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Enumeration and Isolation of Listeria species from Dairy Environmental Samples

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ABSTRACT: In the present study, listeria in dairy environmental samples were enumerated and isolated in order to find the source of its entry into milk. Based on that soil, dung, fodder and feed considered under solid dairy environmental samples collected from Livestock Farm Complex, Veterinary college, KVAFSU, Bengaluru showed listeria counts ranging from 2.49 to 4.56 log_{10}cfu/g when serially diluted and PALCAM agar was used as selective medium. Soil had listeria count of 79% followed by fodder of 60%, dung of 52% and feed of 51% when compared with total bacterial count. The liquid non-milk dairy environmental samples like swab of udder, swab of handler, can rinse, pail rinse, water, urine and air exhibited 0.00 to 1.53 log_{10}cfu/ml of listeria. Swab of udder had more listeria count compared to other samples, while air of milking parlour did not show the presence of listeria. Among liquid milk dairy environmental samples like aseptic milk, pail milk, can milk, chilled milk and pasteurized milk, listeria count ranged from 0.00 to 1.89 log_{10}cfu/ml. Aseptic milk, pail milk and pasteurized milk samples did not reveal the presence of listeria while can milk and chilled milk samples had listeria. The presence of listeria in raw milk may be introduced through soil, dung, fodder, feed, udder swab, urine that showed the presence of listeria. A total of eighteen listeria isolates were obtained that was inclusive of three isolates each from soil, dung, chilled milk followed by two isolates from fodder; feed; swab of udder; can milk and one isolate from cow urine sample.

Keywords: PALCAM, Listeria, Swab, Isolates, Milking Parlour.

INTRODUCTION

Bacteria are microscopic, single celled living organisms and can be grouped on their role as beneficial, defect causing and pathogens. Pathogenic bacteria cause diseases in plants, animals and humans by producing their metabolites and toxins. The word pathogen is derived from a Greek word in which “Pathos” means disease and “Gen” means born of, means they are born to cause the disease, the term pathogen came into use in the 1880s (Vouga and Greub 2016). There are four levels in the occurrence of disease like exposure, entry through food, air, water and carriers, incubation and disease transmission by the etiological agent. The pathogens emerging recently from an inferior condition and now causing diseases which are of major public health threat are termed as “Emerging Pathogens”. The diseases caused by emerging pathogens are called as Emerging Infectious Diseases. (NIAID, 2018). National Institute of Allergy and Infectious Diseases classified emerging pathogens based on the mortality into Category A, B and C priority pathogens. Listeria is one of the emerging pathogen belonging to B category which is transmitted through food, contaminated water and cause the disease called as listeriosis. Species of Listeria considered as food borne pathogens such as Listeria monocytogenes, *Listeria ivanovii* and *Listeria seeligeri* cause listeriosis, a lethal disease in humans and animals. Listeria spp. widely distributed in nature are psychrophilic to psychrotrophic in nature, occurring in vegetation, sewage, human, animal carriers and infect mainly warm-blooded ruminants as well human beings, causing huge economic loss. Listeria was named after Lord Lister, English surgeon and pioneer of antisepsis. They are regular, short rods having 0.4 – 0.5 × 1–2µm with parallel sides and blunt ends, usually occurred singly or in short chains. These bacteria are Gram-positive with even staining, non acid-fast, non-capsuled, non- spore formers, catalase-positive and oxidase-negative. The pathogen produces toxin called Listeriolysin O (LLO) which is mainly responsible for the cause of disease coded by the gene hly A (Chen *et al.*, 2017).

Listeriosis has emerged as the typical foodborne disease of major public health concern that predominantly affect pregnant women, neonates, elderly or immunocompromised people. It manifests as abortion, septicaemia, meningitis and meningoencephalitis and potentially life threatening because of the mortality rate (20 – 30 per cent) and hospitalization (91 per cent) following infection. The epidemiological data on listeriosis in India available to date, are not adequate for assessing the extent of disease. The disease largely remains undiagnosed because of the lack of a suitable and rapid detection test. Human infections with listeria arise mainly from the consumption of contaminated

food like milk, icecream, contaminated water, meat products, ready to eat foods and so on (de Noordhout *et al*., 2014).

