**Original Research Article**

**Phylogeny of the *Listeria* species obtained from dairy environmental samples**

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| --- |
| **ABSTRACT:****Aims:** To establish the phylogeny of the species of *Listeria* isolated from dairy environmental samples**Study design:** Isolates of *Listeria* were isolated from dairy environmental samples and subjected for pheno and genotypic identity. Based on genotypic identification, phylogeny of the species of *Listeria* was created based on neighbour joining method.**Place and Duration of Study**: The study was conducted in a laboratory (controlled setting) between June 2020 to July 2021**Methodology:** Isolation of *Listeria* species were carried out on PALCAM agar. Isolates were maintained on the same agar slants and working cultures were prepared in the broth medium. The isolates were identified through various biochemical tests and confirmed using rRNA sequencing. The neighbour joining method was adopted to establish the phylogeny of all the *Listeria* species obtained from dairy environmental samples**Results:** A total of 18 numbers of isolates of Listeria were obtained from the dairy environmental samples such as about 3 isolates from soil, dung, chilled milk each followed by 2 from fodder; feed; swab of udder; can milk and one from urine. The pheno and genotypic identity of *Listeria* revealed as *Listeria ivanovii* (6 nos; 50.0%), followed byListeria grayi (5 nos;41.6 %) and Listeria seeligeri (1 no; 8.40%). Phylogenetic analysis through Neighbour joining method revealed that *L. ivanovii* L18, L8, L16 and *L. grayi* L9, L10, L12 were having relatedness about 87% while *L. ivanovii* L15, L7, L17, *L. grayi* L11, L13 and *Listeria seeligeri* L14 had about 92% homology. Blasting the obtained sequences in NCBI of the *Listeria* isolates revealed that there was a significant genetic correlation between *Listeria* isolate L7, L14, L15, L16 and L17 with NewYork isolate of *Listeria*, isolates L8 and L12 with Nigerian isolate, L9 and L11 with American isolate, L10 with German isolate, L13 with Himachal Pradesh isolate and L18 with Greece isolate.**Conclusion:** The *Listeria* isolates obtained from dairy environmental samples in the current study unravelled the genetic relatedness with the isolates from New York, Nigeria, Germany, Himachal Pradesh and Greece indicating their geographical distribution and divergence.  |

*Keywords: Listeria spp.; rRNA sequencing; Phylogeny; Neighbour joining method; Dairy environmental samples*

1. INTRODUCTION

The genus Listeria currently includes six species: L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, and L. grayi. Two of these species, L. monocytogenes and L. ivanovii, are potentially pathogenic. The infectious disease caused by these bacteria is known as listeriosis. L. monocytogenes causes serious localized and generalized infections in humans and a variety of other vertebrates, including domesticated and wild birds and mammals. They are widely distributed in nature, psychrotrophs present in vegetation, sewage, human and animal carrier and infect mainly warm-blooded ruminants, causing economic loss. The pathogens produce toxin called Listeriolysin O (LLO), coded by the gene *hly A* and responsible for the cause of disease (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, septicemia, 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 (Karus et al., 2024). Health problems related to food and drinks are highly concerning at global level. Listeria monocytogenes, a Gram positive rod shaped bacteria that enter through contaminated milk, milk products, fruit juices and water involved in causing Listeriosis, is of public health concern. This bacterium in milk or water medium when exposed to high voltage-pulsed electric field of 100kV led to decrease in survival ratio of 0.001 (Ansari et al., 2022; 2023).

The isolated typical colonies of *L. monocytogenes* isolated from food products were identified by performing Gram’s staining, catalase reaction, oxidase reaction, tumbling motility at 20–25 °C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *Staphylococcus aureus* in which *Listeria* isolate was streaked perpendicular to a streak of S. aureus on sheep blood agar. Gram positive motile short rods with catalase positive and oxidase negative, MR-VP tests positive and a positive reaction that appeared as an arrow head zone of haemolysis adjacent to the place where the two streak lines come into proximity, haemolysis on sheep blood agar, nitrate reduction and fermentation of sugars (rhamnose, xylose) confirmed the presence of *Listeria* spp. (Mary and Shrinithivihahshini, 2017; Shamloo *et al.,* 2019; Sushmita et al., 2023 and Chaithra et al., 2024).

