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Pervasiveness of *Listeria monocytogenes* in Milk and Dairy Products

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Abstract

The study was carried out in milk and dairy products from Tiruchirappalli city, Tamil Nadu, India, to determine the incidence of *Listeria monocytogenes*. A total of 415 milk and dairy products were studied and *L. monocytogenes* were isolated from 219 (52.7%) samples. Among the positive samples, the raw milk and flavored milk were 100% contaminated by *L. monocytogenes* followed by branded milk (65.9%), cheese (62.5%), ice-cream (49.2%), milk powder (26.6%), milk sweets (20%), ghee and paneer (13.3%) and yoghurt (6.6%). Conversely, curd and butter were free from *L. monocytogenes*. In this study, the 23 isolates were identified through biochemical test, among the 23 isolates only 14 isolates were further confirmed by the detection of phosphatidylinositol phospholipase C gene (plcA) of *L. monocytogenes* through polymerase chain reaction. From the results concluded the milk and dairy products were more vulnerable for *L. monocytogenes* in Tiruchirappalli city would create awareness among people especially elderly people and children about the pathogen.

Keywords: *L. monocytogenes*; Food safety; Milk; Dairy products

Introduction

Majority of the food sickness are related to the microbial contamination, which is caused by improper food-handling and food processing. The illness is more pronounced in developing regions than the developed counterpart; poverty, illiteracy, unhygienic habits, poor environmental sanitation, and lack of infrastructure could be the leading factors for such contamination, and these are common in economically backward areas [1]. Listeriosis is serious health impairment, caused by *Listeria monocytogenes*, developed due to consumption of contaminated foodstuffs. The infection is manifested in the form of chillness, mild to heavy fever, vomiting, diarrhea, and death. Immune suppressed pregnant women, elderly people, patients underwent organ transplantation, people with HIV/AIDS or autoimmune disease, cancer, chronic kidney disease, liver disease and diabetes are vulnerable for listeriosis. Death rate among the infected individuals is 20-30% (www.foodsafety.gov).

Listeria monocytogenes is a gram positive, intracellular non spore forming, motile, rod-shaped, facultative anaerobic bacterium, found in all segments of the environment. Human body acquires *L. monocytogenes* through many routes, via environment, raw food, unhygienic food handlers, improper food heating, keeping foods more than prescribed time in the refrigerators, bringing food to both refrigerated and normal temperature very frequently [2].

Its ability to grow under adverse conditions, challenges of food industries. Compared to raw foods, Ready-To-Eat foods (Foods available for immediate human consumption (RTEs)) are more vulnerable [3] as they are in preprocessed form, free from other competing organism. Re-heating the food kept under cold temperature sometimes, fails to eliminate the pathogens survive in the cold packets, left during re-heating and cause infection. The U.S has announced 'zero tolerance" policy for *L. monocytogenes* in ready-to-eat (RTE) foods, as it can grow at temperatures as low as 1°C [4].

Reports of listeriosis in countries of South East Asia are scarce, either because of failure to detect, failure to report, or low incidence rate or failure to consider listeriosis for differential diagnosis by clinicians. The disease remains largely undiagnosed and under-reported. However, Listeria monocytogenes has been found to be one of the etiological factors in causing spontaneous abortions and premature births in India.

In India the milk and dairy products are largely consumed by risk groups (immune suppressed people), which are vulnerable to *L. monocytogenes*. There is no accessible adequate data available in India, especially Tamil Nadu on the risk posed by dairy products. Therefore there is no objective picture of the situation and presence of this bacterium in domestic products, for that reason the present study was carried out from milk and dairy products being contaminated by *L. monocytogenes* would be of immense important in minimizing such incidents and safeguarding the life of innocent groups whose lives are already under risk.

Materials and Methods

Bacteria

The standard strains of *L. monocytogenes* 4b (MTCC1143) used in this study were obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

Sample collection

Total of 415 milk and milk products were randomly collected from different street vendors, super markets, retail shops and petty-shops in and around the city and were studied for the presence of *L. monocytogenes*. The milk samples included: raw milk (n=35), pasteurized (branded) milk (n=144) and flavored milk (n=30). The dairy products included: butter (n=24), cheese (n=24), ghee (n=15), curd (n=15), yogurt (n=15), A pannier (n=15), ice-creams (n=65), milk powder (n=15) and milk sweets (n=18). All these milk and dairy products were collected and transferred in ice boxes (4°C) and investigated immediately after arrival at the laboratory.

