenes* (Ellin Doyle, 2001). However, the mortality rate (20-30%) by *L. monocytogenes* far exceeds other common foodborne pathogens, such as Salmonella enteritidis (with a mortality of 0.38%), *Campylobacter*
species (0.02-0.1%) and *Vibrio* species (0.005-0.01%) (Mead *et al*., 1999; Liu, 2006). The disease has been estimated to cost much more per case than other more common but less serious foodborne illnesses (Todd, 1989). The economic losses due to listeriosis in USA have been estimated at $480 millions (Roberts and Pinner, 1990). In Indian context, however, no such data is available. *L. monocytogenes* infections are responsible for the highest hospitalisation rates (91%) amongst known food-borne pathogens (Jemmi and Stephan, 2006).

Outbreaks from *L. monocytogenes* are not common compared with those caused by pathogens like Salmonella. However, they receive considerable attention when they do occur because of high mortality rates often caused by errors made by workers in manufacturing plants (Todd and Notermans, 2011). The outbreaks have major economic consequences, especially as the products may affect international trade. The Maple Leaf deli meat listeriosis outbreak in Canada cost the company $43 million (Wordsnark, 2008) and had to settle for $27 million in law suits (Anonymous, 2009). Soft cheeses and deli meats were the most frequently contaminated products in recent outbreaks (EFSA, 2007; EFSA 2009; CDC, 2009).

In 2005, South Australia experienced two cases of meat borne listeriosis (ABC, 2005). In 2006, Germany reported a *L. monocytogenes* outbreak affecting at least 6 persons. The source was a contaminated hard cheese with cheese samples containing 52,000 1-120,000 *L. monocytogenes* CFU/g (EFSA, 2007). Also, soft cheese was identified as the source of infection in three outbreaks in the Czech Republic, with a total of 78 people affected; all were hospitalised and 13 persons died. An outbreak in Norway in
2007 was caused by a soft cheese produced on a small dairy farm (EFSA, 2009).

In India, there are few studies on the prevalence of *L. monocytogenes* in food samples, and presently no documented clusters and outbreaks of human listeriosis have been documented (Barbuddhe *et al.*, 2011).

The occurrence of listeric infections in the Indian subcontinent has been extensively reviewed by Malik *et al.* (2002). In India, *L. monocytogenes* strains have been isolated from the meat and milk of goats, sheep and buffaloes (Barbuddhe *et al.*, 2000, 2002). The microorganism has been isolated from fish and fishery products (Jayasekaran *et al.*, 1996; Karunasagar and Karunasagar, 2000; Parihar *et al.*, 2008). *L. monocytogenes* has been isolated from cases of mastitis, reproductive disorders and septicaemia in animals (Shakuntala *et al.*, 2006; Rawool *et al.* 2007).

### 2.4.5 Isolation from foods

The ability of *L. monocytogenes* to grow at low temperatures is important in the bacterium’s persistence in food processing environments. Further biofilm forming abilities (Di Bonaventura *et al.*, 2008) and sanitizer resistance (Lunden *et al.*, 2003) contribute to the persistence of *L. monocytogenes* in food processing environments.

The incidence of contaminated milk samples varies among countries, being 1.2% in Denmark (Jensen *et al.*, 1996), 3.62% in Spain (Gaya *et al.*, 1998) and 3.48% as calculated by Ryser and Marth (1991) in the USA. *Listeria monocytogenes* was detected in 4.6 of 131 bulk tank milk samples in eastern South Dakota and western Minnesota, respectively. All isolates of *L.
were identified as O antigen type 1 (Jayarao and Henning, 2001). In a study in UK, the incidence of *Listeria* from milk processing equipments was found to be 18.8% (6.3% *L. monocytogenes*), while in the environment and raw milk was 54.7% (40.6% *L. monocytogenes*) and 44.4% (22.2% *L. monocytogenes*), respectively. On one occasion, *L. welshimeri* was isolated from pasteurised milk, probably demonstrating post-pasteurisation contamination of product. The main environmental sources of *L. monocytogenes* were considered to be a floor drain and stainless steel steps (Kells and Gilmour, 2004). Analysis of a total of 594 samples of various milk and dairy products from selected retail stores in Iran revealed 55 samples (9.3%) to be positive for *Listeria* spp. The most common species recovered was *L. innocua* (58.2%); the remaining isolates were *L. monocytogenes* (32.7%) and *L. seeligeri* (9.1%) (Rahimi et al., 2010).