Evolutionary relatedness of species of *Listeria* may help in finding the lineage and source which is possible through construction of dendrograms of phylogenetic tree for them.  In molecular [phylogenetic](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/phylogeny) analysis, the sequence of a common gene or protein can be used to assess the evolutionary relationship of species. By establishing phylogenesis of *Listeria*, the evolutionary relationship of *Listeria* species with another species can be known from different sources. Phylogenesis of *Listeria* was first constructed by Weidmann in 2002. Many phylogenetic works had been carried out on *Listeria* species isolated from different food and environmental samples by using MEGA software and most of the research on phylogeny was carried out for *Listeria monocytogenes*. (Jarvis *et al.,* 2017). All the *L. monocytogenes* isolates from different environmental, food and clinical samples showed similarity with the isolates from the milk product (KF894986.1), in India and sludge and waste water (AJ535697.1) in France. Additionally, most of the isolates from vegetables also showed similarity with those from food (KF588562.1), in Chile and majority of the isolates from water and human clinical samples showed similarity with the isolates obtained from intestine (HM007564.1) in Germany and chicken (KF956739.1) in Turkey. The phylogenetic relatedness of the isolates of *L. monocytogenes* obtained from different samples of humans, vegetable, soil, water and cow milk in Varanasi was estimated using the neighbour-joining method. (Soni and Dubey, 2014). The typical isolates (23) were biochemically confirmed by subjecting to PCR using primers Forward 5′- CTGCTTGAG CGTTCATGTCTCATCCCCC-3 and Reverse 5′ CATGGGTTTCACTCTCCTTCTAC-3. Out of 23 isolates, 14 were confirmed as *Listeria monocytogenes* while nine isolates showed negative reaction. Phylogeny for 14 isolates of *Listeria monocytogenes* obtained was constructed by using UPGMA (unweighted pair group method with arithmetic mean) method in MEGA 6.0 software that indicated *Listeria monocytogenes* from raw milk showed relatedness with *Listeria ivanovii* and *Listeria innocua*. (Mary and Shrinithivihahshini, 2017)

The results of amplified 16S rRNA of 13 isolates of *Listeria monocytogenes* obtained from clinical and food samples in Iraqconcluded that the Iraqi isolates of *Listeria monocytogenes* showed that there was a significant genetic correlation and high homology between isolate No.1 with Indian isolate (HM589603.1), isolate No.2 with Indian isolate (HM589597.1), isolate No. 3 with US isolate (M24199.1), isolate No.4 with Indian isolate (HM589599.1), and isolate No.5 with Italian isolate (AF253320.1). (Yousif and Alshamari 2018). In the present study attempt has been made to isolate and characterize the *Listeria* species from dairy environmental samples of university dairy farm, KVAFSU, Bengaluru, Karnataka state as well as to find the genetic relatedness and homology of the identified isolates with the global isolates.

2. material and methods

**2.1 Phenotypic identification of *Listeria* isolates**

Isolates of *Listeria* (12 nos.) obtained from dairy environmental samples (33 nos.) were propagated in sterile nutrient broth as working cultures and maintained on PALCAM agar slants. The working cultures of the isolates were prepared fresh (18-hour old) whenever required for biochemical tests while stock cultures were subcultured once in 15 days and after growth stored at 5oC All the isolates were subjected for preliminary identification tests like Gram’s staining, spore staining, catalase test, oxidase test, motility test, methyl red test and nitrate reduction test as reported by Harrigan (1998). The species level identification was possible after conducting specific tests that included acid from ribose and rhamnose, coagulase test, Christie Atkin Munch Peterson (CAMP) test, lecithinase activity, growth in peptone water with 10 per cent NaCl and at 4 °C. The results of biochemical tests obtained for the *Listeria* isolates were compared with identification key developed from Volume 3 of Bergey’s manual of systematic bacteriology (II edition) to place them under the species. Further 16s rRNA sequencing was performed for all the phenotyped *Listeria* isolates to confirm the identity.

