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Journal of Infection and Public Health 17 (2024) 696–703

Contents lists available at ScienceDirect

Journal of Infection and Public Health

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

Original Article

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ARTICLE INFO

Article history:
Received 3 December 2023
Received in revised form 7 February 2024
Accepted 25 February 2024

Keywords:
Detection
Food
DNA Sequencing
Listeria
Molecular
Pathogens

ABSTRACT

Background: Food-borne pathogen *Listeria monocytogenes* is abundantly present in nature and accountable for sporadic and epidemic cases of listeriosis in humans. The objective of this study was to screen common food sources for *L. monocytogenes* using biochemical and molecular methods to detect and characterise its toxin genes as well as for biofilm formation.

Methods: A total of 92 samples, comprising dairy and street food products, were randomly collected from various sources for this investigation. The collected samples were processed for biochemical and molecular methods to detect *L. monocytogenes*. Additionally, virulence factors associated genes, antibiogram profiles and biofilm formation related assays were determined.

Results: *L. monocytogenes* presence was confirmed using molecular detection methods targeting *prs* and *lmo*1030 genes, along with MALDI-TOF MS. Following 16 S rRNA sequencing, the identified *Listeria* species were further categorised into two groups. *L. monocytogenes* was detected in two (2.17%) food samples tested (L-23 and L-74). Multiplex PCR indicated the presence of seven virulence-related genes in *L. monocytogenes* isolates, i.e., *inl*A, *inl*B, *prf*A, *iap*, *act*A, *plc*B, and *hly*A. In addition, 17 antibiotics were tested, whereby two isolates showed resistance to clindamycin and azithromycin, while one isolate (L-74) was also resistant to nalidixic acid, co-trimoxazole, ampicillin, norfloxacin, and cefotaxime. L-23 and L-74 isolates showed biofilm formation, especially at pH 8.6 and 37°C.

Conclusions: Besides the demonstration of the presence of *L. monocytogenes* in some dairy and street food products, this study underscores the need to increase the standards of hygiene on the one hand and the importance of the surveillance of food-borne pathogens on the other.

1. Introduction

Listeria monocytogenes is a worldwide perilous food-borne pathogen detected in various foodstuffs, from ready-to-eat (RTE) food to vegetables, meat, fish, and dairy products [1,2]. It is responsible for outbreaks of listeriosis in humans, especially young, old, pregnant, and immunocompromised people, and can engage with meningitis and septicemia [3–5]. *L. monocytogenes* can survive in different adverse conditions, including low temperature and pH and high concentrations of salt [6]. This Gram-positive facultative intracellular bacterium belongs to the genus *Listeria*, which comprises a total of 18 species with only two pathogenic species, i.e., *L. monocytogenes* and *Listeria ivanovii* [7]. The fundamental spread of *L. monocytogenes* from the gastrointestinal tract relies on its capacity to cross digestive, blood-brain, and placental boundaries and its infection is mediated by numerous virulence factors [8]. Diverse *Listeria* determinants are well-known to play a significant role in *L.*

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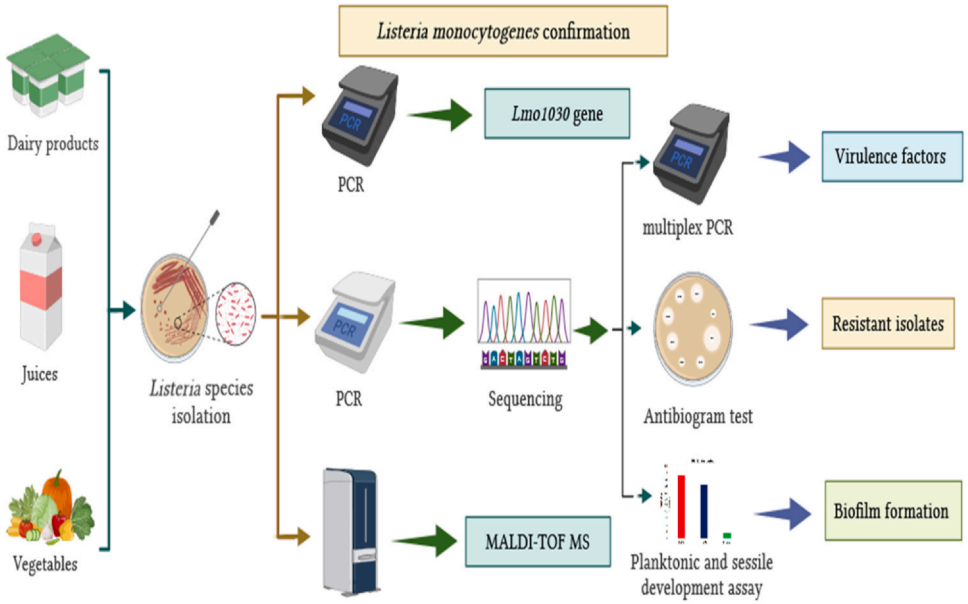
https://doi.org/10.1016/j.jiph.2024.02.014



Scheme 1. Schematic diagram illustrating the work flow of detection and analysis of *Listeria monocytogens* from food sources.

monocytogenes' pathogenicity. These virulence potentials are also influenced by temperature, the presence or absence of oxygen, osmotic stress, and pH [8].