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Isolation and identification of *L. monocytogenes*

After sampling the milk and dairy products were pretreated in the buffered peptone water; by allowing 25 gr or 25 ml portions of the sample in 225 ml of buffered peptone-water for 1 h at room temperature (37°C). After 1 h standing period 1 ml of the enrichment buffer was pipetted out into a sterile Petri-plate in duplicates and the recovered *L. monocytogenes* were cultured on, PALCAM-agar (HIMEDIA) medium as per the NF EN ISO 11290-2 procedure [5]. The plates were incubated at 37°C for 48 h. Grey colored Colonies with black hallo zone were counted and isolated in Tripticase Soy-Agar with Yeast extract (TSAY) (HIMEDIA) and identified through Gram-staining, Cell-motility, Oxidase, Catalase, Indole, Methyl-red, Voges-Proskauer tests, Urease-production, Simon citrate agar, and various sugar-fermentation tests (mannitol, rahmnose, and xylose). Colonies of *L. monocytogenes* were further confirmed by β-hemolytic activity and CAMP-tests as per the Bergey' Manual of Systematic Bacteriology [6].

Confirmation of *L. monocytogenes* by PCR

Isolation of genomic DNA

The confirmed *L. monocytogenes* strains and also the standard strain of *L. monocytogenes* (MTCC 1143) were grown overnight in brain heart infusion plates at 37°C. The culture were taken into sterile toothpick and then centrifuged at 10000 xg for 5 min. The recovered pellet was resuspended in 100sss of sterilized DNAse and RNAse-free milliQ water, heated in a boiling water bath for 15 min and then snap chilled in crushed ice [7].

PCR amplification of *plcA* gene

Gradient Thermo cycler with a pre-heated lid is used. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 min followed by 35 cycles each of 15 s denaturation at 95°C, 30s annealing at 60°C and 1 min 30s extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The resultant PCR product was further analyzed by agarose gel electrophoresis (1.5%); low melting temperature in this study was synthesized from Shrimpex-Biotech, Chennai. The primer sequences used are given in Table 1. PCR was standardized for the detection of virulence associated gene namely plc A of *L. monocytogenes* as per the method described by Notermans et al. [8]. After isolation genomic DNA, the obtained lysate (2 µl) was used as a DNA template in the PCR reaction mixture. The standardized PCR protocol for 50 µl reaction mixture included 5.0 µl of 10X PCR buffer (100 mM Tris–HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 0.2 mM dNTP mix, 2 mM $MgCl_2$ and 0.1 µM of a primer set containing forward and reverse primers, 1.25 units of Taq DNA polymerase, 5.0 µl of cell lysate and sterilized milliQ The primer for the detection of phosphatidylinositol phospholipase C gene (plcA) of *L. monocytogenes* used water to make up the reaction volume. The DNA amplification reaction was performed in a Master Cycler agarose and stained with ethidium bromide (0.5 µg/ml) and visualized by a UV transilluminator.

Results

Prevalence of *L. monocytogenes* in milk and milk products

To test the quality of milk and milk products sold in the city, in terms of the absence of *L. monocytogenes*, 12 different products were selected based on the background information. The products included were, Raw milk (Unpasteurized milk) (n=35), Pasteurized milk (n=144), Milk powder (n=15), Flavored milk (n=30), Curd (n=15), Butter (n=24), Ghee (n=15), Cheese (n=24), Yoghurt (n=15), Paneer (n=15), Ice-cream (n=65), and Milk sweets (n=18). The results of each food items are presented (Table 2).

Target gene	Primer sequence	Product size (bp)	Reference
Plc A	Forward5'-CTGCTTGAGCGTTCATGTCTCATCCCCC-3' Reverse'–CATGGGTTTCACTCTCCTTCTAC-3'	1484	Notermans et al. [8]

Table 1: Primer sequences of the virulence genes of *L. monocytogenes*.

