Chapter 15
Prevalence of *Listeria* in Milk from Farm to Table

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15.1 Introduction

Food safety is at the centre of one's health as food is an excellent vehicle by which many pathogens can reach an appropriate colonization site in a new host. Many, if not most, of all important zoonoses relate in some way to animals in the food production chain. One of the major issues in food safety over the latest decennial has been the lack of cross-sectoral collaboration across this chain (Wielinga and Schlundt 2012).

Dairying plays a dynamic role in agro-based economy and milk is a high value source of nutrients for the urban and rural population. Milk as it is secreted by the gland of the mammals is free of microorganisms but can get contaminated with bacteria during or after milking. Mastitis is one of the most economically important diseases affecting the dairy industry and reduces milk production and alters milk composition (Bansal and Gupta 2009). Although many studies are reported on the analysis of milk collected at different stages of processing, data are lacking on the analysis of milk in production chain, i.e. from farm to table (Anon 2011). Hence, there is an urgent need from the dairy industry to understand the introduction of emerging food-borne pathogens in milk and thus in turn control and prevent milk-borne epidemics and outbreaks.

Human illness from milk-borne pathogens is usually associated with consumption of raw milk or products made from raw milk. Occasionally, this has also been linked to pasteurised milk products but these cases usually have been a result of contamination of the product after pasteurisation or improper pasteurisation (Oliver et al. 2005). In the past 20 years, food-borne illnesses from dairy product consumption have been predominantly associated with *Salmonella enterica, Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

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S. Borkar (ed.), *Bioprospects of Coastal Eubacteria,*
DOI 10.1007/978-3-319-12910-5_15

The microbiological safety of food remains a dynamic situation heavily influenced by multiple factors. Given the recent spate of food-borne illness outbreaks, *Listeria monocytogenes* is once again in the spotlight as an important bacterial pathogen (Newell et al. 2011). Although the number of people infected by food-borne *Listeria* is comparatively small, this bacterium is one of the leading causes of death from food-borne illness due to its toxin listeriolysin O which is rightly referred to the Swiss army knife of *Listeria* (Hamon et al. 2012).

15.2 *Listeria* spp. and *Listeria monocytogenes*

Listeria spp. are ubiquitous bacteria widely distributed in the environment (Liu et al. 2006). They are Gram-positive, nonsporulating, 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 they show typical tumbling motility at 20–25 °C (Topley and Wilson 1990) which is a characteristic feature of the organism.

The genus *Listeria* includes various species such as *L. monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshmeri*, *Listeria seeligeri*, *Listeria grayi*, *Listeria marthii* (Graves et al. 2010), *Listeria rocourtiae* (Leclercq et al. 2010), *Listeria weihenstephanensis* (Lang et al. 2013), *Listeria fleischmannii* (Bertsch et al. 2013), *Listeria floridensis*, *Listeria aquatica*, *L. cornellensis*, *L. riparia* and *L. grandensis*. (Bakker et al. 2014). Among the 15 species of *Listeria*,only *L. monocytogenes* is pathogenic for humans whereas *L. ivanovii* mainly affects ruminants.

L. monocytogenes is a food-borne opportunistic pathogen of great concern for the food industry and milk-producing companies. Due to its physiological characteristics, such as resistance to acidic and sodium chloride stress, ability to grow at low temperature and possibility to form biofilms (Harvey et al. 2007), it can persist and/or recontaminate food products, thereby representing an important risk for the safety of the consumers (Gardan et al. 2003; Liu et al. 2002; Olesen et al. 2009; Pan et al. 2006). The term "*Listeria hysteria*" was coined towards the end of 1980s following a series of listeriosis outbreaks due to the consumption of soft cheese and ready-to-eat (RTE) meats in the UK. Recently, this emerged again in the large outbreaks in Canada caused by deli meats (Warriner and Namvar 2009) and also in USA (Anon 2011). Although human listeriosisoccurs only sporadically (Farber and Peterkin 1991; Schuchat et al. 1991) several outbreakshave been observed during the last two decades (McLauchlin et al. 2004).It is established that food-borne transmission constitutesthe main route of acquisition of listeriosis (Farber and Peterkin 1991). Although the incidence of the first human case of listeriosis was reported by Nyfeldt (1929), it is only since 1981, after the three well investigated listeriosis epidemics, first caused by coleslaw (Schlech et al. 1983), second caused by whole and 2 % fat milk (Fleming et al. 1985) and third caused by consumption of soft Mexican-style cheese (Linnan et al. 1988) that this organism came to be considered as a food-borne pathogen. Multinational outbreak from dairy products was reported

by Fretz et al. (2010). *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 of the cases.