The putative phophoribosyl pyrophosphate synthetase (*prs*) gene is present in all *Listeria* species used to determine the genus *Listeria* [9]. Several genes encode virulence factors responsible for the pathogenicity of *L. monocytogenes*. Those virulence factors are linked to crucial phases of infection, including adhesion, invasion, reproduction, motility, and intercellular spread into host cells [10]. The *prf*A gene encodes a transcription regulator controlling the expression of several virulence factors in *L. monocytogenes*, including the internalins A and B (*inl*A, *inl*B), the listeriolysin O (*hly*A), the actin assembly-inducing protein (*act*A), or the phosphatidylinositol-phospholipase C (PI-PLC, *plc*A) [10]. The pathogenicity of the bacteria and the result of the infection are significantly influenced by these virulence factors. The major factors affect virulence potentials are pH, osmotic stress, oxygen presence or absence and temperature [11]. Besides additional associated virulence factors, such as the invasion-associated protein (*iap*), some genetic markers for antibiotic resistance can also be present in *L. monocytogenes* [6]. The virulence factor PI-PLC, which is only present in *L. monocytogenes* and *Listeria ivanovii*, is a valuable marker for differentiating between pathogenic and non-pathogenic *Listeria* species [11].

The existence of multidrug-resistant *L. monocytogenes* in RTE foods is viewed as a public health indicator, particularly among high-risk groups. Information about the importance of food safety standards, as well as medicines used in public and in animals, is strongly advised [12]. The World Health Organization (WHO) produced a World Road Map (WRM) on Antimicrobial Resistance (AMR) to resolve the issue of increasing morbidity and mortality rates [13]. The emergence of Antibiotic Resistance Genes (ARGs) in *L. monocytogenes* and antimicrobial resistance have the potential to become major public health issues, especially in relation to the food industry. Reports of resistant strains of *L. monocytogenes* in milk and milk products are frequent [14]. However, studies on the occurrence of *Listeria* infections are very limited in India, particularly because of difficulties in isolation and identification. Conventional detection of *Listeria* species via morphological or biochemical based approaches is extensive and time-consuming. Whereas molecular methods like PCR target specific target genes and DNA sequencing is much more rapid, accurate, and hence more suitable to detect *L. monocytogenes* [15]. Besides, morphological and biochemical similarities existed between the *Listeria* species, which often limits the robustness of *L. monocytogenes* identification [16]. Amplification of 16S rRNA has been used for the recognition, identification, and distinction of *L. monocytogenes* from various sources [16]. Another quick method for identifying bacteria is Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). This technique uses the simultaneous desorption and soft ionisation of a co-crystallized sample-matrix mixture to detect unfragmented peptides from the sample [17]. The capacity of *L. monocytogenes* to attach to surfaces and form biofilms is crucial for their survival, persistence, and it may result in food contamination [18]. Biofilm formation most certainly contributes to the contamination of the food supply by *L. monocytogenes*. The different street or RTE foods, including the very popular ice creams and drinks, can be a source of infection. Unfortunately, enumeration of such popular food sources has not been carried out for *Listeria* in most parts of the country. Large listeriosis outbreaks are usually linked to consumption of RTE food products, such as dairy and meat products, fish, and poultry. Consumption of products, such as fresh fruit and frozen vegetables, at home has also been linked to outbreaks [19]. The purpose of the current investigation was to enumerate and understand the extent of *Listeria* contamination in popular food products in a city in North Karnataka, India, which is a cosmopolitan city serving a variety of foods. The secondary objective was to characterize isolated *Listeria* species using molecular approaches and enumerate the genes encoding key virulence factors as well as antibiotic resistance. Additionally, it was determined how biofilm development varied between strains and how pH affected biofilm formation and growth. The work flow of the study has been represented in a schematic diagram (Scheme 1).

2. Materials and methods

2.1. Bacterial strain

L. monocytogenes 5260 was used as a standard strain and obtained from the Microbial Type Culture Collection, National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune (411008), Maharashtra, India.

2.2. Sample size and collection of samples

The sample size was estimated based on the method for sample size estimation for a single proportion. The formula for the sample size is given by,

$$n = Z_{1-\alpha/2}{}^2 \frac{p.(1-p)}{d^2}$$

(Z = Level of significance, at 5% =1.96; p: Anticipated prevalence; d: absolute precision).

The anticipated prevalence of *Listeria* in all food items would be approximately 8%, and therefore, at the 5% level of significance and 5% absolute precision, a total sample size of 114 was estimated. The sampling technique that was used in the study was convenient sampling, which was randomly chosen across the geographical location from the list of sampling frames. Due to COVID-19 restrictions, sample collection was limited to 92 only for this study (Supplementary Table S1). These samples, comprising dairy and street food products, were randomly collected from different street vendors and dairy booths in and around the city of Belagavi, North Karnataka, India, from 2020 to 2021. The milk samples included milk (n = 30), curd, buttermilk, paneer, and ghee (n = 22), street food (panipuries, sauces, and juices) (n = 33), as well as salad (fruit and vegetables) (n = 7). All the collected samples were processed for storage in ice boxes and examined instantly after arrival at the laboratory.