The results obtained for raw milks, collected from 35 locations showed that local milks were highly (100%) contaminated. The cfu value of those milks was ranging from 10 to 155/ml of the sample. The 17 brands of the pasteurized milks, comprised of standardized milk (n=51), toned milk (n=48), double toned milk (n=24) and full cream milk (n=21) were tested for *L .monocytogenes*. The test has revealed that, the standardized milk, were highly contaminated (82.3%) followed by toned milk (69%), full cream milk (42.8%) and double toned milk (41.6%). The overall % of *L. monocytogenes*, observed in the milk powder of the selected brands (B1-B5) was 26.6%. The average number of colony forming units in the above five brands was between 1 and 5 cfu. ml^{-1}. Of them, B1, B2 and B4 were positive for *Listeria monocytogenes*, whereas B3 and B5 were completely free from the contamination.

Test results of the six different flavored-milk brands revealed that all brands were highly (100%) contaminated. The overall pathogenic load was 3-44 cfu.ml^{-1}. Maximum contamination was noticed in the badam milk (22-44 cfu.ml^{-1}) followed by rose milk, whereas it was minimum in cardamom and pista. The results of curd and butter revealed that all the samples were free from *L. monocytogenes*. Results of ghee samples showed that 13.3% of 15 samples were contaminated with *L. monocytogenes* and overall load of pathogen were ranging from 3-4 cfu.ml^{-1}. Out of the 5 popular brands studied, two brands were positive for *L. monocytogenes* whereas in three brands viz., B3, B4 and B5 it was completely absent.

The reults of *L. monocytogenes* observed in eight different cheese varieties revealed that 62.5% of the 24 samples were contaminated by the pathogen. The contamination was ranging from 1 to 15 cfu. ml^{-1}. All the samples of brand B1, B3 and B5 gave positive (100%) for *L. monocytogenes*; in B2 and B8, it was 66.6%. Whereas in one brand (B7) *L. monocytogenes* was completely absent. In yoghurt, out of 5 brands tested, *L. monocytogenes* was detected in one brand (B2) only and the pathogen load was 5 cfu.ml^{-1}. In paneer, out of 5 popular brands, two brands gave positive result and the average load of the pathogen was ranging between 2 and 4 cfu.ml^{-1}. Brands B1, B2 and B5 were free from *L. monocytogenes*. The results of ice-cream showed that out of 13 brands tested, 11 brands contained *L. monocytogenes*. Brands B1, B4, B9 and L1 were 100% contaminated. Whereas B2, B7 and B10 were free from *L. monocytogenes* contamination. The overall load of the pathogen was 1-42 cfu.ml^{-1}. Sweets were tested for *L. monocytogenes*, and the results revealed that 16.6% of the 18 samples were contaminated. Of the 6 sweet types, 66.6% of rasagulla and 33.3 percent of gulabjamun, alone were contaminated and the rest were free from *L. monocytogenes*. Overall, the pathogen load was less (4 -10 cfu.ml^{-1}) in the sweets.

Polymerase chain reaction (PCR)

In this study 23 typical isolates were identified, ten from milk samples and 13 from milk products. These typical isolates were biochemically confirmed by *L. monocytogenes*. Hence, the biochemically confirmed isolates were subjected to PCR. Out of 23 isolates 14 were positive reaction and nine isolates were negative reaction. The amplification product 1484bp was seen for *L. monocytogenes* specific reaction with primer *plcA* (Figure 1). These results showed that PCR is important in

