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RESEARCH ARTICLE

Prevalence and Antimicrobial Resistance Pattern of *Listeria Monocytogenes* in Ready t o Eat Foods in Tamil Nadu, India

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Abstract

Objectives: To examine the prevalence and antibiotic resistance pattern of *Listeria monocytogenes* in RTE food to understand the risk of contamination in the food industries. **Methods:** A total of 105 different samples of RTE foods, including curd, cheese, almond milk, banana milkshakes, chocolate, and strawberry milkshakes were collected from street vendors and departmental stores in and around Kanchipuram, Tamil Nadu. As per ISO 11290-1:2017(E) protocol suspected *Listeria* colonies were selected and identified using standard biochemical tests. Positive colonies were then confirmed by PCR, and isolated confirmed strains were tested for their susceptibility to 12 widely-used antibiotics. **Findings:** Of the 105 samples, 15 (14.28%) showed a positive, and the overall extent of *Listeria monocytogenes* in RTE foods was about 14.3%. The results indicate that curd (n=10) and cheese (n=10) had the highest prevalence of *L. monocytogenes* (30%), followed by almond milk (25%), banana milkshake (13.33%), chocolate, and strawberry, which had a 10% prevalence each. Moreover, antibacterial susceptibility to 12 antimicrobial agents of 25 isolates of *L. monocytogenes* screened from fifteen RTE foods were tested and highlighted the emergence of multi-drug resistant patterns. **Novelty:** Our findings reveal that the Kanchipuram district has a high incidence of *L. monocytogenes* in RTE foods, particularly cheese, curd, and almond milk, which may cause multidrug resistance to the antibiotics used to treat listeriosis.

Keywords: Foodborne Pathogen; Listeria monocytogenes; Ready to eat Food; Antimicrobial Resistance

1 Introduction

Foodborne pathogens are not only the key source of foodborne disease and also the main issue of global food safety. They are emerging and becoming a significant public health problem worldwide. It is the etiological agent responsible for animal and human listeriosis *Listeria monocytogenes* is a foodborne pathogen, widely found in milk,

meat, aquatic products, frozen and chilled foods, and ready-to-eat foods[1]. *L. monocytogenes* is the etiological representative of listeriosis, a food-borne pathogen that causes 23,000 cases of aggressive human infections every year worldwide[2]. Usually, the pathogen contaminates the RTE products during handling and processing measures such as slicing, cutting, or packaging. The contamination would happen at any stage during manufacturing and handling in retail packaging[3]. For these reasons, *L. monocytogenes* poses a significant risk to the food industry, meat products, and particularly producers of RTE foods continue to be one of the three most RTE food categories typically associated with human listeriosis[4]. Food safety regulations in several countries have inclined to implement a zero-tolerance policy for the occurrence of *L. monocytogenes* in RTE foods against human listeriosis outbreaks and sporadic cases of illness, predominately related to the manufacture and commercialization of these products[5]. Symptoms of listeriosis such as encephalitis, septicaemia, and mild symptoms may include fever, nausea, vomiting, muscle aches, and diarrhoea. They are moderate for adults but are aggravated for new offspring, pregnant women, aged persons, and immuno-compromised persons[6]. *L. monocytogenes* are gram-positive, facultatively anaerobic, rod-shaped bacterium. This bacterium is able to motile at the temperature between 22–28°C but non-motile above the temperature of 30°C, non-spore-forming. *L. monocytogenes* is able to grow at the range from -0.4°C to 45°C temperature, with an optimal temperature of 37°C. This organism can grow under proper environments like low pH, high salt concentration, and low temperature. The bacterium is pervasive in the atmosphere and also be found in humid locations, soil, moldy foliage, and eatables. Moreover, this bacterium is also able to form biofilm on several surfaces. which makes it problematic to eliminate[7]. Up to the present time, the genus *Listeria* has been identified with 20 species of bacteria. Some of the prominent species *L. monocytogenes* have the most significant impact on public health because of their ability to cause listeriosis both in humans and animals[8]. The conventional method to diagnose *Listeria* through isolation and biochemical characterization is time - consuming and molecular approaches like PCR-specific techniques for the diagnosis of *L. monocytogenes* target specific genes[9].