2.3. Isolation and characterization of L. monocytogenes

After the sampling process, the dairy products and street food were processed with 225 mL of Fraser broth (HiMedia). Further, preculturing positive broth was spread onto *Listeria* palcam agar (HiMedia) and incubated at 37 °C for 24 h [20]. Biochemical identification was performed using gram staining, oxidase, catalase, the methyl-red test, and Voges-Proskauer tests [21]. Colonies of *L. monocytogenes* were further confirmed by β-hemolytic activity (CAMP-test: Christie-Atkins-Munch-Peterson) using blood agar with the hemolytic activity of Staphylococcus aureus used as a positive control. The plates were then incubated at 37 °C for 18–24 h to observe the signs of β-hemolysis, wherein CAMP is a positive species [22].

2.4. Molecular analysis

2.4.1. DNA extraction, quality and quantity analysis

Bacterial DNA extraction through a conventional modified CTAB (Cetyltrimethylammonium bromide) protocol was conducted. To confirm the presence of DNA, a 1% agarose gel was used. An Eppendorf BioPhotometer Plus (Hamburg, Germany) was used to determine the concentration and purity of (1.8 - 2, A260/A280) the isolated genomic DNA. A precise amount of genomic DNA was diluted in TE buffer to a concentration of 50 ng/μL and maintained at − 20 °C.

2.5. PCR identification of Listeria genus

Listeria genus primer (*prs* gene) procured from Integrated DNA Technologies (IDT, India) was amplified with 11 *Listeria* spp. Samples, which were obtained in selective media [9]. The total reaction volume was 25 μL, which contained 2.5 μL PCR buffer, 1 μL (50 ng/μL) template DNA, 1 μL (10pmol/μL) of each primer (forward and reverse), 1 μL dNTPs (0.1 mM), 1 μL Taq polymerase (3 U), and the remaining volume was adjusted with nuclease-free water (GeNei™, India). The PCR reaction conditions for the *prs* gene was optimised using the Eppendorf Nexus GX2 Mastercycler (Hamburg, Germany) (Table 1) [9]. Amplified products were electrophoresed on 1.5% agarose gels in 1X TAE buffer along with size indicators of 100 bp DNA ladders (GeNei™, India) and recorded using a gel documentation system (Syngene G: Box F3, UK).

2.6. PCR identification of Listeria species

For *L. monocytogenes* identification, a primer of *lmo*1030 was used (Table 1). Primers *nam*A, *lin* 0464 Oxidoreductasi, *lmo* 0333, and *scr*A are used for the other *Listeria* species detection, which include *Listeria innocua*, *Listeria ivanovii*, *Listeria grayi*, *Listeria seeligeri*, and *Listeria welshimeri* [16,21] (Supplementary Table 2). All the primers were commercially synthesized and procured from IDT, India. The PCR reaction consisting of total reaction volume 25 μL, which contained 2.5 μL PCR buffer, 1 μL (50 ng/μL) template DNA, 1 × 6 μL (10pmol/μL) of each primer, 1 μL dNTPs (0.1 mM), 1 μL Taq polymerase (3 U), and the remaining volume was adjusted with nuclease-free water. An Eppendorf Nexus GX2 thermal cycler (Hamburg, Germany) was used to amplify the DNA samples extracted from *Listeria* species. The reaction conditions involved initial denaturation at 94 °C for 2 min, followed by 35 cycles in series of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 45 s, with a final step of one cycle at 72 °C for 2 min to final extension [22]. Amplified products were electrophoresed on 1.5% agarose gels in 1X TAE buffer along with size indicators of 100 bp DNA ladders (GeNei™, India) and recorded using a gel documentation system (Syngene G: Box F3, UK).

2.7. Detection of virulence associated genes of L. monocytogenes

Using multiplex PCR, virulence factor genes including *act*A, *hly*A, *plc*A, *inl*A, *prf*A and *iap* were identified. Although seven total genes were attempted for multiplex PCR, only six genes were amplified by the multiplex PCR and the remaining one (*plc*A) was used in a separate PCR reaction. For that, primers were commercially synthesized and procured from IDT, India, and the PCR condition was optimized for all virulence genes (Table 2) [23]. Amplified products were electrophoresed on 1.5% agarose gels in 1X TAE buffer along with size indicators of 100 bp DNA ladders (GeNei™, India) and recorded using a gel documentation system (Syngene G: Box F3, UK).

3. Sequencing of *Listeria* species

Sequencing was performed on the amplified products of 16 S rRNA, which was commercially synthesised (IDT India) for this study. The primer sequences and PCR conditions are presented in Table 1. The QIAquick PCR purification kit (Qiagen) was used to purify the PCR product prior to sequencing (Eurofins Genomics, India). Further, nucleotide sequences were mined using the BLAST tool (NCBI) and sequences were aligned via BioEdit software. The molecular-phylogenetic analysis was performed using MEGA version 11.0.13 [24]. All the obtained sequences were submitted to GenBank® (Table 3).