15.3 Listeriosis

Listeriosis is a serious invasive bacterial zoonotic disease characterised by neural, visceral and reproductive disorders. It is usually manifested as septicaemia, 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 food-borne bacterial disease and a nagging public health hazard caused by ingestion of contaminated food and water. To manage the problem of food-borne listeriosis, it requires an understanding of the burden of the disease on a worldwide scale as food 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; Doijad et al. 2011; Sauders and Wiedmann 2007).

Listeriosis was first recognized as a disease in 1926 during a spontaneous outbreak of infection among laboratory rabbits and guinea pigs in Cambridge characterised 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).

Although Listeriosis was first recognized as a disease of animals and 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 food-borne human disease that promised intense research activity (Low and Donachie 1997).

Human listeriosis is a public health problem of low incidence but high mortality, requiring prompt diagnosis and adequate antibiotic therapy. Antibiotic resistance and inefficient empirical treatment of *Listeria* infections could be responsible for this increased mortality (Rodas-Suárez et al. 2006).

Milk-Borne Listeriosis

The food-borne pathogens in raw milk originate from the farm environment and direct excretion from animals' infected udder and poor silage quality, whereas, in dairy plants the pathogens may enter via contaminated raw milk, colonize the dairy plant environment and consequently contaminate dairy products. Important sources of contamination during the handling and processing might be the workers as well (Bemrah et al. 1998; Kousta et al. 2010), with *Listeria* also being shed in the faeces (Van Kessel et al. 2004). The prolonged excretion of the organism in milk, the

apparently normal appearance of the milk in majority cases and the consumption of raw milk, especially on farms, could be important factors in the transmission and epidemiology of *Listeria* infection. The sources of contamination of *Listeria spp*. in raw milk are probably insufficient hygiene during milking, storage and transport of milk. *L. monocytogenes* may directly contaminate milk as a consequence *of listerial mastitis*, encephalitis or *Listeria*-related abortion in cattle. Rawool et al. (2007) reported overall occurrence of *L. monocytogenes* in 0.55 % of 243 cattle and buffaloes with subclinical mastitis in India. The contamination of food by *L. monocytogenes* occurs along the food chain from farm-to-fork (Farber and Peterkin 1991). 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 (Lundén et al. 2003) also contribute to the persistence of *L. monocytogenes*.

Globally, cases of human listeriosis are on the increase which is evident from the major outbreaks recorded in various countries. The first proof that milk products could be responsible for listeriosis outbreaks was corroborated by Fleming et al.in 1985which involved 49 cases, seven of them in the fetus and 42 in immunocompromised adults. Listeriosis outbreaks have mostly been linked to consumption of raw milk or cheese made of unpasteurized milk (Fleming et al. 1985; Linnan et al. 1988; Lyytikäinen et al. 2000; Rebagliati et al. 2009).

When cattle are infected with *L. monocytogenes*, the organism is excreted in the milk. *L. monocytogenes* is quite resistant to heat and milk's postpasteurisation storage at a refrigeration temperature might allow the selective growth of the remaining organisms (Dalton et al. 1997). Extensive work has been ongoing in many countries during the last decade to prevent outbreaks and decrease the incidence of listeriosis (Rossi et al. 2008).

The occurrence of listeric infections in the Indian subcontinent has been extensively reviewed by Malik et al. (2002). In Indian context, few studies have been carried out to study the incidence of *Listeria* in food. *L. monocytogenes* was isolated from 8.1 % of raw milk samples (Bhilegaonkar et al. 1997). *L. monocytogenes* could not be isolated from pasteurised 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 RTE indigenous milk products (627) and detected *L. monocytogenes* isolates. *L. monocytogenes* has been isolated from cases of mastitis, reproductive disorders and septicaemia in animals (Shakuntala et al. 2006; Rawool et al. 2007).