*L. monocytogenes* was found in soft and semi soft cheeses made from raw or low heat treated cow milk in 0.1% of samples overall (range, 0% - 3.2%) (Todd and Notermans, 2011). In EU, smoked fish was the food item most often containing *L. monocytogenes* (18.3%), and at levels exceeding 100 CFU/g (2.4%). The highest proportions of positive samples of fishery products (all smoked fish) were reported from Poland (29.6%), the Netherlands (22.6%), Italy (14.5%), the Czech Republic (13.8%) and Germany (11.4% and 9.3%) at retail and processing, respectively (Todd and Notermans, 2011).

The prevalence of *L. monocytogenes* in various meat products was less; pork, 2.2%; red, mixed or unspecified meat, 2.5%; 3.0% in RTE broiler meat (Todd and Notermans, 2011). However, samples with much more contamination were reported from Germany, Greece, Italy, Poland, and
Slovenia with the reported presence of *L. monocytogenes* in samples of 25 g in 11.0%, 20.7%, 13.6%, 62.9%, and 16.7%, respectively. In France, *L. monocytogenes* was detected in 25 of the 144 raw egg samples collected from 3 different egg-breaking plants, in 4 of the 144 pasteurized egg samples stored at 2 °C for two days and in 2 of the 144 analysed at shelf-life date (Rivoal *et al.*, 2010). Contamination of raw egg products appeared to be season dependant and was higher during summer and winter than during autumn.

Four out of 153 (2.61%) farm milk samples and 6 out of 80 (7.50%) tankers' samples tested positive for *L. monocytogenes* collected in Algiers and Blida. However, all samples of whey and curdled milk tested negative for *L. monocytogenes* (Hamdi *et al.*, 2007). Bulk tank milk (BTM) samples (536) and in-line milk filters (519) collected from dairy farms across the United States during the National Animal Health Monitoring System's Dairy 2007 study were analyzed by culture techniques for the presence of *L. monocytogenes*. *L. monocytogenes* was isolated from 7.1% of the dairy operations, and the 1/2a complex was the most common serotype, followed by 1/2b and 4b (lineage 1) (Van Kessel *et al.*, 2011).

In Indian context, few studies have been carried out to study the incidence of *Listeria* in foods. *L. monocytogenes* was isolated from 8.1 % of raw milk samples (Bhilegaonkar *et al.*, 1997). *L. monocytogenes* could not be isolated from pasteurized bulk milk tanks. Isolation of pathogenic *L. monocytogenes* strains was reported from milk of 1.56% goats (Barbuddhe *et al.*, 2000) and 6.25% buffaloes (Barbuddhe *et al.*, 2002). In an extensive study involving central India, *Listeria* spp. were isolated from 139 (6.75%) samples out of 2060 samples collected from dairy cows; 105 (5.1%) were positive for *L.
monocytogenes (Kalorey et al., 2008). Aurora et al. (2008) analysed milk (471) and ready-to-eat indigenous milk products (627) and detected *L. monocytogenes* isolates (eight from milk and 10 from milk products).

In India, analysis of meat samples revealed *L. monocytogenes* isolates from 6.66% to 7.08% goat (Barbuddhe et al., 2000; Rekha et al., 2006), 7.4% sheep (Barbuddhe et al., 2000), and 3.07 % to 6% buffalo (Brahmbhatt and Anjaria, 1993; Barbuddhe et al., 2002) and 8.1 % poultry meat samples (Barbuddhe et al., 2003).