3.1. MALDI-TOF MS

Genus or species-level identification of isolates was accomplished by MALDI-TOF MS (VITEK® MS, BioMerieux, France) based on the manufacturer's instructions. Briefly, using a sterile stick, a single colony from a fresh overnight culture plate of the bacteria was smeared as a thin film directly onto a MALDI target plate and allowed to dry at room temperature. One microliter α-cyano-4-hydroxycinnamic acid (CHCA) matrix (dissolved in 50% acetonitrile and 2.5% trifluoro acetic acid) was added, allowed to dry, and then the slide was loaded in the VITEK MS system for identification, which uses a laser to ionize the sample molecules [17]. The mass spectra were analysed using Vitek® MS preparation software linked to MYLA

Table 1
Primer pairs sequences and PCR condition used for the *Listeria* detection.

Primer	Primer sequence (5′-3′)	Amplification condition					
prs	GCTGAAGAGATTGCGAAAGAAG	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
	CAAAGAAACCTTGGATTTGCGG	94 °C	94 °C	54 °C	72 °C	32	72 °C
		4 min	30 s	20 s	1 min		10 min
*lmo*1030	GCTTGTATTCACTTGGATTTGTCTGG	94 °C	94 °C	60 °C	72 °C	35	72 °C
	ACCATCCGCATATCTCAGCCAACT	2 min	30 s	20 s	45 s		2 min
16 S rRNA	AGAGTTTGATCMTGGCTCAG	94 °C	94 °C	54 °C	72 °C	32	72 °C
	TACGGYTACCTTGTTACGACTT	7 min	45 s	30 s	1 min		7 min

software (BioMerieux, France). The system displays a green spot for organism identification with a confidence value of 99.9%, indicating reliable identification at the species level and a red spot indicates no organism identification. The samples were analysed in duplicate. ATCC *Escherichia coli* 8739 was used as an internal quality and calibration control [25].

3.2. Determination of antimicrobial resistance

The standard Kirby-Bauer disc diffusion method [26] was carried out for the testing of samples [26]. The total of seventeen antibiotics with specified concentrations (HiMedia) were applied to samples for formation of zone of inhibition diameter for Doxycycline (DO-30 µg), Penicillin (P-10 µg), Cefotaxime (CTX-30 µg), Chloramphenicol (C-30 µg), Azithromycin (AZM-15 µg), Nalidixic acid (NA-30 µg), Levofloxacin (LE-5 µg), Ciprofloxacin (CIP-5 µg), Co-trimoxazole (COT-25 µg), Gentamicin (GEN-10 µg), Clindamycin (CD-2 µg), Tetracycline (T-30 µg), Vancomycin (VA-30 µg), Cefepime (CPM-30 µg), Amikacin (AK-30 µg), Ampicillin (AMP-10 µg) and Norfloxacin (NX-10 µg) [13,27].

3.3. Biofilm related assays

3.3.1. Planktonic and sessile development assays

L. monocytogenes isolates were further examined for biofilm formation. The sessile development assay was performed following the classical staining method with crystal violet [28]. Briefly, overnight cultures of *Listeria* isolates were adjusted at 0.09 (OD at 600 nm) in sterile Brain Heart Infusion (BHI) medium and 200 µL loaded into the wells of a 96-well polystyrene microtiter plate prior to incubation at 37 °C. Further, the supernatant was removed from the wells, which were washed with Phosphate Buffer Saline (PBS). Absolute ethanol was then applied for fixation (30 min). After emptying and air drying the wells, 200 µL of an aqueous solution of crystal violet (0.1%) was added and left for 15 min. After washing with water, the bound dye was solubilized with 200 µL of an aqueous solution of acetic acid (33%). The contents of each well (150 µL) were transferred to a sterile polystyrene microtiter plate, and absorbance was finally measured using a microtiter plate reader (EPOCH, BioTek, USA) set to 595 nm. The experiment was repeated at least three times.

Growth curves were performed on growing L. monocytogenes isolates at 37 °C in BHI medium. A 100 µL inoculum was collected from diluted overnight cultures and added to 10 mL of BHI. 200 µL of each cell suspension was added to triplicate wells in a 96 well microtitre plate. The plates were incubated under shaking at 37 °C. The OD at 595 nm was recorded (0.5, 1, 2, 3, and 6 h) using a microplate reader (EPOCH, BioTek, USA). To investigate the effect of pH on growth, bacteria were grown in the same conditions with pH adjusted to 4.5, 5.5, 7.5, and 8.6 and OD at 595 nm recorded after different times [29].

Table 3
Listeria spp. and *Listeria monocytogenes* from diary and street food samples.

Sample Code	Sample name	*Listeria* genus (P: Present)	*Listeria monocytogenes* (A: Absent)	GenBank® accession number
L-1	Curd	P	A	OP615949
L-4	Milk	P	A	OP615950
L-6	Milk	P	A	OP615951
L-7	Milk	P	A	OP615952
L-21	Shreekhand	P	A	OP615953
L-23	Buttermilk	P	P	OP615954
L-24	Milk	P	A	OP615955
L-70	Milk	P	A	OP615956
L-72	Milk	P	A	OP615957
L-74	Milk	P	P	OP615958
L-84	Panipuri	P	A	OP615959

4. Results

4.1. Identification of Listeria monocytogenes isolates from different dairy and street food sources

A total of 92 samples comprising diary (milk, curd, buttermilk, paneer and ghee) and street food (panipuries, sauces, juices, fruit and vegetables) products collected were screened for the presence of

Table 2
Primer pairs sequences and thermo cycling conditions used for *Listeria monocytogenes* virulence genes detection.