In addition, a further concern is due to the augmented detection of antimicrobial-resistant *L. monocytogenes* isolates, mainly for antibiotics frequently used for the treatment of listeriosis, specifically a combination of penicillin or ampicillin with gentamicin and trimethoprim-sulfamethoxazole. Delay in treatment in addition to the wide dispersal of multidrug-resistant (MDR) *L. monocytogenes*, which harbour various virulence factors, are considered the main causes of emergent infection. Therefore, it is vital to perform the in vitro antimicrobial pattern before starting the treatment[6]. *L. monocytogenes* isolates showed resistance to ciprofloxacin and tetracycline, a high prevalence of clindamycin and oxacillin resistance was found in meat and fish production chains and significant percentages of resistance against ampicillin, tetracycline, and penicillin G were reported in *L. monocytogenes* strains isolated from meat, fish, and dairy production chains[10]. The multidrug-resistant *L. monocytogenes* predominantly found in RTE foods is being considered a public health indicator, suggesting building awareness about the reputation of food safety regulations as well as drugs used in humans and animals[9]. Further outbreaks may be more difficult to accomplish because of the rise of antimicrobial resistance among *L. monocytogenes* strains isolated from food products[6,10].

Currently, in India, lack of information about the antimicrobial susceptibility and predominance of *Listeria* species presents in RTE foods. Spoilage of RTE food products with *L. monocytogenes* has been shown in several investigations from various countries and different regions of India, but not well studied, particularly in Tamil Nadu, only a few studies were reported in Tamil Nadu Shrinithivihahshini et al., (2011) reported 51/134 (38.1%) samples were positive for *L. monocytogenes* from RTE foods in Tamil Nadu, and Vinothkumar et al., (2013) reported 20/30 (66.66%) samples were positive and Madharsha et al., (2018) 20/40 (50%) samples were positive isolates *L. monocytogenes* from fish samples. Dairy products mainly, milkshakes, almond milk, and ice creams are frequently consumed RTE, such milk products and almond milk can be a potential source of pathogenic *L. monocytogenes* in humans. This study describes the prevalence and antibiotic resistance profiles of *L. monocytogenes* detected in various ready-to-eat food products around Kanchipuram, Tamil Nadu. In addition, the study of partial nucleotide sequence analysis and genetic similarities of the16S rRNA gene of *L. monocytogenes* isolates to various global clones implies potential public health implications[9].

2 Methodology

Samples collection and processing

In our study, a total of 105 different samples from RTE foods were randomly collected from departmental stores and street vendors in and around Kanchipuram, Tamil Nadu. All the RTE samples were carried in polythene bags and kept at (4°C) on an icebox and shifted to the laboratory and the bacteriological analysis was made within 2 hours.

2.1. L. monocytogenes screening

1g of each sample was transferred aseptically to 9 ml of half Fraser broth (HFB) (Himedia, India) for primary enrichment and incubated for 24h at 37°C. After that, 0.1ml of culture from HFB was again inoculated into 10ml of Fraser broth (FB) and allowed to incubate for 24 to 48hrs at 37°C. The culture was then streaked over Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar in a loop (Himedia, India) and incubated at 37°C for 24 to 48hrs. After incubation, about 3 to 5 selected *Listeria* colonies from PALCAM agar were picked up carefully and re-streaked over Tryptic Soy Agar (TSAYE) plates and allowed to incubate for 24hrs at 37°C [11].

2.2 Identification of *L. monocytogenes*

All the suspected colonies of *L. monocytogenes* present in the isolation media were carried out based on biochemical and morphological characteristics including tumbling motility, catalase reaction, haemolytic activity by sheep blood agar through CAMP test, and sugar fermentation test (0.5% L- rhamnose and 0.5% D - xylose) [12,13].

2.3 Antimicrobial resistance profile of *L. monocytogenes* isolated from RTE foods

The antimicrobial-resistance pattern of all *L. monocytogenes* was done by following the disk diffusion method. 12 different antibiotic disks were used (Himedia, India). The antibiotics used were 1. Penicillin G - P (10 unit), 2. Amoxicillin - AMX (10 mcg), 3. Carbenicillin - CB (100 mcg), 4. Methicillin - MET (5 mcg), 5. Azithromycin - AZM (15 mcg), 6. Clindamycin -CD (2 mcg), 7. Lincomycin -L (2), 8. Vancomycin - VA (30 mcg), 9. Rifampicin -RIF (5 mcg), 10. Roxithromycin - RO (15 mcg), 11. Teicoplanin - TEI (30 mcg), and 12. Linezolid - LZ (30 mcg). The result was construed as resistant, susceptible, or intermediate as described by CLSI guidelines value [14].