The organism has been isolated from fish and fishery products from different parts of the world and interestingly the incidence rate reported from tropical fish is rather low (Karunasagar and Karunasagar, 2000). Earlier reports from India suggested the absence of *L. monocytogenes* in fishes (Fuchs and Surendran, 1989; Karunasagar et al., 1992; Manoj et al., 1991). *L. monocytogenes* has been isolated from 12.1% fresh shell fishes and 17.2% fresh fin fishes from India (Jeyasekaran et al., 1996). *L. innocua* (30.8%) and *L. monocytogenes* (1.3%) were isolated from fresh raw fish samples from Mangalore (Dhanashree et al. 2003). *L. monocytogenes* was detected in 10 of 115 seafood samples from markets in Goa (Parihar et al., 2008). *L. innocua* was the most common *Listeria* species recovered and was detected in 18 samples. Jallewar et al. (2007) isolated 39 strains of *Listeria* spp. from samples (200) of fresh water fish, walking catfish (*Clarias batrachus*). Of these 26 (67%), 8 (21%), 3 (8%) and 2 (5%) were *L. monocytogenes*, *L. seeligeri*, *L. grayi* and *L. welshimeri*, respectively.

### 2.4.6 Detection of *Listeria*

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Listeria monocytogenes and other Listeria species closely resemble morphologically and biochemically and the clinical manifestations of listeriosis are non-specific (Vazquez-Boland et al., 2001), therefore, rapid, specific and sensitive diagnostic tests capable of distinguishing L. monocytogenes from other Listeria species are essential for the effective control of the disease (Liu, 2006).

Detection and enumeration of L. monocytogenes from environments such as food, which can be heavily contaminated with other organisms, are often difficult (Golden et al., 1988). The L. monocytogenes demonstrates strain variations in virulence and pathogenicity (Liu et al., 2003; Roche et al., 2003), therefore, the ability to determine accurately and rapidly the pathogenic potential of L. monocytogenes isolates is essential to limit the spread of listeriosis and reduce unnecessary recalls of food products. The pathogenicity of L. monocytogenes is thought to be a multifactorial phenomenon (Ralovich, 1984). Therefore, the pathogenic potential of the L. monocytogenes isolates from foods need to be ascertained. The pathogenic potential of Listeria isolates can be assessed by in-vitro pathogenicity tests like β-haemolysis on sheep or horse blood agar (Schonberg, 1989), phosphatidylinositol-specific phospholipase C (PI-PLC) assay (Notermans et al., 1991b) and by use of chromogenic media (Greenwood et al., 2005), and by in vivo methods namely, chick embryo and mouse inoculation (Menudier et al., 1991). The in vivo methods remain objectionable from ethical point of view and need skilled personnel to perform. Therefore, the application of molecular techniques has facilitated the identification and characterization of L. monocytogenes (Liu, 2006). Among the several approaches to nucleic acid amplification, PCR was
the first and remains the most widely applied technique in both research and clinical laboratories (Liu, 2006).

A number of factors have been shown to be associated with the virulence of *L. monocytogenes* (Portnoy *et al*., 1992). The hemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. Subsequently, the virulence gene cluster in which most of the genetic determinants required for the intracellular life cycle of pathogenic *Listeria* spp. reside was discovered (Vazquez-Boland *et al*., 2001). The listeriolysin O-encoding gene (*hlyA*) is present only in virulent strains of the species and is required for virulence. LLO is a secreted protein toxin that can be detected easily with the use of blood agar or haemolysis assays and it is well characterized and understood (Churchill *et al*., 2005). A strong correlation between hemolytic activity and pathogenicity in the genus *Listeria* has been demonstrated (Seeliger and Jones, 1986; Skalka *et al*., 1982).

Differentiation of *L. ivanovii* from *L. monocytogenes* and other *Listeria* species can be achieved by its production of a wide, clear or double zone of haemolysis on sheep or horse blood agar, a positive Christie–Atkins–Munch-Petersen (CAMP) reaction with *Rhodococcus equi* but not with haemolytic *Staphylococcus aureus* (Rocourt and Catimel, 1985). A positive CAMP reaction or fermentation of rhamnose and non fermentation of xylose can be used to identify pathogenic *Listeria* spp. with the exception of *L. seeligeri* which is haemolytic but non pathogenic (Seeliger, 1981).

