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# Short Communication

## Prevalence of *Listeria* Species in Environment and Milk Samples

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ABSTRACT

The previous notion that infection by pathogenic *Listeria* (listeriosis) is not an important health problem in India starts to be revised now, mainly due to changing habits of the production, distribution and storage of food, favoring the multiplication and spread of cold–tolerant bacteria like *Listeria*. Despite this altering scenario, data on the prevalence of *Listeria* on the Indian subcontinent are still rather sporadic. We investigated the occurrence of *Listeria* species in environmental and raw milk samples from Mysore city (moderately hot, semi–arid climate). Environmental samples included cow dung from cowshed, grazing pasture and soil samples from vegetable–cultivation land. Cold enrichment was used to recover *Listeria* species from raw cow milk (from individual animals) (n=130), cow dung (collected from individual animals) (n=130) and soil (n=100) samples. 10 g sample in case of cowdung and soil and 10mL in case of milk were transferred to 90 mL BHI broth and incubated at 4°C for two weeks. Aliquots from the enriched broth were streaked on Oxford and PALCAM plates, at weekly intervals. *Listeria* isolates were subjected to phenotypic and genotypic characterization. Phenotypic characterization included standard biochemical tests such as catalase test, motility at 25 °C and 37 °C, acid production from the canonical panel of carbohydrates, nitrate reduction, esculin hydrolysis, methyl red and Voges Proskauer reaction. Genus– and species–specific primers were used for PCR differentiation of the isolates. *L. ivanovii* was isolated from 1% of the soil samples, *L. seeligeri* from 0.76% of the cowdung samples and *L. innocua* from 0.76% of the raw milk samples tested. Strategies to reduce the incidence of *Listeria* in environment and milk samples should be implemented.



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*Listeria* is a ubiquitous organism and has been isolated from variety of sources like environmental sources, soil, sewage; surface water, animals etc., Variety of foods like raw vegetables, fruits, meat, fish, poultry, raw milk and its processing environments may also be contaminated with *Listeria* spp., and act as a vehicle for the dissemination of the *Listeria* spp. The genus *Listeria* has fifteen species namely *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. rocourtiae*, *L. marthii*, *L. fleishmanii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, and *L. grandensis*) (Khelef et al., 2006; McLauchlin and Rees 2009; Bertsch et al., 2013; Halter et al., 2013; den Bakker et al., 2014) *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals (Liu 2006).

Milk is a nutritious food which is easily contaminated during processing and acts as a good medium for the growth and multiplication of the pathogens (Agarwal et al., 2012). India is one of the largest producers of milk in the world with the highest number of cattle (Kalorey et al., 2008). Numerous outbreaks of listeriosis due to consumption of milk and milk products contaminated with *L. monocytogenes* are reported all over the world (Fleming et al., 1985, Dalton et al., 1997, Koch et al., 2010, CDC, 2008). Both pathogenic and nonpathogenic species of *Listeria* has been isolated from raw milk samples (Hayes et al., 1986; Massa et al., 1990; Kozak et al., 1996; Gaya et al., 1998; Unlu et al., 1998; Yoshida et al., 1998; Carlos et al., 2001; Aygun et al., 2006; Nero et al., 2008; Aurora et al., 2009; Vanegas et al., 2009; Sarangi et al., 2009; Jami et al., 2010; Soni et al., 2013; Rahimi et al., 2010; Jamali et al., 2013), milk products (Molla et al., 2004) and its processing environments (Chambel et al., 2007; Doijad et al., 2011).

*L. monocytogenes* has been isolated from organically fertilized soils (Szymczak et al., 2013) and Botzler et al., (1974) reported that *L. monocytogenes* can survive in soil and water. *Listeria* spp. has been isolated from the fecal samples of the mammals and birds (Yadav et al., 2009; Yadav et al., 2011).

The presence of *Listeria* spp. in variety of foods, milk and environmental samples has been reported from all over the world and few reports are available on the incidence of *Listeria* in India. The present study was undertaken to study the incidence of *Listeria* spp. in raw cow milk, cow dung and soil from vegetable–cultivation land and grazing Pasteur.



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Raw milk (n=130), soil (n=100) and cow dung (n=130) samples from Mysore city were studied for the presence of *Listeria* spp. Raw cow milk and cow dung samples from the individual cow and soil samples from vegetable–cultivation land and Pasture were collected. All the samples were collected in UV sterilized polythene covers and brought to the laboratory. The samples were processed on the same day of collection.