15.3.1 Symptoms of Listeriosis

L. monocytogenes causes two forms of listeriosis: noninvasive gastrointestinal listeriosis and invasive listeriosis (Allerberger and Wagner 2010). *L. monocytogenes*

infects normally sterile parts of the body such as liver, spleen, cerebro-spinal fluid and blood, and most cases end up being hospitalized (Todd and Notermans 2011). Persons with a predisposed 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 Notermans 2011). Most cases of confirmed listeriosis fall into the ageing category, especially over 65 years old. Case-fatality rates can be high (20–30 %). It is estimated that 10 % of the population in the developed world carry the bacterium in their gastrointestinal tract (Swaminathan and Gerner-Smidt 2007).

15.4 Isolation of *Listeria* Species

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 Notermans 2011). A number of methods and media currently exist for the detection and enumeration of *L. monocytogenes*. For the 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 Food and Drug Administration (FDA) Bacteriological and Analytical Method (BAM), and the International Organization for Standardization (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 United States Department of Agriculture (USDA) and the Association of Analytical Chemists (AOAC) are also used.

15.4.1 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 the inhibition of the growth of background flora by adding inhibitory agents such as lithium chloride, nalidixic acid, acriflavine, cefotetan, ceftazidime, colistin, cycloheximide, fosfomycin and polymyxin B (Gasanov et al. 2005).

A number of media such as polymixin acriflavin lithium chloride ceftazidime aesculin mannitol egg yolk (L-PALCAMY; Van Netten et al. 1989), Lovett (FDA; Lovett et al. 1987), University of Vermont Medium (UVM; 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, followed by transfer of an aliquot 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 AOAC/International Dairy Federation (IDF) methods use a modification of UVM (Donnely 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).

15.4.1.1 Selective or Differential Plating Media

A number of media have been developed which include Oxford agar (Curtis et al. 1989); lithium chloride–ceftazidime agar (LCAM; Lachica 1990); polymixin–acriflavin–lithium chloride–ceftazidime–asculin–mannitol (PALCAM) agar (Van Netten et al. 1989); Dominguez-Rodriguez isolation agar (DRIA; Dominguez-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. 1989) and MVJ modified further (MVJM; Smith and Buchanan 1990). *L. monocytogenes* blood agar (LMBA) is a a very useful tool to detect *L. monocytogenes.*

15.4.1.2 Chromogenic Media

The chromogenic media commercially available include Agar *Listeria* according to Ottaviani and Agosti (ALOA), the BCM *L. monocytogenes* detection system, CHROM agar, and rapid *L. monocytogenes*. Chromogenic media are simple, cost effective and easy to interpret (Gasanov et al. 2005). 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).

15.4.2 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).

Differentiation of *innocua* and *monocytogenes* (DIM), 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 (Kämpfer 1992). This reaction has been modified by using DL-alanine ß-napthylamide (DLABN) as the substrate and has successfully been carried out for identification of *Listeria* within 5 h (Clark and McLaughlin 1997; McLauchlin 1997). However, despite the availability of alternative identification techniques, conventional and haemolytic reactions are most commonly used (McLauchlin 1997).

15.4.3 Detection of Listeria Species

L. monocytogenes and other *Listeria* species closely resemble morphologically and biochemically and the clinical manifestations of listeriosis are nonspecific (Vázquez-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 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 the 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 an ethical point of view and need skilled personnel to perform. Therefore, the application of molecular techniques has facilitated the identification and characterisation of *L. monocytogenes* (Liu 2006). Among the several approaches to nucleic acid amplification, The polymerase chain reaction (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 haemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. Subsequently, the virulencegene cluster in which most of the genetic determinants requiredfor the intracellular life cycle of pathogenic *Listeria* spp. residewas discovered(Vázquez-Boland et al. 2001). The listeriolysin O(LLO)-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 characterised and understood (Churchill et al. 2005). A strong correlation between haemolytic 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 nonfermentation of xylose can be used to identify pathogenic *Listeria* spp. with the exception of *L. seeligeri* which is haemolytic but nonpathogenic (Seeliger and Jones 1986).

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* (Vázquez-Boland et al. 2001). The activity of virulence factor called phosphatidylinositol-specific phospholipase C (PI-PLC), encoded by the *plc*A 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).