Pathogenic *Listeria* spp. exhibit three different enzymes with phospholipase C (PLC) activity, PlcA and PlcB activities are shown by *L. monocytogenes* and SmcL, is specific to *L. ivanovii* (Vazquez- Boland *et al*.,
The activity of virulence factor called phosphatidylinositol-specific phospholipase C (PI-PLC), encoded by the plcA gene, has been reported to be expressed by the pathogenic species of Listeria (Notermans et al., 1991b). The plcA-deficient strains have been reported to be less virulent in mice (Camilli et al., 1991). The PI-PLC and PCR assays are reliable in vitro alternatives to in vivo pathogenicity tests for L. monocytogenes (Kaur et al., 2010).

Recently, the efficacy of PI-PLC as a diagnostic antigen and the behavior of PI-PLC producing Listeria strains in the in vivo pathogenicity test have been studied in detail (Chaudhari et al., 2004). PI-PLC assay has been used as a reliable method to differentiate between pathogenic and non-pathogenic Listeria (Shakuntala et al., 2006; Rawool et al., 2007). Presently, a variety of chromogenic agars are commercially available which aim at shortening time for the detection of pathogenic L. monocytogenes (Greenwood et al., 2005). A chromogenic media (ALOA) has been developed based on PI-PLC activity (Ottaviani et al., 1997). ALOA medium has proven to be a useful and significantly better assay than other media for the differentiation of L. monocytogenes from non-pathogenic Listeria species (Vlaemynck et al., 2000, Beumer and Hazeleger, 2003). All the Listeria species form bluish green colonies due to the presence of a chromogenic compound X-glucosidase which detects β-glucosidase. Further, pathogenic Listeria spp. can be distinguished from other Listeria species through the production opaque halo around the colonies (Ottaviani et al., 1997).

Virulence of L. monocytogenes for humans has been correlated with pathogenicity in mice (Mainou-Fowler et al., 1988) particularly in immuno-compromised mice (Stelma et al., 1987). Mice inoculation is capable of
providing an in vivo measurement of all virulent determinants; therefore, it is regarded as the gold standard for any newly developed tests for *L. monocytogenes* virulence (Roche et al., 2001; Liu et al., 2003). The mouse virulence assay is conducted by inoculating mice with various doses of *L. monocytogenes* via the oral, nasal, intraperitoneal, intravenous or subcutaneous routes. The virulence of a given *L. monocytogenes* strain is determined by the mouse mortality resulting from infection, or by the number of *L. monocytogenes* bacteria that reach the spleen following experimental infection (Liu, 2006).

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

An array of virulence-associated genes associated with the pathogenicity of *Listeria* spp., include *plcA* encoding phosphatidylinositol phospholipase-C (PI-PLC), *plcB* encoding phosphatidycholine phospholipase-C, *hlyA* encoding a haemolysin, *mpl* encoding a metalloprotease and *actA* encoding the surface actin polymerisation protein ActA. All of these genes are physically linked in a 9 kb chromosomal island referred to as *Listeria* pathogenicity island-1 (LIPI-1) (Vazquez-Boland et al., 2001).

One of the biggest problems associated with detection of *L. monocytogenes* is the low numbers at which the bacteria are normally found in contaminated
food samples (Hoffman and Weidmann, 2001). DNA-based methods of detection employ ways of amplifying the specific genetic signals from a few cells. PCR is the basis of many nucleic acid-based detection systems (Churchill et al., 2005). Among the target genes for PCR detection of *L. monocytogenes* are the *hlyA* gene (Norton et al., 2001; Thimothe et al., 2004), the *iap* gene (Cocolin et al., 2002), *inlB* (encoding internalin B) (Lunge et al., 2002) and 16S rRNA (Call et al., 2003). Among these genes, the *hlyA* gene has been used most commonly (Aznar and Alarcón, 2002). While comparing PCR with the ISO culturing method 11290-1 to detect *L. monocytogenes* in salmon gave comparable results in spiked samples if culture enrichment is used prior to PCR to lower the detection limit for *L. monocytogenes* (Wan et al., 2003).