**2.2 Key for the identification of *Listeria* species**

The identification key for *Listeria* species was prepared by referring to Bergey’s manual of systematic bacteriology (II edition) Volume 3 - the firmicutes edited by Ludwig *et al.* (2009).

LIST 1 : Identification of Listeria species

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Biochemical tests** | ***L. monocytogenes*** | ***L. ivanovii*** | ***L. innocua*** | ***L. seeligeri*** | ***L. welshimeri*** | ***L. grayi*** |
| **Specific Tests** |
| Acid from ribose | - | + | - | - | - | + |
| Acid from L-Rhamnose | + | - | d | - | D | + |
| β-haemolysis  | + | + | - | + | - | - |
| CAMP test | + | - | - | + | - | - |
| Coagulase test | + | + | + | + | + | + |
| Lecithinase activity | + | + | - | D | - | - |
| Growth in peptone water with 10 per cent NaCl | + | + | + | + | + | + |
| Growth at 4 °C | + | + | + | + | + | + |

**Note:**

 \* d – variable

* Among the preliminary tests, all the *Listeria* species were Gram positive motile short non-spore forming rods with catalase and methyl red tests positive while oxidase negative, nitrate reduced by all species except *L.grayi*

**2.3 Genotyping of obtained *Listeria* isolates**

All the obtained isolates of *Listeria* spp. were genotyped. The DNA extraction of the isolates were carried out by using DNA extraction kit (HiMedia) and further subjected to PCR for 16s rRNA sequencing as per the procedure prescribed by Wachiralurpan *et al.* (2016). Forward and reverse primers of about 5 µl was diluted 10 times by using 45 µl biological grade water. All the ingredients like sterile milliQ water (12.0 µl), 2X PCR master mix (12.5 µl), forward primer (0.5 µl) 27F AGAGTTTTGATCCTGGCTCAG and reverse primer (0.5 µl) 1492R GGTTACCTTGTTACGACTT were mixed together in the above order in an eppendorf tube using a vertex mixer. Accurately 1.0 µl of DNA was added to the above 25.5 µl mixture and transferred to thermocycler to perform PCR and set up the PCR cycling parameters that included cycles each consisting of 95oC for 1 min, 58.8oC for 30 sec and 72oC for 30 sec, and confirmed the amplification of DNA through agarose gel electrophoresis by using 2 per cent agarose gel at 80 V for 1 h as per the standard procedure. After obtaining PCR product, it was outsourced for gene sequencing at Barcode scientific, Whitefield, Bengaluru. The gene sequence obtained was blasted in NCBI (http://www.ncbi.nlm. nih.gov) and final identity was obtained. The identified culture was submitted for accession number in NCBI website (http://www.ncbi.nlm. nih.gov).

**2.4 Establishment of phylogeny of obtained *Listeria* isolates**

Phylogeny was constructed to know the evolutionary development of the organism by using the obtained gene sequence of the isolate after outsourcing of DNA sequence. MEGA 10X software was used to construct phylogenesis by using Neighbour joining method and the obtained tree was analyzed. Relatedness among the strains of *Listeria* was given by the per cent. (

[www.megasoftware.net).](http://www.megasoftware.net).)

3. results and discussion

 Both pheno and genotypic characterization were carried out for 12 Listeria isolates as well based on genotypic identity of the isolates, phylogeny was determined

**3.1 Phenotypic characterization of *Listeria* isolates**

A total of 12 *Listeria* isolates were obtained from various dairy environmental samples (33 nos.) comprised 3 isolates from chilled milk; 2 from fodder; feed; swab of udder; can milk and one from urine through pour plate technique using PALCAM agar medium (Table 1). These isolates, when subjected for preliminary tests revealed as Gram positive, catalase positive, oxidase negative, methyl red positive motile rods. Among them 6 *Listeria* isolates (L7, L8, L15, L16, L17, L18) reduced nitrate, produced acid from ribose, no acid from rhamnose, haemolysin positive, lecithinase positive, coagulase positive, CAMP test negative with *Staphylococcus aureus* and showed growth at 4°C and in 10 percent NaCl, L14 was positive for CAMP test with *Staphylococcus aureus* whereas 5 isolates of *Listeria* (L9, L10, L11, L12, L13 ) did not reduce the nitrate, produced acid from ribose as well from rhmnose, haemolysin negative, lecithinase negative, coagulase positive, CAMP test negative with *Staphylococcus aureus* and showed growth at 4ºC and in 10 percent NaCl. Based on the results of biochemical tests (Table 2) of 12 *Listeria* isolates of dairy environmental samples, 6 isolates were *Listeria ivanovii* at 50% predomination followed by 5 isolates as *Listeria grayi* (41.60%) and 1 isolate as *Listeria seeligeri* (8.40%).