A chromogenic medium (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 nonpathogenic *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 of 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 (Liu et al. 2003; Roche et al. 2001). 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 h while nonpathogenic species fail to do so (Terplan and Steinmeyer 1989), and the test has been reported to agree with mouse bioassay (Notermans et al. 1991a). Because of nonspecific 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 *plc*A encoding phosphatidylinositol phospholipase-C (PI-PLC),

*plc*B encoding phosphatidycholine phospholipase-C, *hly*A encoding a haemolysin, *mpl* encoding a metalloprotease and *act*A encoding the surface actin polymerisation protein ActA. All these genes are physically linked in a 9 kb chromosomal island referred to as *Listeria* pathogenicity island-1 (LIPI-1; Vázquez-Boland et al. 2001).

One of the biggest problems associated with the 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), *inl*B (encoding internalin B; Lunge et al. 2002) and 16S rRNA (Call et al. 2003). Among these genes, the *hlyA* gene has been most commonly used (Aznar and Alarcón 2002).

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). An mPCR assay employing four genes, the *hly*A, *plc*A, *iap* and *act*A for the 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 *L. 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 (Rodríguez-Lázaro et al. 2004). 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-min assay based on **m**atrix-**a**ssisted **l**aser **d**esorption/**i**onisation-**t**ime **o**f **f**light (MALDI-TOF) spectroscopy directly from colonies on agar plates has been reported. The method allows not only discrimination between pathogenic and nonpathogenic *Listeria* spp. but also permits resolution up to the level of the PCR serotype analysis (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.

15.5 Subtyping of *Listeria* Species

L. 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 a method depends most on the performance criteria of the 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). Serotyping may potentially be useful for tracking *L. monocytogenes* strains involved in disease outbreaks. Indeed, it has been observed that *L. monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for 98 % of documented human listeriosis cases, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease (Wiedmann et al. 1996; Jacquet et al. 2002). The development of PCR-based serotyping procedures has provided additional tools for the identification and grouping of *L. monocytogenes* (Borucki and Call 2003; Doumith et al. 2004).

15.5.1 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 enzyme-linked immunosorbent assay (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 predominant 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 nontypeable (Rocourt and Catimel 1985).

Multi locus enzyme electrophoresis (MLEE) is a protein-based, isoenzyme typing method that correlates specific protein band patterns with genotypes (Liu 2006).

15.5.2 Genotypic Methods

PFGE is a molecular subtyping method that has been successfully used to characterise *L. monocytogenes* isolates (Kerouanton et al. 2010; Mammina et al. 2009) and is considered to be the gold standard subtyping method because of the documented reproducibility in previous epidemiological studies and its high discriminatory power (Autio et al. 2002; Graves and Swaminathan 2001). 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 epidemiological investigations (Wiedmann 2002); however, it is expensive and not as discriminatory as PFGE (Inglis et al. 2003). 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 labour intensive. Even recently developed rapid protocols take approximately 30 h 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).

The application of PFGE in the characterisation of *L. monocytogenes* isolated from the food can provide a significant insight into the presence of endemic strains and valuable information on potential sites of cross-contamination. Epidemiological tracking of strains over a period of time is required to enable more precise identification of sites of cross-contamination, or critical control points, and to enable to take some measures to avoid the persistence of individual strains within the processing environment.

Several typing methods involving PCR have been developed. The methods employ either just PCR amplification or random amplified polymorphic DNA-PCR (RAPD-PCR) amplification is performed either before polymerase chain reaction-restriction enzyme analysis (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. A total of 30 *L. monocytogenes* isolates from human patients and food 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 *plc*B (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, 2005). 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 %) and 4c (4.4 % and 1⁄2c (6.7 %).

15.6 Studies on *Listeria* spp. from Milk Samples in Goa

15.6.1 Isolation and Characterisation

A total of 767 milk samples from dairy cows were taken at different levels of collection and processing (udder, from milking utensils/cans, dairy cooperative society, receiving dock and market) and were processed for the isolation of *Listeria* as per the USDA method described by McClain and Lee (1988) after making necessary modifications. Double enrichment of the samples was carried out using UVM medium. The enriched inoculum from UVM-2 was streaked on PALCAM agar (Himedia Labs, Mumbai, India). The inoculated plates were incubated at 37 °C for 24–48 h. From the isolation media, suspected colonies of *Listeria* were subcultured on 5 % sheep blood agar. Morphologically typical colonies were verified by Gram's staining, catalase and oxidase reaction, tumbling motility at 25 °C, methyl red-Voges Proskauer (MR-VP) reactions, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl- D-mannopyranoside), hemolysis and Christie-Atkins Munch Petersen (CAMP) test with *S. aureus* and *Rhodococcus equi*.