Multiplex PCR is a variation of the traditional PCR. This method makes use of multiple sets of primers to amplify a number of genes or gene fragments simultaneously (Churchill et al., 2005). mPCR was used to simultaneously detect *L. monocytogenes*, *S. typhimurium*, and *E. coli* O157:H7 from artificially contaminated produce (Bhagwat, 2003). This identification procedure is effective, but not conducive to high-throughput screening because of the need to analyze the PCR products by agarose gel electrophoresis. A mPCR assay employing four genes, the *hlyA*, *plcA*, *iap* and *actA* for detection of *L. monocytogenes* from clinical samples has been developed (Kaur et al., 2007). Rawool et al. (2007) detected multiple virulence-associated genes (the *plcA*, *prfA*, *hlyA*, *actA* and *iap*) in *Listeria monocytogenes* isolated from bovine mastitis cases. The development of PCR-based serotyping procedures, such as the use of group-specific PCR primers, has provided additional tools for the
identification and grouping of *L. monocytogenes* (Borucki and Call, 2003; Doumith *et al.*, 2004).

Use of real-time PCR in a 96-well PCR format eliminates the need for agarose gel electrophoresis. In this method, a fluorescent dye, such as SYBR Green I is used to follow the PCR amplification in real-time and can be used to detect the amplified products from a number of genes at the same time (Bhagwat, 2003). Primers for real-time PCR can be designed to simultaneously detect both *Listeria* spp. and *L. monocytogenes* by amplifying the 23S rRNA gene (conserved in all *Listeria* spp.) at the same time as the *hlyA* gene (Rodriguez-Lázaro *et al.*, 2004a). Real time PCR can be used with the proper primers to quantify the number of pathogens present in a sample by measuring the level of fluorescence as compared to a standard. The adaptation of conventional PCR to the reverse transcription PCR (RT-PCR) format also permits the detection of viable *L. monocytogenes* organisms in specimens (Liu, 2006).

The development of a 10-minute assay based on **Matrix Assisted Laser Desorption/Ionisation-Time Of Flight** (MALDI-TOF) spectroscopy directly from colonies on agar plates has been reported. The method allows not only discrimination between pathogenic and non-pathogenic *Listeria* spp. but also permits resolution up to the level of the PCR serotype analysis described above (Barbuddhe *et al.*, 2008). Nevertheless, high cost of the capital equipment involved, despite being offset by cheap running costs of the assay, puts this method beyond the means of smaller diagnostic laboratories.

**2.4.7 Subtyping of *Listeria***
Listeria monocytogenes is a ubiquitous organism and exhibits diversity of strains. The subtyping procedures are used to track individual strains involved in listeriosis outbreaks, and to examine the epidemiology and population genetics of L. monocytogenes. The subtyping is integral to control and prevention programmes aimed at listeriosis. Two major subtyping approaches are in common use: Phenotypic and Genotypic (molecular or DNA subtyping).

The choice of method depends most on the performance criteria of a method, such as typeability, discriminatory power, reproducibility, rapidity and ease of use, and the purpose of subtyping, e.g. phylogenetic analysis, epidemiological surveillance, outbreak investigations or food processing contamination analysis (Struelens et al., 1996).

L. monocytogenes can be classified into four lineages: lineage I encompasses serotypes 1/2b, 3b, 4b and 3c; lineage II includes serotypes 1/2a, 1/2c, 3a, lineage III comprises serotypes 4a, 4b and 4c and lineage IV comprises 4a, 4b, 4c (Orsi et al., 2011). Several subtyping procedures including serotyping, multilocus enzyme electrophoresis (MLEE), DNA restriction endonuclease analysis, ribotyping, DNA sequencing-based subtyping techniques [e.g. multilocus sequence typing (MLST)] and PFGE have been developed for L. monocytogenes (Borucki and Call, 2003; Liu, 2006).