 **Table 1: Number of *Listeria* isolates obtained from dairy environmental samples**

|  |  |  |
| --- | --- | --- |
| **Name of the Dairy environmental sample** | **Codes of *Listeria* isolates** | **Number of isolates obtained** |
| Fodder | L7, L8 | 2 |
| Feed | L9, L10 | 2 |
| Urine | L11 | 1 |
| Swab of udder | L12, L13 | 2 |
| Can milk | L14, L15 | 2 |
| Chilled milk | L16, L17, L18 | 3 |
|  Total | 12 |

**Table 2: Phenotypic identification of *Listeria* spp. isolated from dairy environmental samples**

|  |  |  |
| --- | --- | --- |
| **Codes of *Listeria* isolates****(Total number of isolates)** | **Specific biochemical tests carried out** | **Identity** |
| **Nitrate reduction** | **Acid from ribose** | **Acid from** **L-Rhamnose** | **Haemolysis** | **Lecithinase activity** | **Coagulase test** | **CAMP test** | **Growth** |
| **4** **ºC** | **10 % NaCl** |
| L7, L8, L15, L16, L17, L18(6 nos.) | - | + |  | + | + | + | - | + | + | *Listeria ivanovii* |
| L14(1 no.) | - | + |  | + | + | + | + | + | + | *L. seeligeri* |
| L9, L10, L11, L12, L13(5 Nos.) | + | + |  | - | - | + | - | + | + | *L. grayi* |

 **Note:**

* The results mentioned in the table are confirmed after 3 replications
* All the 12 isolates were Gram positive, non-spore forming rods, motile, catalase positive, oxidase negative and methyl red positive

Nayak *et al.* (2015) found that out of 200, 18 samples of milk and milk products from Navsari, Gujrat were found positive for *Listeria* spp*.* which were identified as *Listeria
seeligeri* (6, 33.3 percent), *Listeria innocua* (5, 27.7 percent), *Listeria welshimeri* (4, 22.2 percent), and *L. monocytogenes* (3, 16.6 percent). Similar to the present study, Matto *et al.* (2018) collected dairy farm samples like soil, dung, water, bulk milk to study the prevalence of *Listeria* species. They isolated only one isolate of *L. monocytogenes* from pasture. No isolation of *Listeria* spp. was retrieved from the bulk tank milk or drinking water from of the farms. *Listeria innocua* was detected in 13 feedstuffs and seven samples of soil from the entry and exit points of the milking parlour. EL-Naenaeey *et al.* (2019) determined the overall isolation rate of *Listeria* spp. as 16 per cent in feaces of dairy cows; 8 per cent *Listeria monocytogenes* from normal milk and mastitis milk; 4 percent and 2 percent in feaces of dairy cows, normal milk and mastitis milk, respectively. The prevalence of *L. ivanovii* and *L. welshimeri* in milk of dairy cows were 6 percent and 4 percent, respectively. On the contrary to the present study, Chaitra et al (2024), identified 23 isolates of *Listeria* obtained from environmental samples of university dairy farm in Bengaluru as *Listeria monocytogenes* (56.50%) followed by*L. innocua* (17.39%**)**, *L. ivanovii* (8.70%); *L. seeligeri*  (4.35%) and 13.06 % of unidentified  *Listeria* spp.