The grey green colonies with black sunken centres from PALCAM (Fig. 15.1), Gram-positive, coccobacillary forms (Fig. 15.2) with characteristic tumbling motility at 20–25 °C were considered as to be of *Listeria*. Overall, 10.56 % of the samples (81 of 767) were positive for *Listeria* species. The catalase positivity and oxidase negativity was observed in all the 81 isolates. On further testing, 37 isolates produced acid from rhamnose and α-methyl D-mannopyranoside but not from xylose, and therefore were tentatively designated as *L. monocytogenes.*

On streaking of 81 confirmed *Listeria* isolates onto 5 % sheep blood agar, a varying degree of haemolysis was observed (Fig. 15.3) and a total of 38 isolates showed haemolysis. Unlike a typical β-haemolysis with broad and clear zones exhibited by the isolate of *L. ivanovii*, the degree of haemolysis shown by *L. monocytogenes* isolates was moderate.

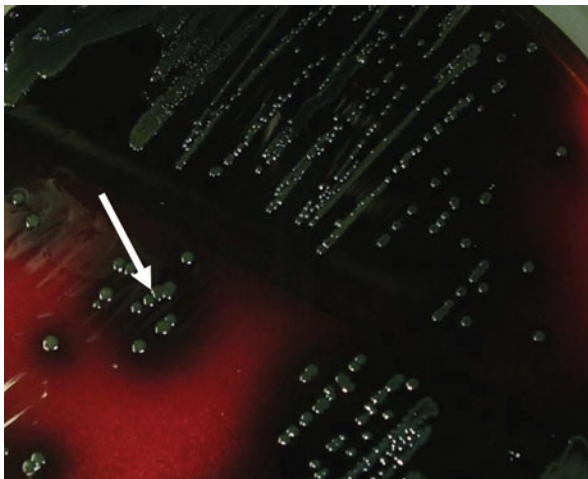

Fig. 15.1 Colonies of *Listeria* species on PALCAM agar

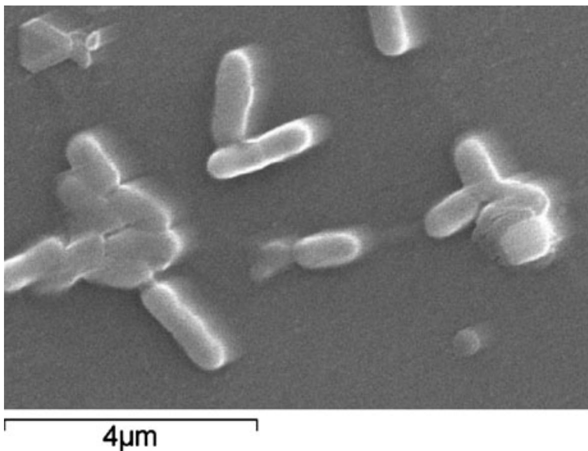

Fig. 15.2 Scanning electron microscopy (*SEM*) of *L. monocytogenes*

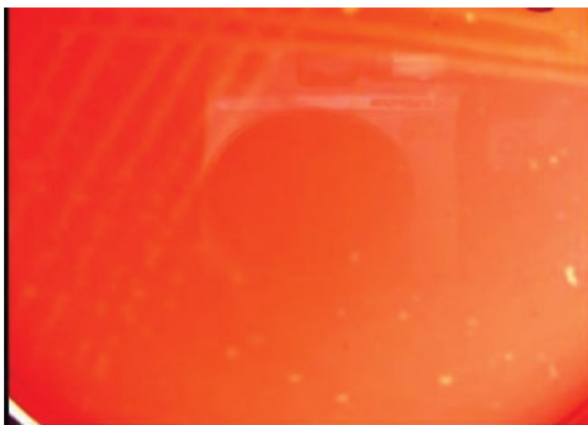

Fig. 15.3 *L. monocytogenes* hemolysis on sheep blood agar

The 81 *Listeria* isolates from milk were then analysed by CAMP test. 37 isolates showed characteristic enhancement of haemolytic zone with *S. aureus* indicating the presence of *L. monocytogenes* while one isolate showed enhanced haemolytic zone typically with *R. equi* confirming the presence of *L. ivanovii* (Fig. 15.4).