2.4.7.1 Phenotypic methods

Phenotypic methods often have a low power of discrimination in strains, suffer from biologic variability (e.g., phage typing), and may not be
applicable to all strains (Graves et al., 1999). Serotyping has been a classical tool in subtyping of *L. monocytogenes*. Based on somatic (O) and flagellar (H) antigens, *L. monocytogenes* strains are divided into 12 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). The major drawbacks of serotyping include cost, availability and standardization of reagents, as well as the technical expertise needed to perform the assay (Borucki and Call, 2003). The usefulness of serotyping in epidemiological investigations is limited as more than 95% of strains isolated from human cases and foods belong to serotypes 1/2a, 1/2b and 4b (Liu, 2006). The ELISA method of serotyping described by Palumbo et al. (2003) is a cost-effective method. The reduced variability of the antiserum quality by using a commercially available antisera and also the reduction of inconsistencies in judgement associated with weakly agglutinating antigen–antiserum combinations are overcome by this method (Palumbo et al. 2003). Studies have found that serotype 1/2a was the predominate serotype of *L. monocytogenes* food and environmental isolates (Corcoran et al. 2006; Gilbreth et al. 2005; Lukinmaa et al. 2003).

Phage typing has been shown to be an efficient method for large scale subtyping of *L. monocytogenes* (Audurier and Martin 1989), and a standard phage set with a standardized method has been described (McLauchlin et al., 1996). Even though phage typing shows high discrimination power, the high number of strains remains non-typeable (Rocourt et al., 1985). In an investigation to test the validity of the international phage set and 13 experimental phages for subtyping *L. monocytogenes* strains isolated from poultry in Spain, the phage set used was not effective for typing *L.
*monocytogenes* strains (Capita *et al.*, 2002). MLEE is a protein-based, isoenzyme typing method that correlates specific protein band patterns with genotypes (Liu, 2006).

### 2.4.7.2 Genotypic methods

Pulsed-field gel electrophoresis (PFGE) is a molecular subtyping method that has been successfully used to characterize *L. monocytogenes* isolates (Mammina *et al.* 2009; Kerouanton *et al.* 2010) and is considered to be the gold standard subtyping method because of the documented reproducibility in previous epidemiological studies and its high discriminatory power (Graves and Swaminathan 2001; Autio *et al.* 2002). Ribotyping is based on the use of nucleic acid probes targeting ribosomal genes after restriction enzyme analysis of chromosomal DNA (Grimont and Grimont, 1986). Automated ribotyping was previously used for rapid subtyping *L. monocytogenes* for source tracking, population genetics–based studies, and epidemiologic investigations (Wiedmann, 2002); however, it is expensive and not as discriminatory as PFGE (Inglis *et al.*, 2002). PFGE provides sensitive subtype discrimination and is often considered the standard subtyping method for *L. monocytogenes* (Graves and Swaminathan, 2001). However, this method is not automated and is labor intensive. Even recently developed rapid protocols take approximately 30 hours to perform (Graves and Swaminathan, 2001). Computer-assisted data analysis of large and diverse PFGE type databases can improve the correct interpretation of subtyping data in epidemiological studies and in tracing routes and sources of contamination in the food industry (Neves *et al.*, 2008).
Several typing methods involving PCR have been developed. The methods employ either just PCR amplification or RAPD or PCR amplification is performed either before (PCR-REA) or after (amplified fragment length polymorphism, AFLP) restriction enzyme analysis. RAPD is more economical and faster than other typing methods, and is particularly suitable for testing fewer than 50 strains (Liu, 2006). It employs short (9–10 bp) primers with sequences chosen at random, thus prior sequence knowledge of template DNA is not needed. Several workers have focused on the use of this typing method (Dhanashree et al., 2003; Ertas and Seker, 2005). Thirty *L. monocytogenes* isolates from human patients and foods originated from two different geographic locations without any epidemiological relations showed 92–99% genetic homogeneity and contained virulence genes, *inlA, inlB, actA, hlyA, plcA* and *plcB* (Jaradat et al., 2002).

A rapid multiplex-PCR serotyping assay has been developed which separated the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c and 4b) into distinct groups (Doumith et al., 2004; Doumith et al., 2005). Multiplex PCR assay grouped *L. monocytogenes* isolates from Algiers and Blida to the PCR-group IVb corresponding to serovars 4b, 4d and 4e. The combination of Asci and ApaI macrorestriction patterns yielded five different pulsovars (I to V) (Hamdi et al., 2007).

