Food Control 22 (2011) 1900–1904

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Incidence and genetic variability of *Listeria* species from three milk processing plants

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ARTICLE INFO

Article history:
Received 1 February 2011
Received in revised form
28 April 2011
Accepted 7 May 2011

Keywords:
Listeria monocytogenes
Milk processing plant
Serovars
PFGE

ABSTRACT

The presence of *Listeria* in three milk processing environments as a potential source of milk contamination was assessed. Swab samples ($n = 210$) taken from milk processing plants were examined. Sample sites included the milk processing equipment, besides areas handling raw and pasteurized milk. The USDA *Listeria*-selective enrichment procedure was used to process the samples. Forty one (19.52%) *Listeria* isolates were recovered. The isolates were further subjected to biochemical and genotypic characterization. Out of 41 isolates, 16 (7.62%) were confirmed as *Listeria monocytogenes*, 2 (0.95%) as *L. ivanovii*, 19 (9.05%) as *L. innocua*. 1 (0.48%) as *L. seeligeri* and 3 (1.43%) as *L. grayi*. All the *L. monocytogenes* isolates were positive for the *hly*A gene. PCR based serotyping revealed all *L. monocytogenes* to be of 1/2a, 1/2c, 3a and 3c serovar group. *Asc*I and *Apa*I restriction analysis yielded four PFGE clusters for 16 *L. monocytogenes* isolates obtained from raw milk collector, milk silos, buttermilk mixer, cheese and other milk product processor. No predominant PFGE cluster was observed among these *L. monocytogenes* isolates. The main sources of *L. monocytogenes* were found to be raw milk collector and milk silos. In the present study *L. monocytogenes* was isolated from milk and milk products processing plants which could cross-contaminate the processed products and may possess a potential threat to public health.

1. Introduction

Listeria monocytogenes is a gram-positive bacterium capable of causing morbidity and mortality in both humans and animals. Due to the ubiquitous nature and hardy growth characteristics of this bacterium, L. monocytogenes is able to contaminate and thrive in the food processing environment (Donnelly, 2001). Earlier, the genus *Listeria* comprised of six species of which *L. monocytogenes* is the species of public health significance (Faber & Peterkin, 1991; Swaminathan, Barrett, Hunter, & Tauxe, 2001). Recently two new species of *Listeria*, *Listeria marthii* (Graves et al., 2010) and *Listeria rocourtiae* (Leclercq et al., 2010) have been described. Listeriosis constitutes a serious threat to elderly, immunocompromised people and pregnant women as a cause of septicemia and abortion (Vazquez-Boland et al., 2001). *Listeria monocytogenes* has been recognized as an important human foodborne pathogen, since manufactured ready to eat foods are being consumed in increasing quantities (Liu, 2006).

Dairy products have been incriminated in several early cases of listeriosis outbreaks. Pasteurized milk (Fleming et al., 1985), chocolate milk (Dalton et al., 1997), Mexican-type cheese (Linnan et al., 1988), the Vacherin Mont d'Or cheese (Bille et al., 2005), and soft cheeses (Goulet et al., 1995) were involved in listeriosis epidemics. Multinational outbreak from dairy product was reported recently by Fretz et al. (2010).

Furthermore, various studies have indicated that certain strains of *L. monocytogenes* survive within the food processing environment (Senczek, Stephan, & Untermann, 2000; Unnerstad et al., 1996). The ability of *L. monocytogenes* to form biofilms (Harvey, Keenan, & Gilmour, 2007) may contribute to its persistence in food processing plants (Thimothe, Nightingale, Gall, Scott, & Wiedmann, 2004).

The milk processing environment and handling practices change may vary among the processing plants. About 4–7% surface of hand of the food handlers and 16% on the processing tables were found to carry *L. monocytogenes* (Kerr, Birkenhead, Seale, Major, & Hawkey, 1993; Jeyasekaran, Karunasagar, & Karunasagar, 1996). Therefore, the chances of cross contamination increase. As per requirements of the US-FDA, *L. monocytogenes* should be absent in ready to eat foods (Fusch & Reilly, 1992).