Agar *Listeria* according to Ottaviani and Agosti (ALOA) assay, an alternative way to assess phophatidyl inositol phospho lipase C activity (PI-PLC) was carried out using chromogenic ALOA (Himedia, Mumbai, India) medium which helps to differentiate pathogenic *Listeria* spp. From nonpathogenic ones. (Ottaviani et al. 1997). Out of the 81 isolates of *Listeria*, 38 isolates exhibited halo formation on ALOA (Fig. 15.5). The enzymatic activity expressed by *L. monocytogenes* isolates on ALOA agar was reckoned as high (with 8–9 mm zones), moderate (with 5–6 mm zones) and low (with > 4 mm zone) in case of 11, 18 and 8 isolates, respectively. The only isolate of *L. ivanovii* showed a low enzymatic activity.

Among the virulence genes of *Listeria*, hemolysin gene (*hly*A) of *L. monocytogenes* has been used most commonly for confirmation of the isolates (Aznar and Alarcón 2002). All the isolates were subjected to PCR assay for amplification of the *hly*A gene as per the method described by Paziak-Domańska et al. (1999) and Notermans et al. (1991b). Out of 81 *Listeria* isolates, the *hly*A gene was detected in 37 isolates while all the other *Listeria spp*. were negative in PCR analysis.