Serotyping of 145 *L. monocytogenes* isolates revealed serovar 1/2a to be the most frequent (57.4%) followed by 4b (14.1%), 1/2b (9.7%), 4c (4.4%) and 1/2c (6.7%). Eleven isolates were identified as non-*Listeria* spp., the remaining ten *L. monocytogenes* isolates were nontypeable (O’Connor et al., 2010).
Serotyping of 196 *L. monocytogenes* isolates from food sources revealed 3 serovars with 1/2a to be dominant serovar presented by 94.4% of the isolates (Rivoal et al., 2010). Assessment of diversity of 60 strains of *L. monocytogenes* isolated from sludge gave 44 different combined *Apa*I / *Asc*I PFGE patterns. The PFGE patterns of most isolates were similar or very similar to those of epidemic isolates. The majority (93%) of isolates were found to be virulent by plaque-forming assay and by mouse virulence assay representing a potential health hazard (Kerouanton et al., 2010).

Characterization of 378 *Listeria* spp. isolates by PFGE revealed PFGE profile I (n = 14.5%) to be the most prevalent pulse-type consisting of mainly of environmental *Listeria* spp. samples (O’Connor et al., 2010).

PFGE typing of 196 *L. monocytogenes* isolates was carried out by macrorestriction of the genomic DNA with *Apa*I and *Asc*I enzymes showed a large diversity with 21 genotypes of *L. monocytogenes* (Rivoal et al., 2010). *L. monocytogenes* isolates from eastern China were grouped in 17 to 19 subtypes using PFGE with *Sma*I digestion, and multilocus sequence typing (MLST) based on three virulence genes (*act*A, *inl*A and *inl*B) and four housekeeping genes (*bet*L, *dat*, *rec*A and *sig*B). The virulence genes based MLST had better discriminatory power than that targeting the housekeeping genes (0.990 vs 0.895), similar to PFGE (0.976) (Jiang et al., 2008). MLST, a DNA sequence-based method developed by Chan et al. (2001) has been used extensively in subtyping of *L. monocytogenes* (Chen et al., 2005; Nightingale et al., 2005).

A longitudinal study aimed to detect *L. monocytogenes* on a New York State dairy farm was conducted (Latorre et al., 2009). Twenty two (7.3%) of 303 environmental samples, 73 (67.6%) of 108 in-line milk filter samples and
34 (19.7%) of 172 bulk tank milk samples were positive for *L. monocytogenes*. *L. monocytogenes* was isolated from 6 of 40 (15%) sampling sites in the milking parlour and milking equipment. Analysis of 60 isolates by PFGE yielded 23 PFGE types after digestion with *Ascl* and *ApaI* endonucleases. Three PFGE types of *L. monocytogenes* were repeatedly found in longitudinally collected samples from bulk tank milk and in-line milk filters indicating a reservoir in milking equipment (Latorre *et al.*, 2009).

Investigation of PFGE type diversity of a total of 495 temporally and geographically matched *L. monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments revealed 310 PFGE types by two-enzyme (*Ascl* and *ApaI*) PFGE and exhibited higher overall discriminatory power than either *EcoRI* ribotyping or *Ascl* or *ApaI* single-enzyme PFGE. Seven PFGE types showed significant associations with specific sources, including one and four PFGE types, respectively, associated with human clinical cases and foods. Nine PFGE types were geographically widespread and occurred among isolates from multiple sources. It was opined that large PFGE type databases representing isolates from different sources are needed to appropriately interpret subtype data in epidemiological investigations and to identify common as well as source-specific PFGE types (Fugett *et al.*, 2007).

Five *L. monocytogenes* serogroups were identified among isolates recovered from retail RTE meats, raw chickens and fresh produce. Of the 167 isolates 68 (41%) belonged to serogroup 1/2b, 3b; 53 (32%) belonged to serogroup 4b, 4d, 4e; 43 (26%) belonged to serogroup 1/2a, 3a; 2 (1.2%) belonged to serogroup 1/2c, 3c; and 1 (0.6%) belonged to serogroup 4a, 4c.
PFGE generated 120 patterns which correlated well with PCR serogrouping (Zhang et al., 2007).