Isolation of *Listeria* spp. was done by following cold enrichment method as per Dhanashree et al., 2003 with slight modifications for the isolation of *Listeria* spp. 10 ml in case of cowdung and soil and 10mL in case of milk were transferred to 90 mL of Brain Heart Infusion Broth (BHI, Hi–Media Laboratories, Mumbai). The sample was incubated at 4 °C for 48 h to six week. The enriched broth was streaked on Oxford and PALCAM agar plates and incubated at 30 °C for 24 h.

Grey green colonies with black sunken centres from PALCAM agar plates and black colonies with black sunken centre from Oxford agar, suspected to be *Listeria* spp. were picked up and cultured on Brain Heart Infusion Agar (BHI, Hi–Media Laboratories, Mumbai). All the suspected isolates were subjected to phenotypic and genotypic characterization. Phenotypic characterization included standard biochemical tests such as catalase test, motility at 25 °C and 37 °C, acid production from glucose, mannitol, rhamnose, xylose and α methyl D mannoside, nitrate reduction, hydrolysis of esculin, methyl red test and Voges Proskauer test.

The confirmation of the *Listeria* isolates was done by Polymerase Chain Reaction (PCR). Lis1A and Lis1B primer pairs were used for the identification of genus *Listeria* (Bubert et al., 1992). Then the positive isolates were subjected to species identification by using primer pairs Mono A and Mono B for *L. monocytogenes*, Ino 2 and Lis1B for *L. innocua*, Wel 1 and Lis 1B for *L. welshimeri*, Sel 1 and Lis1B for *L. seeligeri*, Iva1 and Lis 1B for *L. ivanovii* (Bubert et al., 1999).

The isolates were grown on BHI agar plates for 24 h at 30 °C. A single colony was transferred to 100 µl of sterile distilled water and heated at 100 °C for 10 minutes in a dry bath (Bangalore Genei Pvt. Ltd., Bangalore) followed by cooling at 4 °C. This served as crude DNA lysate.

PCR amplification was performed in 50 µl reaction mixture containing 5 µl of 10X PCR buffer; 1 µl of 10 mM dNTP mix; 0.5 µl of 10 µM of each primer; 0.25 µl of 5U/µl of Taq polymerase; 4 µl of 25 mM $MgCl_2$; 2 µl DNA template; 36.75 µl of distilled water. All the reagents used in PCR were purchased from Fermentas.

The DNA amplification reaction was performed in a Master Cycler gradient thermocycler (Eppendorf, Hamburg, Germany) with a pre–heated lid in PCR tubes (0.5 ml). The cycling conditions for PCR with the primer pair Lis1A and Lis1B included an initial denaturation of DNA at 94 °C for 5 min followed by 30 cycles each of 45 s denaturation at 94 °C, 60 s annealing at 50 °C and 3 min extension at 72 °C, followed by a final extension of 10 min at 72 °C.

The PCR conditions for identification of species started with an initial denaturation temperature of 94 °C for 5 min and were completed with the final elongation step at 72 °C for 8 min. Amplification conditions varied in the denaturation, annealing and elongation step with the different primer pairs. The details of the primers used in the study are as given in the Table 1.

For Mono A and Mono B primer pair denaturation temperature is 94 °C for 45 s, annealing temperature of 55 °C for 60 s and elongation step at 72 °C for 60 s. For Ino2 and Lis1B pair denaturation temperature is 94 °C for 45 s, annealing temperature of 62 °C for 60 s and elongation step at 72 °C for 45 s. For Sell, Lis1B pair, Well, Lis1B pair and Ival, Lis 1B pair denaturation temperature is 95 °C for 30 s, annealing temperature of 62 °C for 30 s and elongation step at 72 °C for 90 s.

Table 1: Primers used in the study and the species identified. (Bubert et al., 1999; Bubert et al., 1992)

| No | Primer name | Primer sequence (5´–3´) | Species identified |
|---|---|---|---|
| 1 | Lis 1B<br>Lis1B | (TTATACGCGACCGAAGCCAA)<br>(ATGAATATGAAAAAAGCAA) | All *Listeria* species |
| 2 | Mono A<br>Mono B | (CAAACTGCTAACACAGCTACT)<br>(GCACTTGAATTGCTCTTATTG) | *L. monocytogenes* |
| 3 | Ino2<br>Lis1B | (ACTAGCACTCCAGTTGTTAAAC)<br>(ATGAATATGAAAAAAGCAA) | *L. innocua* |
| 4 | Iva 1<br>Lis1B | (CTACTCAAGCGCAAGCGGCAC)<br>(ATGAATATGAAAAAAGCAA) | *L. ivanovii* |
| 5 | Wel 1<br>Lis1B | (CCCTACTGCTCCAAAAGCAGCG)<br>(ATGAATATGAAAAAAGCAA) | *L. welshimeri* |
| 6 | Sel 1<br>Lis1B | (TACACAAGCGGCTCCTGCTCAAC)<br>(ATGAATATGAAAAAAGCAA) | *L. seeligeri* |