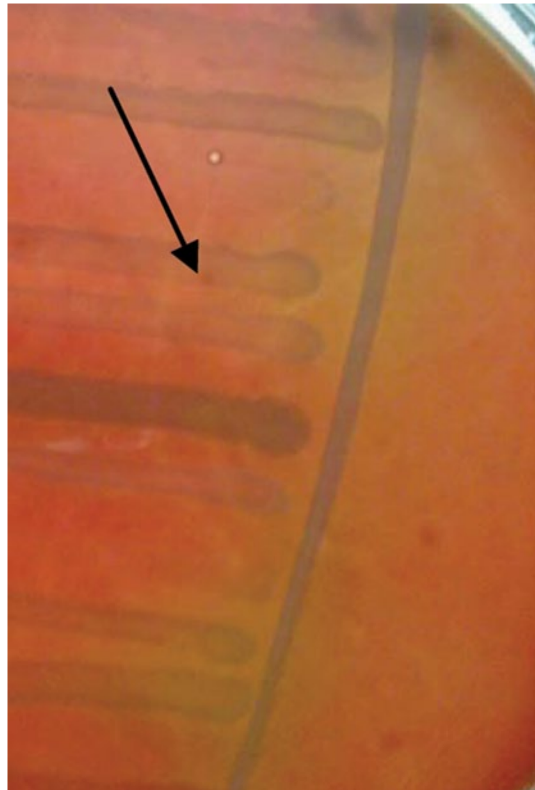

Fig. 15.4 *L. monocytogenes* showing positive CAMP test

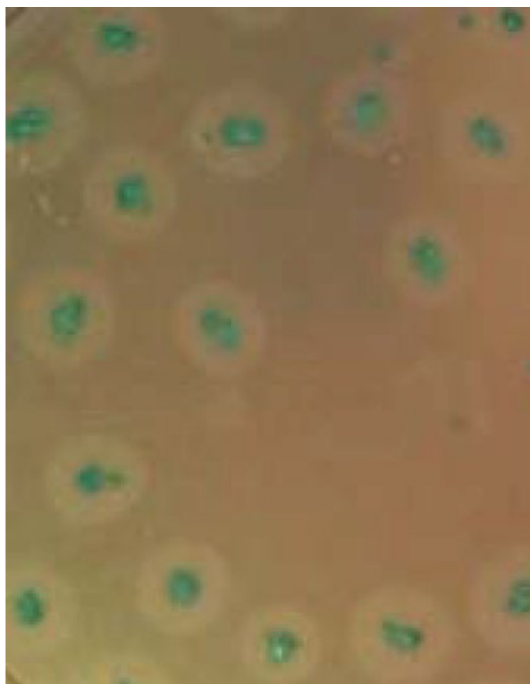

Fig. 15.5 *L. monocytogenes* positive PI–PLC activity on ALOA agar

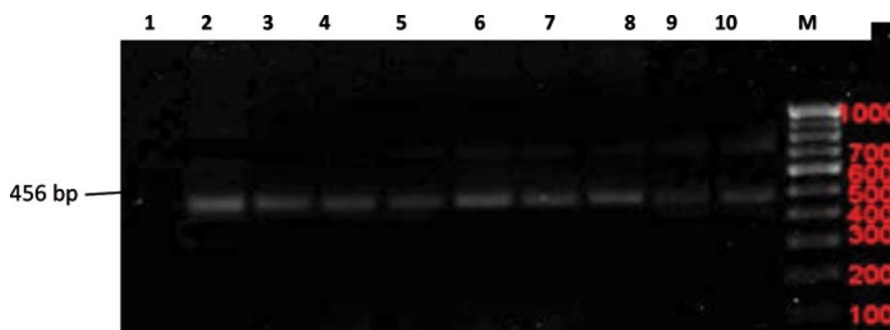

Fig. 15.6 Amplification of the *hly*A gene in *L. monocyotgenes* isolates. *Lane 1*: Negative control; *Lanes 2–9: Listeria monocytogenes* isolates; *Lane 10: Listeria monocytogenes* MTCC 1143

Amplification of the *hlyA* gene of *L. monocytogenes* to its respective 456 bp product represented by a single band in the corresponding region of the DNA marker ladder is as presented in Fig. 15.6.

15.6.2 Serotyping by Multiplex PCR

Typing of *L. monocytogenes* is important in epidemiological studies for investigation of food-borne outbreaks and in the food-processing environment, to identify

the sources of contamination and routes of dissemination. Serotyping by multiplex PCR (mPCR) has been developed which separates the four major *L. monocytogenes* serovars into distinct groups (Doumith et al. 2004, 2005). This assay was employed in the present investigation to serotype *L. monocytogenes* isolates recovered from milk. The genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India) and were subjected to mPCR-based serotyping. The five primer sets for target fragments from genes *lmo0737, lmo1118,* ORF2819, ORF2110 and *prs* were synthesized by Sigma Aldrich, USA (Table 15.1).

Out of 37 *L. monocytogenes* isolates, a larger proportion of isolates (26) belonged to the group corresponding to serovars 1/2a, 1/2c, 3a, and 3c. Serogroup corresponding to serovars 4b, 4d and 4e was detected in two strains while serogroup 1/2b, 3b, 4b, 4d, and 4e was detected in nine strains (D'Costa et al. 2012). The profiles of multiplex PCR serotyping of standard *Listeria* strains and *L. monocytogenes* isolates recovered from milk are as depicted in Fig. 15.7. Our data showed that most of the isolates belonged to 1/2a, which was considered as a sporadic cause for human listeriosis (Liu 2006). Studies have also found that serotype 1⁄2a was the predominant serotype of *L. monocytogenes* food and environmental isolates (Corcoran et al. 2006; Gilbreth et al. 2005; Lukinmaa et al. 2003). Our earlier studies indicated predominance of *L. monocytogenes* serotype 4b in human clinical isolates (Kalekar et al. 2011) and 1/2a in isolates from milk-processing environments (Doijad et al.

Table 15.1 Primer sequences for *L. monocytogenes* used in multiplex PCR serotyping

Target gene	2. Primer sequence	3. Product size (bp)
lmo0737	2. Forward 5′-AGGGCTTCAAGGACTTACCC-3′	3. 691
	Reverse 5′-ACGATTTCTGCTTGCCATTC-3′	
lmo1118	Forward 5′-AGGGGTCTTAAATCCTGGAA-3′	906
	Reverse 5′CGGCTTGTTCGGCATACTTA-3′	
ORF2819	Forward 5′-AGCAAAATGCCAAAACTCGT-3′	471
	Reverse 5′-CATCACTAAAGCCTCCCATTG-3′	
ORF2110	Forward 5′-AGTGGACAATTGATTG-GTGAA-3′	597
	Reverse 5-CATCCATCCCTTACTTTGGAC-3′	
prs	Forward 5′-GCTGAAGAGATTGC-GAAAGAAG-3′	370
	2. Reverse 5′-CAAAGAAACCTTG-GATTTGCGG-3′	