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doi:10.1016/j.foodcont.2011.05.001

India is the largest producer of milk in the world. In the year 2006-07 the total milk production in India was over 94.6 million tonnes. The milk industry had recorded an annual growth of 4% during the period 1993–2005, which is almost 3 times the average growth rate of the dairy industry in the world. Milk processing in India is around 35%. The organized dairy industry account for 13% of the milk produced, while the rest of the milk is either consumed at farm level, or sold as fresh, non-pasteurized milk through unorganized channels. Some of the states of India produce surplus milk and the manufacturing of milk products is obviously high in these milk surplus States. Exports of dairy products have been growing at the rate of 25% per annum in the terms of quantity and 28% in terms of value since 2001. Significant investment is being poured for the manufacturing of value-added milk products like milk powder, packaged milk, butter, ghee, cheese and ready-to-drink milk products (Anon, 2007). *L. monocytogenes* has been isolated from milk and milk products (Aurora, Prakash, Prakash, Rawool, & Barbuddhe, 2008; Barbuddhe, Chaudhari, & Malik, 2002; Kalorey, Warke, Kurkure, Rawool, & Barbuddhe, 2008). However, in India, no data is available on assessment of the presence of *Listeria* spp. in dairy processing environments. Thus, the objective of the present investigation was to study the occurrence of *Listeria* species in milk processing plants and their genetic variability.

2. Materials and methods

2.1. Samples

A total of 210 swab samples from milk processing environment were taken from three different milk processing plants situated in Goa and Maharashtra, India. The samples were collected after the cleaning and sanitation of the plant as per guidelines of Bureau of Indian Standards, IS 7005:1973 Code of hygienic conditions for production, processing, transportation and distribution of milk. Each of the milk processing plant was visited twice for collection of samples. The plants are situated about 250 km apart and managed by different agencies, and no plant workers are exchanged. Sterile cotton swabs from Hi-culture collection device (Hi-Media Labs, Mumbai, India) were moistened with sterile physiological saline (0.85% NaCl) at the sampling place and approx. 50 cm^2 area was swabbed (Graham, 2004). These swabs were then placed back in the collecting device. All the collecting devices were kept in ice-box, transported to laboratory and processed within 24 h of collection. Details of sample locations of swabs are given in Table 1.

Table 1
Incidence of *Listeria* spp. and *L. monocytogenes* (LM) at all sites sampled.

Sample source	No. of samples	No. of isolates	Lm[a]	Li[b]	Lin[c]	Ls[d]	Lg[e]
Raw milk collector	27	9	7	1	1	0	0
Milk filing machine	33	6	0	0	6	0	0
Milk silo	25	10	4	0	4	0	2
Cheese blender machine	27	5	1	1	2	1	0
Product blender	24	5	1	0	4	0	0
Butter storage vessel	25	2	0	0	2	0	0
Buttermilk mixer	8	3	3	0	0	0	0
Floor	6	1	0	0	0	0	1
Drains	8	0	0	0	0	0	0
Milk can	19	0	0	0	0	0	0
Bulk milk Tanker	8	0	0	0	0	0	0
Total	210	41	16	2	19	1	3

[a] *L. monocytogenes.*
[b] *L. ivanovii.*
[c] *L. innocua.*
[d] *L. seeligeri.*
[e] *L. grayi.*

2.2. Isolation and identification

Isolation of *Listeria* was attempted as per USDA method. The swabs were inoculated into 10 ml University of Vermont Medium-1 (UVM-1) and incubated at 37 °C for 18–24 h (Donnelly & Baigent, 1986). Further enrichment of *Listeria* was carried by inoculating 10 ml of UVM-2 containing high amount of acriflavin with 0.1 ml of UVM-1. Inoculated UVM-2 broth was incubated further for 24 h at 37 °C. A loopful of enriched broth of UVM-2 was streaked directly on polymyxin–acriflavine–lithium chloride–ceftazidime–esculin–mannitol agar (PALCAM) agar for selective isolation of listerial colonies. The inoculated agar plates were incubated at 37 °C for 48 h.