**3.2 Genotypic characterization of *Listeria* isolates**

The DNA was extracted from allthe isolates of *Listeria* grown in PALCAM broth and further amplified by PCR. The PCR product was outsourced for sequencing. Based on the gene sequence obtained through nucleotide blast at the website [www.ncbi.com](http://www.ncbi.com), the isolates were confirmed as *Listeria ivanovii* (L7, L8, L15, L16, L17, L18), *Listeria seeligeri* (L14) and *Listeria grayi* (L9, L10, L11, L12, L13) which was matching with phenotypic identity. After confirmation of genotypic identification, the accession numbers were obtained from NCBI. The accession numbers obtained for *Listeria ivanovii* were L7 - KP01000451.1, L8 – MW386242.1, L15 - KP01000447.1, L16 - KP01000445.1, L17 - KP01000434.1 and L18 - MW386243.1 respectively and MW466720 for *Listeria seeligeri* L14 while *Listeria grayi* accession numbers were L9 - AY643840.1, L10 - M80352.1, L11 – AY643839.1, L12 - MW020239.1 and L13 – MW386232.1 respectively (Table 3).

**Table 3: Genotypic identification and Accession numbers of *Listeria* isolated from**

 **dairy environmental samples**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S. No** | **Code of isolate** | **Genotypic identity** | **Source** | **Accession number** | **Data base****(Type)** |
| 1 | L7 | *Listeria ivanovii* | Fodder | KP01000451.1 | GenBank(Direct submissions) |
| 2 | L8 | *Listeria ivanovii* | MW386242.1 |
| 3 | L9 | *Listeria grayi* | Feed | AY643840.1 | EMBL(Patents - Nucleotide) |
| 4 | L10 | *Listeria grayi* | M80352.1 |  GenBank(GSDB Direct submissions) |
| 5 | L11 | *Listeria grayi* | Urine | AY643839.1 | EMBL(Patents - Nucleotide) |
| 6 | L12 | *Listeria grayi* | Swab of udder | MW020239.1 | GenBank(Direct submissions) |
| 7 | L13 | *Listeria grayi* | MW386232.1 |
| 8 | L14 | *Listeria seeligeri* | Can milk | MW466720 |
| 9 | L15 | *Listeria ivanovii* | KP01000447.1 |
| 10 | L16 | *Listeria ivanovii* | Chilled milk | KP01000445.1 |
| 11 | L17 | *Listeria ivanovii* | KP01000434.1 |
| 12 | L18 | *Listeria ivanovii* | MW386243.1 |

In the present study universal bacterial primers were used for genotyping of 12 *Listeria* isolates and Soni and Dubey (2014) as well Mohamed *et al.* (2016) also used bacterial universal primers 27F (50 AGAGTTTGATCMTGGCTCAG-30) and 1492R (50-GGTTA CCTTGTTAC GACTT-30) for genotyping. In case of Soni and Dubey (2014), 80 isolates of *Listeria* spp. obtained from different samples like humans, vegetable, soil, water and cow milk in Varanasi were identified as *Listeria monocytogenes* while as per Mohamed *et al.* (2016), 6 isolates of *Listeria* spp.isolated from Egyptian meat samples were confirmed as *Listeria monocytogenes*. Mary and Shrinithivihahshini (2017) also characterized 23 typical isolates of *Listeria* spp. identified from milk and milk products in Tamil Nadu using universal bacterial primers as *Listeria monocytogenes* (14) while nine isolates showed were as well *L.monocytogenes* but without phospholipase C, a pathogenic characteristic . In an advanced study by Yousif and Alshamari (2018) who genotyped 13 isolates of *Listeria* spp. obtained from different food samples of Iraq such as like chicken, meat and cheese using *hlyA* gene primers and confirmed them as *Listeria monocytogenes*.

**3.3 Phylogeny of the isolates of *Listeria* obtained from dairy environmental samples**

The DNA sequences obtained for 12 *Listeria* isolates were used to construct phylogeny based on Neighbour joining method in MEGA 10X software.

The phylogenetic analysis with Neighbour joining method revealed that *Listeria ivanovii* L18, L8 and *Listeria grayi* L9 had about 79 per cent homology and correlation with each other whereas *Listeria grayi* L12, *Listeria ivanovii* L16 and *Listeria grayi* L10 revealed about 81 per cent correlation. All the above six isolates had about 87 per cent relatedness with each other. The relatedness among *Listeria ivanovii* L15, L7, L17 was 91 per cent while *Listeria grayi* L11, *Listeria seeligeri* L14 and *Listeria grayi* L13 related to each other with 86 per cent and all six isolates related with 92 per cent homology (Fig 1).