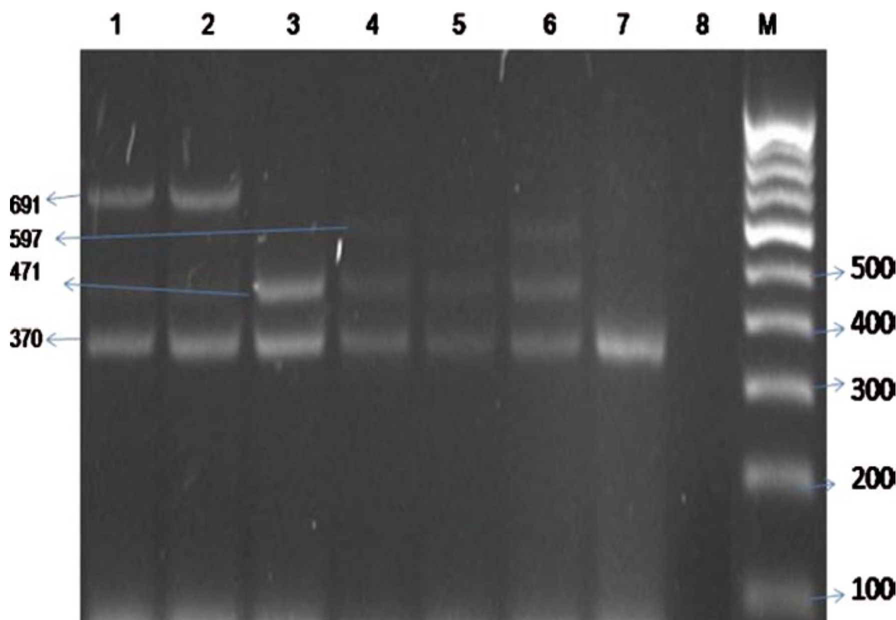

Fig. 15.7 Serotype profile of *Listeria* species by multiplex PCR serotyping. *Lanes 1–5 L. monocytogenes* isolates, *Lane 6 L. monocytogenes,* 4b (NCTC 11994), *Lane 7 L. innocua, Lane 8* Negative control, M – DNA ladder

2011). The observation indicates the potential of milk and milk products to serve as vehicles of transmission of virulent *L. monocytogenes*.

15.6.3 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is considered the gold standard method for subtyping food-borne pathogens, because of its high discriminatory power and reproducibility and is currently used by several public and private laboratories for subtyping and serogrouping *L. monocytogenes*. A total of 36 *Listeria* isolates were subjected to PFGE analysis to cover different sampling areas and different species. PFGE was performed according to the CDC PulseNet standardized procedure (Graves and Swaminathan 2001) used for typing *L. monocytogenes* by using the CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, USA).

PFGE discriminated the *L. monocytogenes* isolates into 5 *Apa*I and 4 *Asc*I PFGE patterns (pulsotypes) at 80 % similarity, but could differentiate serovars within multiplex PCR (MPCR) serogroups, in which isolates from different serovars displaying the same pulsotype were found.

Dendrogram analysis showed that PFGE yielded a good binary division into genetic lineages I (serotypes 1/2b, 3b, 4b, and 4e) and II (serotypes 1/2a, 1/2c, 3a and 3c) a result that is consistent with previous studies (Gilbreth et al. 2005; Nadon et al.

2001) and further confirm that these two lineages represent distinct subgroups. Our data also showed that there was a nearly complete correlation between pulsotypes and serotypes with identical PFGE patterns belonging to the same serotype.

15.7 Conclusions and Future Prospects

Based on the results, it can be concluded that enzymatic assays like CAMP and ALOA tests simultaneously with PCR targeting *hlyA* gene, serotyping by multiplex PCR and PFGE dendrograms can be used for confirming and comparing the pathogenic strains of *L. monocytogenes.*

Farm animals can be asymptomatic or suffer from encephalitis, septicaemia and abortions and thus may be a source of *L. monocytogenes* in the farm environment. Managing the problem of milk-borne listeriosis requires an understanding of the burden of the disease on a worldwide scale as milk that is prone to contamination is consumed widely domestically and traded globally. Surveillance of the disease, caused by *L. monocytogenes*, is typically restricted to developed countries, but many of these do not consider listeriosis as a notifiable disease and estimate the numbers by other means.

Studies on microbiological surveillance of milk and milk products at farm level may help in management and stamping of herds as clean**.** Human illness attribution has been recently recognized as an important tool to better inform food safety decisions. All dairy farmers, suppliers to dairy farmers, milk carriers, dairy product and food manufacturers, distributors and retailers should be part of an integrated food safety and quality assurance management system. Good farming practices underpin the marketing of safe, quality-assured milk-based products. Good dairy-farming practices should contribute to ensuring that milk and milk products are safe and suitable for their intended use.

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