Target genes	Primer sequence (5'–3')	Direction	Amplicon size (bp)	PCR steps	Time & Temp.
*inl*A	CCGCACTCACTAACTTAGAG	F	580	Initial denaturation	4 min at 94 °C
	GTTGTTTCTTTGCCGTCCAC	R			
*inl*B	CCTAAACCTCCGACCAAACA	F	293		
	CCATTTCGCGCTTCTCTATC	R			
*prf*A	CTCAAGCAGAAGAATTCA	F	695	Denaturation	45 s at 94 °C
	TCCCCAAGTAGCAGGACA	R		Annealing	30 s at 54 °C
iap	TTTGCTAAAGCGGGTATCTC	F	205	Extension	1 min at 72 °C
	AGCCGTGGATGTTATCGTAT	R		(32 Cycle)	
*act*A	TGCATTACGATTAACCCCGACA	F	431		
	AGGCTTTCAAGCTCACTATCCG	R			
*plc*B	AGTGTTCTAGTCTTTCCGG	F	792	Final extension	10 min at 72 °C
	ACCTGCCAAAGTTTGCTGT	R			
*hly*A	ACGCAGTAAATACATTAGTG	F	372		
	AATAAACTTGACGGCCATAC	R			

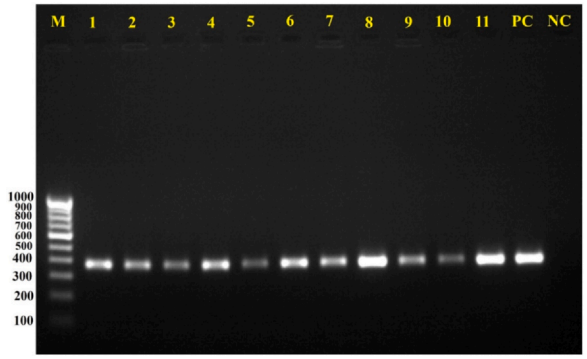


Fig. 1. Amplification of *prs* gene from *Listeria* species isolates at 370 bp [Lane M: 100 bp DNA ladder; Lane 1–11: *Listeria* isolates; PC: Positive Control; NC: Negative Control].

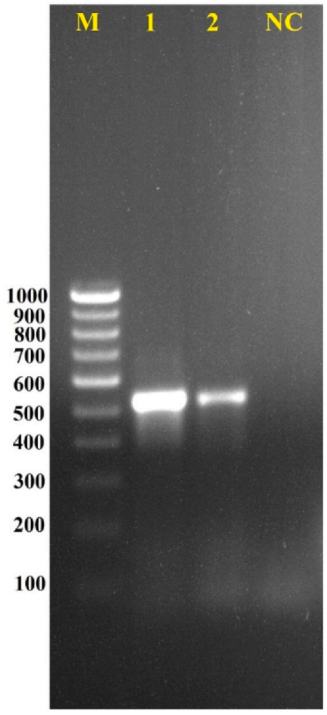


Fig. 2. Amplification profiles of *lmo*1030 gene from *Listeria monocytogenes* strain L-23 and L-74 isolates at 509 bp [Lane M: 100 bp DNA ladder; Lane 1: L-23 strain; Lane 2: L-74 strain; NC: Negative Control].

Listeria spp. on Palcam agar. The typical *Listeria* spp. colony characteristics appeared as grey to black colonies surrounded by a black halo (Supplementary Material Fig. S1). A total of 11 putative *Listeria* spp. colonies were picked and taken through a number of biochemical analyses for further characterization. Out of the 11 isolates, 7 originated from milk, 2 from buttermilk and 2 from street food (Table 3; Supplementary Material Table S3); none was detected from fruits or vegetable salads. Gram staining assay revealed that all 11 colonies contained Gram-positive rod bacterial cells. Besides, MR-VP, oxidase and catalase assays were all positive tests, which are typical of the species *Listeria*. Plating the isolates on blood agar, it appeared that all of them showed either complete or partial haemolysis (Supplementary Material Figs. S2-S5). To confirm the 11 isolates belong to the *Listeria* genus and potentially identify *L. monocytogenes* species, genomic DNA was extracted through a modified CTAB method prior to PCR amplification of a 370 bp region of the *prs* gene (Fig. 1). Two samples were detected of *L. monocytogenes* from the dairy product with the amplification of a 509 bp region of the *lmo*1030 gene (Fig. 2). Additionally, MALDI-TOF MS also confirms the presence of two *L. monocytogenes* from the dairy product. All 11 *Listeria* spp. samples were further processed for 16 S rRNA sequencing. The two isolates L-23 and L-74, originating from milk and buttermilk samples, respectively, were confirmed to belong to *L. monocytogenes* species (Fig. 3).