The isolated pinpoint grayish-green colonies surrounded by black zone of esculin hydrolysis were presumed as *Listeria*. These colonies were further purified on PALCAM agar. Isolates were identified for species using different biochemical tests. 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 reaction, tumbling motility at 25 °C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *S. aureus* and *R. equi*, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and a-methyl- D-mannopyranoside), and hemolysis. Isolates were also cultured on ALOA (Himedia Labs, Mumbai) agar to screen for phosphatidyl inositol specific phospholipase C (PI-PLC) activity.

2.3. PCR

The genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India) and were subjected to multiplex-PCR based serotyping (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004) and virulence marker, the *hly*A gene.

The primers used for multiplex-PCR serotyping were synthesized from Sigma Aldrich, USA. The details of primers used in the study are given in Table 2. The multiplex-PCR serotyping was standardized as per the methodology described by Doumith et al. (2004). Fifty microliter reaction mixtures were prepared each containing 2 units Taq DNA Polymerase, 10x PCR Buffer (50 mM Tris–HCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM MgCl2), 300 μM dNTP mix, 2 mM MgCl2, 2 μM each of primer *lmo0737*, *ORF2819*, *ORF2110* and *prs* and 10 μg/ml of DNA template. PCR was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 54 °C for 1 min 15 s, and 72 °C for1 min 15 s, and one final cycle of 72 °C for 10 min in thermocycler. Samples were held at 4 °C until electrophoresis. Eight microliter of PCR product was separated by electrophoresis in 1.5% agarose gel stained by ethidium bromide.

L. monocytogenes isolates were further processed for PCR amplification of the *hly*A gene as per (Noterman et al., 1991). Reaction was set for 25 μl reaction volume and performed by Master Cycler epGradient (Eppendorf, Germany) with a pre-heated lid. The completed reaction mixture was subjected to an initial denaturation at 95 °C for 2 min followed by 35 cycles each of 15 s denaturation at 95 °C, 30 s annealing at 60 °C and 1 min 30 s extension at 72 °C. It was followed by final extension of 10 min at 72 °C and held at 4 °C.

2.4. Pulsed field gel electrophoresis analysis

Twenty seven isolates were selected for PFGE analysis to cover different sampling areas and different species. PFGE was performed according to the CDC PulseNet standardized procedure (Graves & Swaminathan, 2001) used for typing *L. monocytogenes* by using

Table 2
Details of primers used in present study for amplification of virulence and serotype marker in *Listeria* sp.

Gene target	Primer sequences (5′-3′)	Product Size (bp)	Serovar	Specificity
hlyA	For GCAGTTGCAAGCGCTTGGAGTGAA Rev GCAACGTATCCTCCAGAGTGATCG	456		Not applicable
lmo0737	For AGGGCTTCAAGGACTTACCC Rev ACGATTTCTGCTTGCCATTC	619		*L.monocytogenes* serovars 1/2a, 1/2c, 3a, 3c
lmo1118	For AGGGGTCTTAAATCCTGGAA Rev CGGCTTGTTCGGCATACTTA	906		*L.monocytogenes* serovars 1/2c, 3c
ORF2110	For AGTGGACAATTGATTGGTGAA Rev CATCCATCCCTTACTTTGGAC	597		*L.monocytogenes* serovars 4b, 4d, and 4e
ORF2819	For AGCAAAATGCCAAAACTCGT Rev CATCACTAAAGCCTCCCATTG	471		*L.monocytogenes* serovars 1/2b, 3b, 4b, 4d and 4e
prs	For GCTGAAGAGATTGCGAAAGAAG Rev CAAAGAAACCTTGGATTTGCGG	370		All *Listeria spp*

the CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, USA). Sample plugs were digested with 25 U of *Asc*I (Fermentas, USA) at 37 °C for 3 h or 160–200 U of *Apa*I (New England Biolabs) at 30 °C for 5 h. Electrophoresis was performed in a 1% agarose gel (in 0.5X Tris-borate EDTA buffer). The agarose gel was loaded into the electrophoresis chamber containing 2000 ml of 0.5X buffer. The following electrophoresis conditions were used: voltage, 6 V; initial switch time, 4.0 s; final switch time 40 s; runtime 22 h. Lambda ladder (New England Biolabs, Beverly, MA) was loaded on the gel. After electrophoresis, the gel was stained for 30 min in 400 ml of 0.5x TBE containing 25 μl (10 mg/ml) of ethidium bromide and destained by two washes of 20–30 min each using 400 ml of deionized water and photographed with Alpha Imager. The generated PFGE patterns were analyzed using the Gel Compare II (Applied Maths) software. The pattern clustering was performed by the unweighted-pair group algorithm and the Dice correlation coefficient with a tolerance of 1%. The results of the clustering analysis were confirmed by visual comparison of the PFGE profiles. A similarity coefficient of 60% was selected to define the pulsed field type clusters.