According to Gezali *et al.* (2016), listeria was found to survive for 13 years in milk, 16 years in a brain sample, 12 years in faeces and 12 years in silage. It was reported to persist for 2 years in dry soil, 11.5 months in damp soil, 2 years in dry faeces, 3 months in sheep faeces, 16.5 months in cattle faeces and up to 7 months on dry straw. Listeria could be a common contaminant in the dairy environment, both on the farm and in the processing plant. On the farm, important sources include manure and improperly fermented silage. It was most frequently found in moist environments or areas with condensed or standing water or milk, including drains, floors, coolers, conveyors and case washing areas (Usman *et al*., 2016). Shamloo *et al.* (2014) collected 292 samples of raw milk and traditional dairy products from Isfahan, Iran and analysed using the method recommended by the United States Department of Agriculture (USDA) by pre-enrichment and streaking onto PALCAM agar and the appearance of black colonies with black sunken centers were considered as listeria colonies. The prevalence of Listeria spp. in raw milk, icecream, cream and ferni (4pudding) samples were 5.91 (5.49%), 12.63 (19.04%), 3.27 (11.11%) and 1.25 (4%), respectively. Listeria was not detected in yogurt, butter, Kashk and cheese samples. Nucera *et al.* (2016) detected Listeria spp. in 22 bales of silage (27.5 per cent) out of 80 collected from 20 dairy farms in Italy by enriching the samples and streaking onto selective PALCAM agar. Among 415 milk and milk product samples, 219 (52.7%) showed the presence of *L. monocytogenes by following standard method of* ISO 11290 where in *pre enrichment and streaking was followed on* PALCAM-agar medium. Samples of raw milk and flavoured milk were 100% contaminated by *L. monocytogenes* (grey colonies with black hollow colonies) followed by branded milk (65.9%), cheese (62.5%), icecream (49.2%), milk powder (26.6%), milk sweets (20%), ghee & paneer (13.3%) and yoghurt (6.6%). Conversely, curd and butter were free from *L. monocytogenes* (Mary and Shrinithivihahshini 2017). A study conducted by Chow *et al*. (2021) strikingly revealed high incidence of *Listeria monocytogenes* shedding, in 90% of fecal samples from 20 lactating dairy cows in one Wisconsin farm over a 29-d period. Samples of bulk-tank milk from 444 small ruminant farms (sheep and goat) around Greece were collected and examined by standard microbiological techniques for *Listeria* spp. by enrichment and streaking on PALCAM agar medium (ISO 11290-1:2017) and found the presence of listeria at the rate of 1.2 % (Lianou *et al*., 2022). Out of 200 dairy environmental samples collected from Haramaya University Dairy Farm, Ethiopia, 40 samples (20%) were positive for *Listeria* species. *Listeria* was isolated on PALCAM agar from cow barn, milk supply, silage feed and milk from cow teat, milking operation and milk auditing at 30%, 26.7%, 20%, 18.6% and 10%, respectively (Ahimed *et al.,* 2022). Listeria was found in 10% of pasteurized milk samples collected from retail markets across Sharkia Governorate, Egypt which were pre-enriched and streaked onto Himedia agar *Listeria* Ottaviani Agosti (Abou Elez *et al*., 2023).

MATERIAL AND METHODS

Solid dairy environmental samples, liquid non-moll samples and liquid milk samples were collected, serially diluted, plated for total bacterial count and listeria count using Standard Plate Count Agar (SPCA) and PALCAM agar (HiMedia, 1998), respectively.

Collection of samples

Solid samples such as soil, dung, feed, fodder; Air; liquid samples like water, swab of handler & milch animal udder, pail rinse, can rinse and milk samples such as aseptic milk, pail milk, can milk were collected aseptically from Livestock Farm Complex, Veterinary college while chilled milk, pasteurized milk were collected from Student Experimental Dairy Plant, Dairy Science College, KVAFSU, Hebbal, Bengaluru with prior permission.

Enumeration of total bacterial count and Listeria count from dairy environmental samples

Dairy environmental samples (solid or liquid) of 11 g/ml were weighed and transferred to sterile 99 ml flask containing phosphate buffer solution to make 1st dilution. Air samples were analysed by using air sedimentation method with SPCA and PALCAM agar. Milk samples like aseptic milk, pail milk, can milk, chilled milk and pasteurized milk of 11 ml were pipetted and transferred to the sterile 99 ml flask individually containing physiological saline to make 1st dilution. Further required dilutions were prepared serially using 1st dilution (Harrigan, 1998). Serially diluted samples were transferred to labelled sterile petri plates for the enumeration of total bacterial count and listeria using sterile pipettes. Sterile molten standard plate count agar (SPCA) and listeria identification agar base with supplement (PALCAM), maintained at 50°C water bath was poured to labelled plates containing 1 ml of dilution and mixed thoroughly for the enumeration of total bacterial count and listeria. Later the poured agar plates were allowed to solidify. All the poured plates were incubated at 37°C for 24 h by inverting the plates. All the colonies that appeared on SPCA plates were considered as total bacterial count. Black or black green colony with a black halo and black sunken center colonies were taken as positive for Listeria species and counted. Average count was expressed as $\log_{10}$cfu/ g or ml of the sample. The total bacterial count was enumerated in order to find the per cent occurrence of listeria among the total bacterial population.