4.2. Presence of virulence factor genes in L. monocytogenes isolates

L. monocytogenes virulence genes, including *iap*, *inl*B, *hly*A, *act*A, *inl*A, *prf*A, and *plc*A, were examined in strains L-23 and L-74. The results showed that both strains (L-23 and L-74) possessed the genes *iap*, *inl*B, *hly*A, actA, *inl*A, *prf*A and *plcA* with 205 bp, 293 bp, 372 bp, 431 bp, 580 bp, 695 bp and 792 bp, respectively (Fig. 4a and Fig. 4b; Table 2). Overall, 11/92 samples were contaminated with *Listeria* spp., whereas 2/92 samples were positive for *L. monocytogenes* (Supplementary Table S3).

4.3. Antibiotic susceptibility patterns of L. monocytogenes isolates

The phenotypic antibiotic characteristics of *L. monocytogenes* were detected through disc diffusion method using seventeen antibiotics. Antibiotics were applied to only two samples that confirmed *L. monocytogenes* via PCR (L-23 and L-74). In this study, zone of inhibition observed using a calliper in two samples and analysed the range (resistant, sensitive and intermediate). Samples L-23 and L-74 showed susceptibility to Levofloxacin, Ciprofloxacin, Co-trimoxazole, Gentamicin, Tetracycline, Cefepime, Amikacin, Ampicillin, Norfloxacin, Doxycycline, Vancomycin, Penicillin, Cefotaxime and Chloramphenicol, respectively. While sample L-74 was resistant to Nalidixic acid, Co-trimoxazole, Clindamycin, Ampicillin, Norfloxacin, Cefotaxime and Azithromycin, sample L-23 was only resistant to Clindamycin and Azithromycin. The other antibiotics, such as Levofloxacin, Ciprofloxacin, Vancomycin and Penicillin, showed intermediate results against L-74, and only one antibiotic, Nalidixic acid observed intermediate results against L-23 (Table 4).

4.4. Planktonic and sessile development of L. monocytogenes isolates

To evaluate the sessile development of *L. monocytogenes* over a surface, a crystal violet assay was performed. Both isolates were able to form biofilm on polystyrene surfaces in terms of the number of biofilm-producing strains, the other nine *Listeria* species demonstrated the ability to create biofilms (Fig. 5a; Supplementary Material: Fig. S6). In the tested conditions, no much difference in the sessile biomass reached over 24 h for both *L. monocytogenes* isolates (L-23 OD at 595 = 0.95 ± 0.17; L-74 (OD at 595 = 0.82 ± 0.12). Growing *L. monocytogenes* isolates in planktonic conditions (37 °C at BHI media), it appeared they exhibit a similar maximum specific growth rate (μ max) of 6 h^{-1} and likewise varied growth rates were also shown by other *Listeria* species (Fig. 5b; Supplementary Material Fig. S7). Considering the effect of pH encountered in various food products on bacterial growth, *L. monocytogenes* isolates and *Listeria* species were cultured at pH 4.5, 5.5, 7.5 and 8.6 (Fig. 5c; Supplementary Material Fig. S8). While no growth could be observed in acidic conditions, higher biomass could be reached at pH 8.6 compared to 7.5 (in BHI at 37 °C) with no significant difference between the two *L. monocytogenes* isolates and *Listeria* species (Fig. 5c; Supplementary Material Fig. S8).

5. Discussion

L. monocytogenes can contaminate food products and survive at low temperatures. It has been reported to cause a high mortality rate [3,5]. The overall prevalence of *Listeria* spp. in 92 different food products was 11.95% (11/92), whereas *L. monocytogenes* was detected

Fig. 3. UPGMA-based phylogenetic tree illustrating the relationships among isolates of *Listeria* spp. identified using 16 S rRNA sequencing analysis (see Table 3 for sample label).

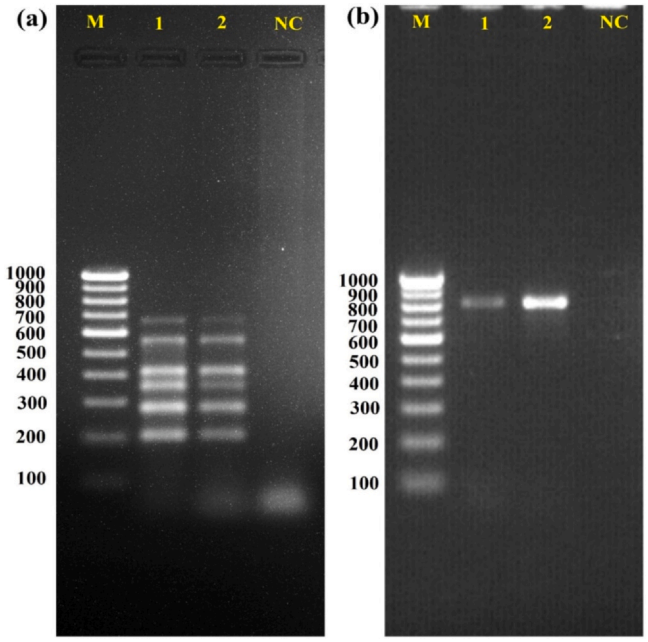


Fig. 4. Amplification profiles of virulence genes from *Listeria monocytogenes* isolates by multiplex PCR. **(a):** Lane 1: L-23 and Lane 2: L-74 have six virulence genes (*iap* (205 bp), *inl*B (293 bp), *hly*A (372 bp), *act*A (431 bp) *inl*A (580 bp) and *prf*A (695 bp); **(b):** Lane 1: L-23; Lane 2: L-74 have virulence gene *plc*A (792 bp) [Lane M: 100 bp DNA ladder and NC: Negative Control].