S. No	Milk and milk products	No. of samples	No. of positive	CFU.ml^{-1}/ (%)
1	Unpasteurized milk	35	35	10-155 (100)
2	Pasteurized milk			
	Standardized milk	51	42	1-63 (82.3)
	Toned milk	48	33	1-28 (69)
	Double toned milk	24	10	1-19 (41.6)
	Full cream	21	9	1-5 (42.8)
	Total	144	94	1-63 (65.2)
3	Milk powder			
	B1	3	1	2 (33.3)
	B2	3	1	2 (33.3)
	B3	3	0	0 (0)
	B4	3	2	1-5 (66.6)
	B5	3	0	0 (0)
	Total	15	4	1-5 (26.6)
4	Flavored milk			
	Rose milk	5	5	16-27 (100)
	Badam milk	5	5	22-44 (100)
	Pista milk	5	5	4-10 (100)
	Chocolate milk	5	5	4-12 (100)
	Strawberry milk	5	5	8-13 (100)
	Cardamoms milk	5	5	3-11 (100)
	Total	30	30	3-44 (100)
5	Curd			
	B1	3	0	0
	B2	3	0	0
	B3	3	0	0
	B4	3	0	0
	B5	3	0	0
	Total	15	0	0
6	Butter			
	B1	3	0	0
	B2	3	0	0
	B3	3	0	0
	B4	3	0	0
	B5	3	0	0
	B6	3	0	0
	B7	3	0	0
	B8	3	0	0
	Total	24	0	0
7	Ghee			
	B1	3	1	4 (33.3)
	B2	3	1	3 (33.3)
	B3	3	0	0 (0)
	B4	3	0	0 (0)
	B5	3	0	0 (0)
	Total	15	2	3-4 (13.3)
8	Cheese			
	B1	3	3	7-15 (100)
	B2	3	2	3-5 (66.6)
	B3	3	3	6-9 (100)
	B4	3	1	3 (33.3)
	B5	3	3	1-11 (100)
	B6	3	1	3 (33.3)
	B7	3	0	0 (0)
	B8	3	2	1-2 (66.6)
	Total	24	15	1-15 (62.5)
9	Yoghurt			
	B1	3	0	0 (0)
	B2	3	1	5 (33.3)
	B3	3	0	0 (0)
	B4	3	0	0 (0)
	B5	3	0	0 (0)
	Total	15	1	5 (6.6)
10	Pannier			
	B1	3	0	0 (0)
	B2	3	0	0 (0)
	B3	3	1	4 (33.3)
	B4	3	1	2 (33.3)
	B5	3	0	0 (0)
	Total	15	2	2-4 (13.3)
11	Ice-creams			
	B1	5	5	2-26 (100)
	B2	5	0	0 (0)
	B3	5	2	7-20 (40)
	B4	5	5	4-31 (100)
	B5	5	2	1-3 (40)
	B6	5	1	4 (20)
	B7	5	0	0 (0)
	B8	5	2	1-5 (40)
	B9	5	5	1-10 (100)
	B10	5	0	0 (0)
	LB1	5	5	6-42 (100)
	LB2	5	3	9-13 (60)
	LB3	5	2	4 (40)
	Total	65	32	1-42 (49.23)
12	Milk sweets			
	Gulab jamun	3	1	4 (33.3)
	Dairy milk	3	0	0 (0)
	Rasagulla	3	2	4-10 (66.6)
	Milky mist	3	0	0 (0)
	Palkova	3	0	0 (0)
	Milky bar	3	0	0 (0)
	Total	18	3	4-10 (16.6)
Total		415	219	1-155 (52.7)

Table 2: Primer sequences of the virulence genes of *L. monocytogenes*.

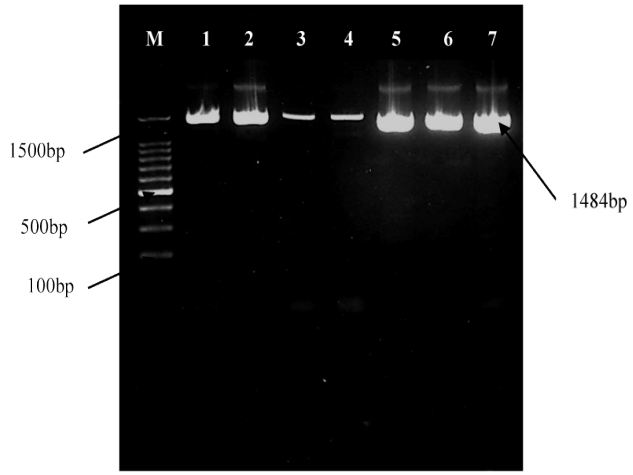

Figure 1: M-1000 bp ladder; Lane 1: MTCC 1143; Lane 2 to 7 isolates from milk and dairy products.

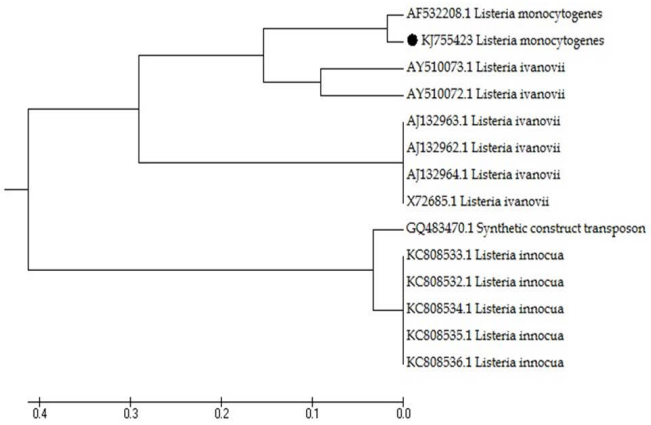

Figure 2: Phylogenetic trees of *L. monocytogenes* KJ755423.

confirming the identity of biochemically strains of *L. monocytogenes*, which otherwise would have been misidentified as *L. monocytogenes*. The *plcA* gene of *L. monocytogenes* isolated from raw milk strain was sequencing and submitted to NCBI and got the accession number KJ755423 (Figure 2).