Isolation and maintenance of obtained Listeria isolates

The typical colonies of listeria that appeared on PALCAM medium from dairy environmental samples were selected based on colony morphology. Each colony selected was considered as listeria isolate and were coded accordingly. These isolates were purified by streaking 3 times onto poured PALCAM agar plates. After the third streak, the discrete colonies were selected and maintained on PALCAM agar slants as

stock cultures. Working cultures were prepared by inoculating the isolate on the slant to sterile listeria broth tubes.

RESULTS AND DISCUSSION

Among the solid dairy environmental samples like soil, dung, fodder and feed, soil had more listeria count of 4.56 log_{10}cfu/g while feed showed lowest count of 2.49 log_{10}cfu/g. Dung, fodder and feed samples showed nearly 2 log viable counts of listeria (Table 1). If total bacterial count was considered, soil had highest count followed by dung, feed and fodder but occurrence of listeria count varied among samples with respect to the trend observed. Total bacterial count helped to know the per cent occurrence of listeria count among the samples plated. Soil, fodder, dung and feed possessed 79, 60, 52 and 51 per cents of listeria count out of total bacterial count, respectively. Significant difference in listeria count was not observed among the samples plated.

Liquid non-milk dairy environmental samples enumerated for total bacteria and listeria counts were swab of udder, swab of handler, can rinse, pail rinse, water, urine and air. The swab of udder of milch animal had more listeria count of 1.53 log_{10}cfu/ml while urine showed lowest count of 0.10 log_{10}cfu/ml (Table 2). Hand swab of milker, can rinse, pail rinse, water and air samples had no viable counts of listeria. Swab of milch animal's udder and hand swab of milker had higher total bacterial count followed by can and pail rinse, water, urine sample of milch animal and air. Significant difference in listeria count was observed among the samples plated as many samples showed nil count. Swab of udder and urine samples of milch animal possessed 37 and 5 per cents of listeria count out of total bacterial count, respectively.

Table 1: Enumeration of Total Bacteria and Listeria from Solid Dairy Environmental Samples.

Name of the Sample	Type of enumeration	
	Total Bacterial Count (Per cent)	Listeria Count (Per cent)
	log_{10} cfu/g	
Soil	5.75[a] (100)	4.56[a] (79.0)
Dung	5.33[a] (100)	2.76[a] (52.0)
Fodder	4.35[a] (100)	2.59[a] (60.0)
Feed	4.82[a] (100)	2.49[a] (51.0)
CD (P=.05)	**1.96**	**3.25**

Note:

- CD – Critical Difference
- All values are average of three trials
- Values in the parenthesis represent per cent
- Medium used -
  - Standard Plate Count Agar (SPCA) - Total Bacterial Count (TBC)
  - PALCAM basal agar with additive – Listeria count
- Plates were incubated at 37 ºC for 24 h
- Higher value in the last column was compared with other values
- Different superscripts within the column indicate significant difference

Table 2: Enumeration of Total Bacteria and Listeria from Liquid Non-Milk Samples of Dairy Farm.

Name of the Sample	Type of enumeration	
	Total Bacterial Count (Per cent)	Listeria Count (Per cent)
	log_{10}cfu/ml	
Swab of udder	4.10[a] (100)	1.53[a] (37.0)
Swab of handler	4.10[a]	0.00[b]
Can rinse	3.56[ab]	0.00[b]
Pail rinse	3.56[ab]	0.00[b]
Water	3.43[ab]	0.00[b]
Urine	2.12[bc] (100)	0.10[b] (5.0)
Air (log_{10} cfu/min) *	1.02[c]	0.00[b]
CD (P=.05)	**1.23**	**1.08**

Note:

***** Air sample of dairy farm included under this non- milk samples and collected by sedimentation method

Among the liquid milk dairy environmental samples like aseptic milk, pail milk, can milk, chilled milk and pasteurized milk, chilled milk possessed higher listeria count of 1.89 log_{10}cfu/ml while can milk showed lowest count of 1.05 log_{10}cfu/ml (Table 3). Many milk samples showed nil count of listeria except can and chilled milk samples and hence significant difference was observed among the samples plated with respect to listeria count. Aseptic milk, pail milk and pasteurized milk samples had no viable counts of listeria. This indicated if milking was done following hygienic practices, presence of listeria could be controlled and one more interesting thing observed was though chilled raw milk showed listeria but killed when milk was pasteurized. This indicated that raw milk might act as a vehicle for transmission of listeria if consumed raw. If total bacterial count was considered, chilled milk and can milk had higher count followed by pail milk and aseptic milk. Chilled milk and can milk samples revealed 28 and 7 per cents of listeria count out of total bacterial count during the winter months October to November. Due to seasonal variation, the occurrence of Listeria species may be varied. Black coloured typical listeria colonies obtained in dairy environmental samples were selected accounting for eighteen isolates that included three isolates each from soil, dung, chilled milk followed by two isolates from fodder, feed, swab of udder, can milk and one isolate from cow urine sample (Table 4). The isolates were maintained on PALCAM agar slants and subcultured once in 21 days.