in 2.17% of our samples. More specifically, *L. monocytogenes* was found in milk and buttermilk samples, respectively, while none of the ice cream, sauces, juices, and street food samples were contaminated with *Listeria.* A similar study showed a high prevalence of *L. monocytogenes* 52.7% (219/541) in branded milk, cheese, ice cream, milk powder, milk sweets, ghee, paneer, and yoghurt [30]. In contrast, *L. monocytogenes* was detected in 6.8% (17/250) of food samples in Egypt [21]. A similar study conducted in Portugal indicated *L. monocytogenes* contaminated 7% (72/1035) of food products (raw food like milk, meat and fish, and processed food like ready-to-eat) [31], whereas *Listeria* spp. and *L. monocytogenes* were detected in 25% (96/384) and 6.25% (24/384) of food products, respectively, in Ethiopia [12]. Thus, there is a large variety in the level of contamination of food products by *L. monocytogenes* depending on the geographical location and certainly related to sample size, food products and/or hygienic conditions. Although a dendrogram based on the 16 S rRNA gene indicated two main groups of *Listeria* detected with their distinct genetic characteristics, it was noticed that both 16 S rRNA gene sequencing and MALDI-TOF MS were shown to be very useful in this study for the identification of *L. monocytogens* because it is a rapid, accurate and straightforward identification technique before the characterisation of pathogenic genes [17,32].

Both of the *L. monocytogenes* isolates that were examined in this investigation using multiplex PCR had all seven of the virulence factor genes, viz., *inl*A, *inl*B, *prf*A, *iap*, *act*A, *plc*B, and *hly*A. Detection of a few virulence genes in *L. monocytogenes* strains from food samples was previously reported in studies from China [23], Iran [33], and Egypt [34] in dairy products. Although further in-depth investigations are required, this study confirms the presence of virulent *L. monocytogenes* strains within the limited sample size. As revealed by the antibiogram, two *L. monocytogenes* isolates showed antibiotic resistance that included nalidixic acid, co-trimoxazole, clindamycin, ampicillin, norfloxacin, cefotaxime, and azithromycin for L-74, and clindamycin and azithromycin for L-23 only (Table 4). The remaining *Listeria* isolates were susceptible to the antibiotics. While *L. monocytogenes* is generally considered susceptible to most antibiotics, some other studies reported resistance of *L. monocytogenes* food isolates to vancomycin, penicillin, gentamycin

Table 4
Antibiogram profiles of *Listeria monocytogenes* strains.

Antibiotics (µg/disc)	Diameter of zone of inhibition (mm)			*L. monocytogenes* strains	
	Resistant (R)	Intermediate (I)	Sensitive (S)	L-23	L-74
Nalidixic acid (NA-30 µg)	≤ 13	17-18	≥ 19	I (18)	R (0)
Levofloxacin (LE-5 µg)	≤ 15	16-18	≥ 19	S (39)	I (17)
Ciprofloxacin (CIP-5 µg)	≤ 15	16-20	≥ 21	S (40)	I (18)
Co-trimoxazole (COT-25 µg)	≤ 13	14-22	≥ 23	S (36)	R (0)
Gentamicin (GEN-10 µg)	≤ 12	13-14	≥ 15	S (36)	S (30)
Clindamycin (CD-2 µg)	≤ 14	15-20	≥ 21	R (0)	R (0)
Tetracycline (T-30 µg)	≤ 14	15-18	≥ 19	S (38)	S (28)
Vancomycin (VA-30 µg)	≤ 14	15-16	≥ 17	S (22)	I (16)
Cefepime (CPM-30 µg)	≤ 21	22-23	≥ 24	S (34)	S (28)
Amikacin (AK-30 µg)	≤ 14	15-16	≥ 17	S (38)	S (29)
Ampicillin (AMP-10 µg)	≤ 13	14-20	≥ 21	S (32)	R (13)
Norfloxacin (NX-10 µg)	≤ 12	13-16	≥ 17	S (33)	R (12)
Doxycycline (DO-30 µg)	≤ 12	13-15	≥ 16	S (34)	S (26)
Penicillin (P-10 µg)	≤ 19	20-27	≥ 28	S (40)	I (20)
Cefotaxime (CTX-30 µg)	≤ 14	15-22	≥ 23	S (28)	R (14)
Chloramphenicol (C-30 µg)	≤ 12	13-17	≥ 18	S (32)	S (21)
Azithromycin (AZM-15 µg)	≤ 13	14-17	≥ 18	R (0)	R (0)

amoxicillin, ampicillin, erythromycin, rifampicin, chloramphenicol, levofloxacin, azithromycin, oxytetracycline and trimethoprim-sulfamethoxazole [21], clindamycin [23] and against neomycin and tetracycline [6].