3. Results and discussion

The presence of *L. monocytogenes* has been demonstrated in a variety of foods. Raw and processed meats, dairy products, hot dogs, seafood products and fresh vegetables have been linked to both sporadic cases and outbreaks of listeriosis (Kathariou, 2002; Swaminathan & Gerner-Smidt, 2007). Though the foods are treated for elimination of pathogens, chances of post-processing contamination cannot be denied. Presence of pathogenic strains in food processing environment may contribute to post-processing contamination. 210 swab samples collected from milk processing plants, 41 showed the presence of *Listeria* (Table 1). The milk processing plants (A and B) included in the study are ISO 9001 and HACCP certified, while plant C is ISO 9001 certified. Plants A and B process 700 thousand liters of milk per day while Plant C processes about 100 thousand liters milk per day. The milk processing plants (A and B) collect raw buffalo and cow milk from nearby villagers and process for market milk and milk products like UHT Tetra pack milk, pasteurized milk, milk powder, ghee, butter, cheese and other Indian sweets, while plant C process pasteurized milk, cheese, butter, flavored milk and indigenous milk products. The milk processing plants are managed by different personnel. The isolates were further characterized biochemically for species identification. Among 41 strains, 16 (7.61%) were confirmed as *L. monocytogenes*, 2(0.95%) as *L. ivanovii,* 19(9.04%) as *L. innocua*, 1(0.48%) as *L. seeligeri* and 3(1.43%) as *L. grayi.* Thus, 8.57% of samples from milk processing plants were found positive for presence of pathogenic *Listeria* species. *L. monocytogenes* strains have been isolated from plants A and B only. *Listeria ivanovii* was isolated from plant C. Earlier studies reported an incidence of *L. monocytogenes* as 5.1% in raw milk samples (Kalorey et al., 2008) from India. Kells and Gilmour (2004) reported overall incidence of 6.3% for *L. monocytogenes* on equipments. Seven samples from 27 raw milk collection areas, four from 25 milk storing area (silo), one from 27 cheese storing area, one from 24 milk product mixer and three from 8 buttermilk mixer were found to be positive for *L. monocytogenes* (Table 3). More number of samples was positive from plant A than B and C. Higher numbers of *Listeria innocua* strains were isolated. *L. innocua* is used as an indicator for *L. monocytogenes*. The presence of either organism is regarded significant (Klauser & Donnelly, 1991).

Sixteen *L. monocytogenes* and two *L. ivanovii* isolates showed hemolytic activity on sheep blood agar and PI-PLC activity on ALOA agar. All the isolates were subjected to PCR detection for presence of the *hlyA* gene. All *L. monocytogenes* and *L. ivanovii* isolates were positive for the *hly A*gene (Table 3).

Serotyping of *L. monocytogenes* is important in epidemiological studies for investigation of foodborne outbreaks (comparison of clinical and food isolates) and, in the food-processing environment, to identify the source of contamination. Isolates identified as *L. monocytogenes* and found to contain virulent genes were further subjected for serotyping by PCR amplification of *lmo0737, lmo1118, ORF2819* and *prs* genes. Serotyping of sixteen isolates of *L. monocytogenes* by PCR revealed all the 16 isolates to be 1/2a, 1/2c, 3a and 3c serovar group. Earlier, all *L. monocytogenes* isolates

Table 3
Isolates identified as *L.monocytogenes* showing presence of hlyA and serotype.