Most of the research studies on the prevalence of listeria in milk and milk products were regarding isolation by using enrichment broth followed by streaking on selective agar medium and not on enumeration. Hence the isolation studies are quoted here. Usman *et al*. (2016) opined that listeria could be a common contaminant in the dairy environment, both on the farm and in the processing plant. On the farm, important sources included manure and improperly fermented silage. It was most frequently found in moist environments or areas of processing unit with condensed or standing water or milk, including drains, floors, coolers, conveyors and can washing areas. Shamloo *et al.* (2014) also agreed that *Listeria* spp prevailed in raw milk at 5.49%. Nucera *et al.* (2016) also detected *Listeria* spp. in 22 bales of silage (27.5 per cent) out of 80 collected from 20 dairy farms in Italy. Mary and Shrinithivihahshini (2017) found contamination of *L. monocytogenes* in raw milk and flavoured milk samples by 65.9%, while Chow *et al.* (2021) revealed high incidence of the same in 90% of fecal samples from 20 lactating dairy cows. Samples of bulk-tank milk of sheep and goat exhibited the presence of listeria at the rate of 1.2% (Lianou *et al.*, 2022). Ahimed *et al*. (2022) found 20% of dairy farm samples collected were positive for *Listeria* species and found in cow barn, milk supply, silage feed and milk from cow teat, milking operation and milk auditing at 30%, 26.7%, 20%, 18.6% and 10%, respectively where as Abou Elez *et al.* (2023) could able to detect listeria in 10% of pasteurized milk samples collected from retail markets across Sharkia Governorate, Egypt which can be ascribed to post pasteurization contamination.

Table 3: Enumeration of Total Bacteria and Listeria from various milk samples of dairy farm.

Name of the Sample	Type of enumeration	
	Total Bacterial Count (Per cent)	Listeria count (Per cent)
	log_{10} cfu/ml	
Aseptic milk	3.34^{ab}	0.00^{b}
Pail milk	5.39^{ab}	0.00^{b}
Can milk	6.30^{ab} (100)	1.05^{a} (17.0)
Chilled milk	6.84^{a} (100)	1.89^{a} (28.0)
Pasteurized milk	0.00^{b}	0.00^{b}
CD (P=.05)	**5.13**	**1.61**

Table 4: Number of Listeria isolates Obtained from Dairy Environmental Samples.

Name of the Dairy environmental sample that showed Listeria count	Codes of listeria isolates	Number of listeria isolates obtained
Soil	L1, L2, L3	3
Dung	L4, L5, L6	3
Fodder	L7, L8	2
Feed	L9, L10	2
Urine	L11	1
Swab of udder of milch animal	L12, L13	2
Can milk	L14, L15	2
Chilled milk	L16, L17, L18	3
Total		**18**

On par with the present research study, Chaitra (2020) had enumerated Listeria species from different dairy environmental samples by plating on Listeria PALCAM agar. Among them, maximum listeria count was observed in fodder samples of about 3.14 log_{10}cfu/g whereas in other solid samples like soil, dung and feed listeria count ranged from 3.04 log_{10}cfu/g to 2.30 log_{10}cfu/g. In liquid samples like swab of udder, pail rinse, pail milk, can milk and chilled milk the listeria count ranged from 0.60 log_{10}cfu/ml to 0.30 log_{10}cfu/ml whereas listeria was not found in air, water, swab of hand of milker, can rinse, aseptic milk and in pasteurized milk during the months of August to September in 2017-2018.

CONCLUSIONS

Among dairy environmental samples, soil, fodder, dung, feed while swab of udder, urine of milch animal, can milk and chilled milk showed the counts of listeria on PALCAM agar. A total of 18 number of isolates of listeria were obtained from the dairy environmental samples that revealed listeria counts. From this study it is evident that listeria may enter from dairy environmental samples into raw milk.

FUTURE SCOPE

Many dairy farms and various dairy environmental samples can be included in order to establish the sources of contamination of listeria in raw milk. Further study to investigate the pathogenicity of listeria isolates with genotyping may also help to know their severity in milk and milk products.

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Conflict of Interest. Authors have declared that no competing interests exist.

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