The primary cause of contamination, post-processing contamination, and cross-contamination of the finished product which mostly results in food spoilage and foodborne illness, is biofilm growth on foods and food contact surfaces [35]. Controlling biofilms is the first step to preventing *L. monocytogenes* contamination [36]. However, factors like temperature, pH, media used for growth and time may affect how quickly biofilm formation [29]. In this study, both *L. moncytogenes* (L-23 and L-74) were grown on the BHI medium for varied lengths of time and pH scales, and it was shown that alkaline media (pH 8.6) produced the most growth after 24 h compared to other tested conditions. It is known that the effects of various factors on the development of biofilms vary depending on the strain and therefore, setting up appropriate risk assessment programs in the food sector requires a thorough understanding of how environmental factors affect the development of *L. monocytogenes* biofilms and their regulating mechanisms [37].

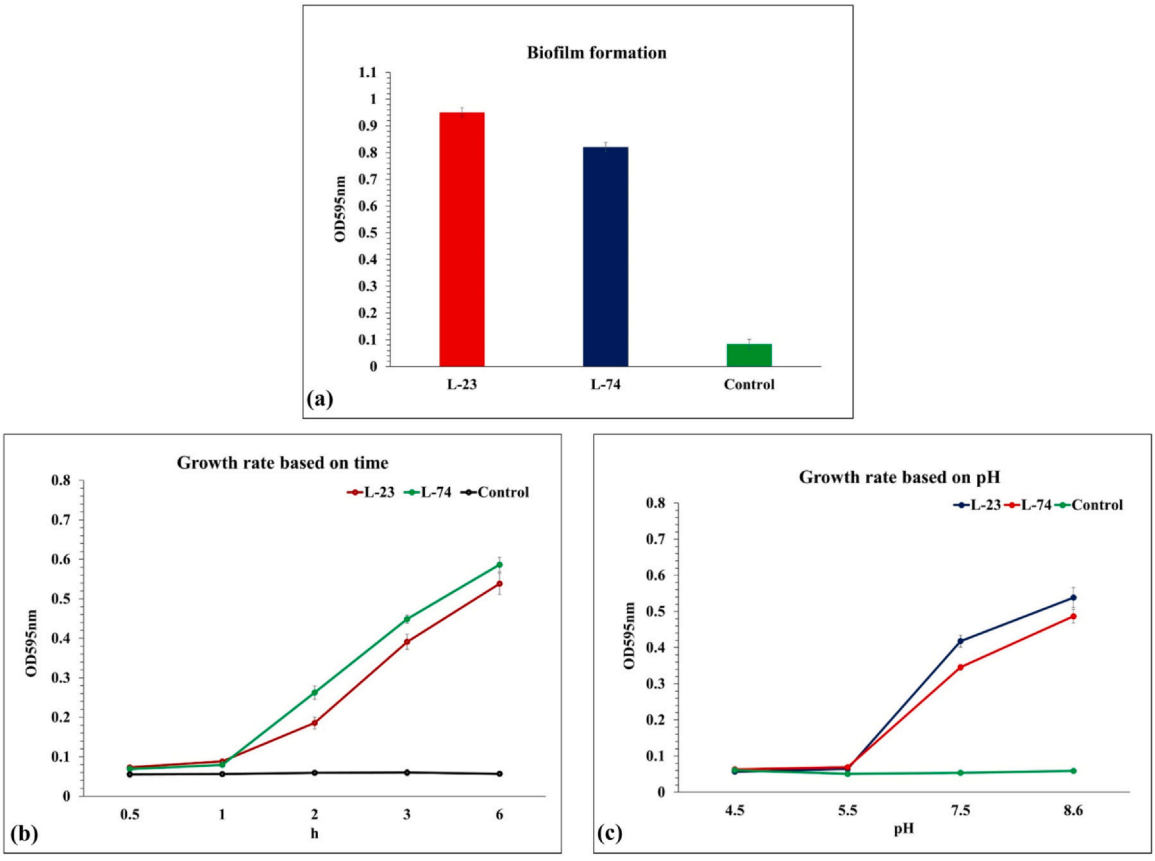


Fig. 5. Growth curves of *Listeria monocytogenes* L-23 and L-74 isolates at (a) Biofilm formation; (b) different pH and (c) different time.

6. Conclusion

Results of this study confirm that, within the limited sample size, the presence of *L. monocytogenes* in dairy and street food products is more prevalent than originally thought. Besides the need to increase the standard of hygienic measures for these food sources, this study highlights the importance of the surveillance of these food-borne pathogens to limit the risk of contamination and infection and ultimately preserve human health, especially for the persons at risk, including pregnant women, children, the elderly and immunocompromised people.

Funding

This research received no external funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors grateful to KLE Academy of Higher Education & Research, Belagavi and Department of Biotechnology, Govt. of India for necessary support. All authors appreciate the financial support provided by Dr. Mickaë Desvaux, INRAE, France. The authors thankful to the MALDI-TOF MS support from Department of Microbiology, JIPMER, Puducherry, India.

Author contributions

RKS carried out the sample collection and laboratory experiments. SH participated in molecular studies and study design. The manuscript was written by SH and RKS. SH, RKS, SJ, SP, SG, MD and SR performed interpretation, analysis of results and participated in correction of the manuscript. The final manuscript was read and approved by all authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2024.02.014.

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