Isolate	Sample Area	Spp. identified	*hlyA*	Serovar group
WS3	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS4	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS7	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS9	Milk silo	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS10	Milk silo	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS12	Milk silo	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
WS44	Milk silo	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS77	Cheese blender machine	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS85a	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GK85b	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GK89a1	Product blender machine	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS90	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS96	Raw milk collector	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS135	Buttermilk processor	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS136	Buttermilk processor	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c
GKS137	Buttermilk processsor	*L.monocytogenes*	+	1/2a, 1/2c, 3a and 3c

from milk processing environment from Brazil were found to be 1/2a serotype (Brito et al., 2008). Whereas an equal frequency of genomic types was reported for serotypes 1/2b or 3b and 1/2a or 3a by Chambel et al. (2007) from Portugal dairy. The observation indicated the potential of milk and milk products to serve as vehicles of transmission of virulent *L. monocytogenes*.

PFGE analysis using *Asc*I and *Apa*I restriction enzymes yielded four clusters for 16 *L. monocytogenes* isolates obtained from raw milk collector, milk silo, buttermilk mixer, cheese and milk product blender.Presence of *L. monocytogenes* in equipments like buttermilk mixer, cheese, milk product blender and pasteurized milk silos from which ready to eat milk products prepared may contaminate the finished product. No predominant PFGE type was observed among these *L. monocytogenes* isolates. Isolates from plant A and B clustered separately, however, the isolates from the same plant showed congruence suggesting the presence of a persistent source of *L. monocytogenes*. Latorre et al. (2010) observed 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment, parlor, bulk tank milk, and milk filters with combined *Asc*I and *Apa*I restriction analysis. A predominant and persistent PFGE type was observed among these *L. monocytogenes* isolates (9/15 isolates). *L. monocytogenes* strains from plants A and B branched separately indicating diversity.

Listeria spp. can survive in aerosols, especially when protected by milk solids, and enable them to be transferred from reservoirs within the processing environment to the finished product (Spurlock & Zottola, 1991). Aerosols are produced by the use of high powered hoses during cleaning, by workers and ventilation systems. These contaminated aerosols circulating within a food processing environment may be a potent source of contamination of finished product with spoilage and pathogenic organisms (Kozak, Balmer, Bryne, & Fisher, 1996; Spurlock & Zottola, 1991).

The focus of this study was to determine the incidence of *L. monocytogenes* within these environments. Certain milk products like cheese, Indian sweets etc. get processed and packaged, during these operations chances of environmental contamination may occur if proper precautions are not taken. The areas within considered to be most likely contaminated were sampled. The strains isolated were then subtyped in order to determine persistence of strains.

Fretz et al. (2010) reported multinational outbreak from 1/2a serotype from milk product. The organism is present ubiquitously, thus food in the processing environment get easily contaminated. *L. monocytogenes* may then persist in these industries because its ability to grow at low temperature, adhere various surfaces and possible adaptation to disinfectants (Salvat, Toquin, Michel, & Colin, 1995). The contamination of a product with *L. monocytogenes* is not acceptable in industry and thus leads to heavy loss. The incidence of *L. monocytogenes* in milk and milk product may have serious impact on its processing as it tends to contaminate the processing table as well as sanitized surfaces of food processing. Personal hygiene practices together with sanitary conditions of food contact surfaces and handling areas should reduce the potential contamination with *L. monocytogenes* at the retail level.

L. monocytogenes will be killed by boiling of milk. But in India, many milk products prepared and consumed without any further process. Also, numbers of products are prepared home-made from milk without boiling.

Genetic characterizationof L. monocytogenes isolates can differentiate single-source clusters of foodborne infection and contribute to the outbreak identification. The high case fatality ratio of listeriosis makes a strong case for the importance and priority of improved surveillance in India. Presence of *Listeria* in raw milk could also pose a risk of contamination for a milk processing plant. The presence of *L. monocytogenes* in a processing plant could lead to post-processing contamination, which also draws attention to the need to reduce the level of contamination of milk that will eventually be transported to a milk processing plant. The present study indicated the incidence of *L. monocytogenes* in milk and milk products processing plants which may possess a potential threat to public health.

Acknowledgments

The authors are thankful to Director, ICAR Research Complex for Goa, Ela, Old Goa, for providing necessary facilities to carry out part of work. Part of work is supported by grants from Department of Biotechnology, Government of India (BT/IN/FRG/01/SBB/2008) to SBB.

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