The serotypes and pulsotypes of 674 L. monocytogenes isolates from human (57), food (558) and environmental (59) sources, collected from different Italian geographical areas were determined to study the possible association with certain foods, and to determine possible geographical or temporal associations. Eleven different L. monocytogenes serotypes with predominant 1/2a, 1/2b, 1/2c, 4b serotypes were found in the food, environmental and human isolates. The isolates were divided into 133 distinct AscI pulsotypes grouped into 26 pulsogroups. The associations between subtypes and isolates were highly significant but not exclusive, indicating that there was no close correlation between specific subtypes and specific food products (Gianfranceschi et al., 2009).

2.4.8 Antibiotic resistance to Listeria

Since the first emergence of multidrug resistant strains in France in 1988, L. monocytogenes has been closely monitored for antimicrobial resistance (Poyart-Salmeron et al. 1990). Currently, the treatment of choice for listeriosis is a combination of ampicillin, rifampin, or penicillin G with an aminoglycoside, such as gentamicin (Charpentier and Courvalin 1999). L. monocytogenes has been reported to be resistant towards tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999). Of major concern are Listeria spp. exhibiting resistance to antibiotics commonly used for the treatment of listeriosis, e.g. ampicillin, penicillin, and
gentamicin (Walsh et al. 2001). The antimicrobial susceptibility of 86 *Listeria* spp. isolated from processed bison carcasses to 25 antimicrobial agents using E-test and National Antimicrobial Resistance Monitoring System (NARMS) panels revealed most *Listeria* isolates (88–98%) to be resistant to bacitracin, oxacillin, cefotaxime, and fosfomycin. Resistance to tetracycline (18.6%) was also common (Li *et al.*, 2006). Strains resistant to one or more antibiotics have been recovered from environmental, food and from sporadic cases of human listeriosis (Arslan and Ozdemir 2008; Davis and Jackson 2009). Hence, the importance of continuous monitoring of environmental, food and clinical *Listeria* isolates for antibiotic resistance is emphasized by the slow and gradual emergence of antimicrobial-resistant strains. Gentamicin (5%) displayed most resistance towards *L. monocytogenes* followed by sulfamethoxazole-trimethoprim (2%), tetracycline and ciprofloxacin (1.5%). The emergence of antimicrobial-resistant *L. monocytogenes* isolates could have serious therapeutic consequences (O’Connor *et al.*, 2010). In the study by Davis and Jackson (2009) majority of *L. monocytogenes* isolates were resistant to oxacillin (99%) and that most of the *L. welshimeri* (67%) were resistant to sulfamethoxazole-trimethoprim. Out of 54 *Listeria* isolates from retail stores in Iran, 98.2% were resistant to one or more antimicrobial agents and resistance to nalidixic acid was the most common finding (96.4%). However, all *Listeria* isolates were susceptible to vancomycin (Rahimi *et al.*, 2010). All the 19 strains of *Listeria monocytogenes* (serovars 1/2a, 1/2b, 4b and 4c), isolated from dairy products in Rio Grande do Sul, Brazil were susceptible to the antimicrobials tested (De Nes *et al.*, 2010).
Most *L. monocytogenes* isolates recovered from retail RTE meats, raw chickens and fresh produce were resistant to sulfonamides (73%) and some were resistant to tetracycline (8.4%) and ciprofloxacin (1.8%) (Zhang *et al.*, 2007).

The susceptibility testing of 120 *L. monocytogenes* strains isolated from food and food-processing environments to 19 antibiotics currently used in veterinary and human therapy using the automated VITEK2 system displayed resistance to at least one antibiotic in 11.7% strains. In particular, resistance to one antibiotic was more common than multiple resistance, i.e., 10 (8.3%) isolates were resistant to one antibiotic, 3 (2.5%) to two antibiotics and one (0.8%) to five antibiotics. Resistance to clindamycin was most common, followed by linezolid, ciprofloxacin, ampicillin and rifampicin, trimethoprim/sulphamethoxazole and, finally, vancomycin and tetracycline (Conter *et al.*, 2009). The study showed susceptibility of *L. monocytogenes* strains from food and food-processing environments to the antibiotics commonly used in veterinary and human listeriosis treatment.