Discussion

Ten of the, twelve milk and milk products tested contained the pathogen *L. monocytogenes***.** Whereas in curd (n=15) and butter (n=24) it was totally absent. When the test result was compared, in terms of the sample size alone, of the 415 samples that belong to 12 different food items falling under the category of milk and milk products studied, 52.7% of the samples carried *L. monocytogenes*; when the food items were compared based on the number of Colony Forming Units (CFU. ml^{-1}), unpasteurized milk (10-155) followed by branded milk (1-63) encountered maximum population load. The 100% contamination found in unpasteurized milk, could be due to several factors. As per the literatures, health of the milch animal, materials adhered over the animals' udder, cleanliness of milking vessels, milk-man, sanitation of dairy unit and cowshed are the leading factor for such contamination [9,10].

A Study conducted at Coimbatore, Tamil Nadu, India reported that, branded milks are more prone to *L. monocytogenes* (53%) than the local milk [11]. In contrast, Dhanashree et al. [12] have reported that branded milks, which are sold in the Dairy/ Milk depots are free from *L. monocytogenes***.**

The reason for the contamination observed in all the six types of flavored milks and ice-creams could be due to the poor quality milk used or use of raw milks in the preparation of such milks. It was reported by the shop owners during the interview schedule that such milks are widely used for ice-creams, milk shakes and flavored milks as they are cheap. In addition, cross contamination during the preparations via added flavoring additives, could have caused the contamination.

Though *L. monocytogenes* has been reported in the curd, by several workers, present study could not find any similar observation. The reason could be due to the nature of the curd produced in the state. If well boiled milk is used for curdling, chances for curd being contaminated by Listeria is less. Similarly, well finished curd will be sour in taste; this is due to the production of lactic acid from the sugar lactose, by Lactobacillus-spp. Most of the Lactobacillus spp. are probiotics and suppress many harmful pathogens. Arquez et al. [13] and Vermeulen et al. [14] have reported that the production of antagonistic compounds by the lactic acid bacteria reduces the *L. monocytogenes* in yoghurt.

Result of enumeration of *L. monocytogenes* in eight different brands of butter revealed, butter samples are free from contamination. Complete absence of the pathogen in the sample indicates that butter can be consumed by risk groups (especially children and pregnant women). Further work is needed in future that what ingredient of butter prevents growth of *Listeria monocytogenes***.**

According to Indian standard the presence of *L. monocytogenes* in 1g or ml of milk and milk products are not acceptable except cheese (25 g) [15]. In this study, 52.7 % of the analyzed samples showed impropriety with Indian food safety regulations. In India, the incidence of *L. monocytogenes* in milk and milk products has been reported in few studies by researchers. A study conducted by Bhilegaonkar et al. [16] 8.1% of the raw milk samples were contain *L. monocytogenes***.** Burbuddhe et al. [17] 1.56% of goats milk and 6.25% of buffalos milk samples were contain *L. monocytogenes*. Similarly Kalorey et al. [18] isolated *L. monocytogenes* in 5.1% of cow milk samples. Aurora et al. has reported 18 isolates of *L. monocytogenes* were present in milk (8 isolates) and milk products (10 isolates). In this study 23 isolates were identified, ten from milk samples and 13 from milk products.

Conclusion

Milk and milk products sold in the city are not up to the satisfactory level, particularly the local milks, and flavored milks. Milk products are to be consumed only from the hygienically sound food shops. Regarding listeriosis-vulnerability, children and old age people in the city are at risk. Shops selling milk and milk products should improve their standard to maintain the food they are selling. Government can look into the above aspects, and should periodically check the shops concerned for ensuring food quality. Additionally cold storage facility and alternate power supply should be made mandatory for shops selling, milk based food products.

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