Table 2: Prevalence of *Listeria* in the samples tested

| No | Samples | Samples tested | Samples positive | Species identified | %incidence |
|---|---|---|---|---|---|
| 1 | Raw milk | 130 | 1 | *L. innocua* | 0.76 |
| 2 | Cow dung | 130 | 1 | *L. seeligeri* | 0.76 |
| 3 | Soil | 100 | 1 | *L. ivanovii* | 1 |

The PCR products were separated in a 1.2% agarose gel along with a DNA ladder (Lambda 1Kb fermentas) and analyzed using a gel documentation system.

In the present study *Listeria* spp. was isolated from 1–2% of the samples tested (Table 2). *Listeria* spp. has been isolated from raw milk, soil and cow dung samples from different parts all over the world. In India few reports are available on


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the incidence of *Listeria*. In our study, ten isolates were suspected to be *Listeria* on PALCAM plates and among them three samples were found to be positive for *Listeria* spp. The isolates were confirmed as *L. innocua*, *L. ivanovii* and *L. seeligeri*. (Figure 1)

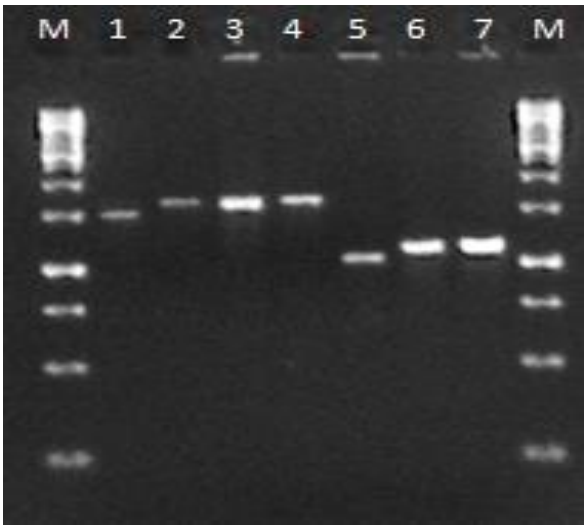


Figure 1: Identification of the isolates using the genus specific and species specific primer pairs; Lane M – 1 Kb Marker; Lane 1– Control *L. monocytogenes* EGD–e; Lane 2 – 4 – Isolates tested with the primer pair Lis1A and Lis1B; Lane 5–7 *Listeria* species confirmed with species specific primers

*L. ivanovii* was isolated from 1% of the soil samples, *L. seeligeri* from 0.76% of the cowdung samples and *L. innocua* from 0.76% of the raw milk samples tested. The presence of *L. innocua* in milk correlates with the results reported by Dhanashree et al. 2003. Singh et al., (2008) found that out 51 isolates from milk 13 isolates were confirmed as *L. monocytogenes*. In 2003 Gianfranceschi et al., reports that 17.4% of dairy products were found positive for *L. monocytogenes*. 60.6% of milk samples from Tiruchirapalli were found positive for *L. monocytogenes* (Shrinithivihanshini et al., 2011). 16.7% of *L. monocytogenes* was isolated from raw milk samples commercialized in Portugal (Mena et al., 2004) Raw milk in Malaysia was assessed for the presence of *Listeria* spp. by Chye et al. (2004). They reported that 4.4% of raw milk samples were positive for *Listeria* spp. Among them 1.9% were *L. monocytogenes*, 2.1% were *L. innocua* and 0.6% were *L. welshimeri*. Latorre et al. (2009) reported the incidence of *L. monocytogenes* in milk and fecal samples of cows. 7.1% of fecal samples and 7.3% of milk samples were positive for *L. monocytogenes*. 16% of the fecal samples of mammals and bird were found positive for *L. monocytogenes* (Kalorey et al., 2006). Same kind of results was reported by Zaytseva et al., (2007). Soil samples from agriculture fields and animal inhabited areas were examined for the presence of *Listeria* by Moshtaghi et al. (2003) and found 17.7% of *Listeria* spp. among them 5.4% were *L. monocytogenes*, 1.5% were *L. ivanovii*, 7.7% were *L. innocua* and 3.1% were *L. welshimeri*.

The results of our study showed the incidence of *Listeria* spp. in raw milk, soil and cow dung samples. Very strict measures should be taken to ensure that milk samples are not contaminated by external sources.

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## CONFLICT OF INTEREST

The author confirms that this article has no conflict of interest.

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