Blasting the sequences with those in database in NCBI of the *Listeria* isolates obtained from university dairy farm, Bengaluru showed that there was a significant genetic correlation between *Listeria* isolate L7, L14, L15, L16 and L17 with NewYork isolate of *Listeria*, isolates L8 and L12 with Nigerian isolate, L9 and L11 with American isolate, L10 with German isolate, L13 with Himachal Pradesh isolate and L18 with Greece isolate (Table 4).

**Fig 1: Phylogenetic tree of *Listeria* isolates obtained from dairy environmental**

 **samples using Neighbour joining method**

**Table 4: *Listeria* isolates obtained in the present study with the available ones in Gene**

 **Bank from different country**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S. No** | **Code of isolate** | **Genotypic identity** | **Accession number** | **% relatedness based on Neighbour joining method** | **Country** |
| 1 | L18 | *Listeria ivanovii* | MW386243.1 | 79 | Greece |
| 2 | L8 | *Listeria ivanovii* | MW386242.1 | Nigeria |
| 3 | L9 | *Listeria grayi* | AY643840.1 | USA |
| 4 | L12 | *Listeria grayi* | MW020239.1 | 81 | Nigeria |
| 5 | L16 | *Listeria ivanovii* | KP01000445.1 | NewYork |
| 6 | L10 | *Listeria grayi* | M80352.1 | Germany |
| 7 | L15 | *Listeria ivanovii* | KP01000447.1 | 91 | NewYork |
| 8 | L7 | *Listeria ivanovii* | KP01000451.1 | NewYork |
| 9 | L17 | *Listeria ivanovii* | KP01000434.1 | NewYork |
| 10 | L11 | *Listeria grayi* | AY643839.1 | 86 | USA |
| 11 | L14 | *Listeria seeligeri* | MW466720 | NewYork |
| 12 | L13 | *Listeria grayi* | MW386232.1 | Himachal Pradesh |

Normally researchers used either neighbour joining or UPGMA methods to determine the relatedness. In the present study neighbour joining method was adopted that showed on an average of 84 percent relatedness among the species of *Listeria* and their strains.

Many researchers carried out phylogeny for *Listeria monocytogenes* hence those studies have been quoted here.Like in the present study,Soni and Dubey (2014) also constructed phylogeny for 80 isolates of *Listeria* spp. using MEGA 4.0isolated from humans, vegetable, soil, water and cow milk samples in Varanasi by using neighbour joining method. The data revealed that the isolates recovered showed high level of 16S rRNA sequences relatedness (97–99 per cent) with *L. monocytogenes.* Mohamed et al*.* (2016) also used neighbour joining method in MEGA 6.0 software for 6 *Listeria monocytogenes* isolates obtained from meat samples in Egypt having high homology with Colombian isolate, except one Egyptian isolate which showed high homology with Indian isolate of *Listeria monocytogenes*. This may be due to the importation of animals and raw meat from the Latin America and India.

While Mary and Shrinithivihahshini (2017) performed phylogeny for 14 isolates of *Listeria monocytogenes* obtained from milk and milk products in Tamil Nadu by using UPGMA method in MEGA 6.0 software and concluded that *Listeria monocytogenes* from raw milk showed relatedness with *Listeria ivanovii* and *Listeria innocua*. Yousif and Alshamari (2018) also conducted phylogenetic analysis on 5 *Listeria monocytogenes* isolated fromchickens, meat, cheeseand clinical samples in Iraq using MEGA 6.0 software by UPGMA tree analysis. The Iraqi isolates showed significant genetic correlation and high homology between isolate No.1 with Indian isolate, No.2 with Indian isolate, No. 3 with US isolate, No.4 with Indian isolate, and isolate No.5 with Italian isolate. The genetic affinity in the isolates of *L. monocytogenes* isolated from chickens, meat and cheese by 70 per cent existed, while there was a genetic difference in the isolates of *L. monocytogenes* isolated from the clinical samples.

Some researchers worked on clonal complex through core genome phylogenetic tree to find the source of contamination. Ricchi et al. (2019) showed that *Listeria* isolates obtained from milk vending machine (2), tank milk(2) and single cow milk samples(5), udder swab (2) and clinical samples (2) of Piacenza Province, Italy belonged to lineage II, serogroup IIa, sequence type 37, clonal complex 37 and harboured some virulence determinants and prolonged milk contamination by *L. monocytogenes* can originate from subclinical and persistently infected cows, posing a health risk to consumers. As per Varasaki et al. (2022), the majority of samples of environment of dairy farm in the Cantabria region (91%) were positive for Listeria spp., of which 54% were positive for L. monocytogenes. The isolates of 95% of belonged to lineage I with high tolerance to low pH. which can be advantageous for the survival of L. monocytogenes in the gastric acidic environment and can be an additional indicator that the presence of L. monocytogenes in the farm environment is due to fecal contamination. According to Merwe et al. (2024), of the 38 strains of L. monocytogenes that persisted in food processing facilities, 31 were assigned to lineage II, more likely to persist in food processing facilities and concluded that among the four lineages of L. monocytogenes, strains of lineage I being most virulent and strains of lineage IV being essentially avirulent. Bolten et al. (2024), detected 6 clusters of *Listeria* spp. from the dairy farm environmental samples (75%) compared with samples collected from the dairy processing samples (24%) when whole-genome sequencing was performed. Of these 6 clusters, 3 (i.e., clusters 2, 4, and 5) contained isolates that were collected from both the dairy processing samples and dairy farm environmental samples, suggesting that transmission between these 2 environments was likely (Bolten et al., 2024).

**3.4** **Occurrence of *Listeria* spp. in dairy environmental samples**

Among 12 isolates of *Listeria* obtained from dairy environmental samples, the species identified after phenotypic and genotypic characterization were*Listeria ivanovii* (6), *Listeria seeligeri* (1)and *Listeria grayi* (5). Among the species of *Listeria*, *Listeria ivanovii* (50.0 per cent) predominated followed by*Listeria grayi* (41.60 per cent) and *Listeria seeligeri* (8.40 per cent) (Fig. 2).

 **Fig. 2: Occurrence of *Listeria* spp. in dairy environmental samples**

On the contrary to the present study, Nayak *et al.* (2015) showed predomination of *Listeria seeligeri* followed by *Listeria innocua*, *Listeria welshimeri* and *L. monocytogenes* in milk and milk products samples collected from Navsari, Gujrat. As per Matto *et al.* (2018) prevalence of *Listeria innocua* in feedstuffs and soil samples of dairy farms of Uruguay followed by *L. monocytogenes* from pasture. EL-Naenaeey *et al.* (2019) found 14 percent *Listeria monocytogenes* in milk samples and feaces of dairy cows and 6 percent of *L. ivanovii* and 4 percent of *L. welshimeri* in milk of dairy cows of Egypt. According to Chaitra et al. (2020), *Listeria monocytogenes* (56.50 percent) predominated followed by  *Listeria innocua* (17.39 per cent)*, Listeria ivanovii* (8.70 per cent)*, Listeria seeligeri* (4.35 per cent) and three as unidentified *Listeria* species (13.06 per cent) out of 23 isolates from the dairy environmental samples of university dairy farm in Bengaluru. The present study did not find the presence of *L. monocytogenes* while none of the above quoted studies revealed the presence of *L.grayi*, a non-pathogenic species of *Listeria*  with natural habitat as soil and vegetation, compared to the present study.

4. Conclusion

Among the 12 dairy environmental *Listeria* isolates, *Listeria ivanovii* was predominant (50.0%), followed by Listeria grayi (41.6 %) and Listeria seeligeri (8.40%). The neighbour joining method of phylogenetic analysis of these isolates depicted genetic correlation with *Listeria* isolates of NewYork, Nigeria, Germany, Himachal Pradesh and Greece, indicating their sources of origin with widespread and ubiquitous presence.

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**COMPETING INTERESTS**

 Authors have declared that no competing interests exist.

Authors’ Contributions

 ‘Author 1’ designed the study, performed the experiment, wrote the first draft of the manuscript. ‘Author 2’ managed the analyses of the study. ‘Author 3’ corrected the article and finally all authors read and approved the final manuscript.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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