2.4 Molecular findings of *L. monocytogenes* present in RTE foods

DNA was isolated by QIAamp DNA mini kit from Qiagen Germany. In PCR analysis, the Taq PCR Master Mix Kit from Qiagen, Germany was used. A 50 μL reaction mixture was prepared which includes 25 μL PCR Master Mix, 1 μL of each primer, 2 μL of DNA, and finally, the reaction mixture was adjusted to 50 μL using distilled water. The approached thermal profile was applied during PCR. The sequence for Forward primer was 5'- GTGCCAGCAGCCGCGGTAA -3', and the sequence for reverse primer was 5'- AGGGTTGCGCTCGTTG -3′. PCR cycle: preliminary denaturation at about 95°C for 3 min; every 35 cycles consist of denaturation at 94°C for the 30s, annealing at 53°C for 15s, extension at 72°C for 90 s, and at 72 °C for 7 min for a final extension. Agarose gel (0.7 %) electrophoresis was used to analyse PCR products (15 μL) and envisioned in a gel documentation system under UV light [10].

2.5 Phylogenetic and sequencing analysis of *L. monocytogenes* present in RTE foods

The 16S rRNA gene was sequenced from *L. monocytogenes* by PCR amplicon and confirmed by Gene JET PCR Purification Kit from Thermo Scientific, EU-Lithuania was used for the gene purification. In both directions, PCR products were examined. The 16s rRNA partial nucleotides and amino acid sequence homology analysis among studied isolates and global strains was performed using NCBI-BLAST. Sequences were aligned, edited, and analyzed using ClustalW and Mega software versions.6 [15]. Evolutionary relationships of taxa were carried out UPGMA method [16]. Evolutionary distances and analysis were done by the Maximum Likelihood method using MEGA 11 software [17]. In the bootstrap test (500 repetitions), the part of replicate trees linked with taxa grouped composed were showed subsequent to the branches [18]. The partial nucleotide sequence of *Listeria monocytogenes* strain SELM23 from RTE foods was submitted to GenBank.

3 Results and Discussion

3.1 The predominance of *L. monocytogenes* in different RTE food products

RTE foods are mostly associated with the outbreak of various diseases and rare cases of listeriosis. In particular, *L. monocytogenes* possesses significant public health issues regarding food contamination owing to the capability of *L. monocytogenes* thus growing in cooling conditions or the refrigerator in varieties of food products which makes the pathogen problematic to control. Detection of the bacteria in food products at retail vents is challenging for quality control measures [10,19]. Therefore, this study was designed to detect the overall incidence of *L. monocytogenes*, isolated from RTE food products.The existence of *L. monocytogenes* is present in several RTE foods list given in Table 1. *L. monocytogenes* was seen in 105 RTE food samples.

Of these, 15 (14.3%) samples showed positive for *L. monocytogenes*. *L. monocytogenes* are found in curd and cheese (30%), almond milk (25%), chocolate milkshake and strawberry milkshake (10%), and Banana milkshake (13.33%). However, no *L. monocytogenes* isolates were found in Rose milk and a vanilla milkshake. Typically, isolated colonies were showed in grey-green shiny colonies and diffused colonies showed black shadows on the selective PALCAM agar plate. These isolated colonies were also streaked on TSYEA slants, and used for biochemical testing. The suspected 36 isolates were examined to perform motility test, catalysis activity, and biochemical characterization like sugar fermentation tests using L-rhamnose and D- xylose, Camp test (haemolysis). Among these, 25 isolates exhibited tumbling motility activity gas bubbles formation in catalysis activity and fermentation of L-rhamnose and D- xylose was observed. Arrowhead haemolysis was observed in the camp test. Based on the results the isolated cultures were confirmed as *L. monocytogenes* and their distribution in processed RTE samples were clearly depicted in Table 2. From our findings, *L. monocytogenes* were found in all the samples of dairy products analyzed, and the highest percentage was seen in curd and cheese (30%). The prevalence rate of *L. monocytogenes* showed high when compared to the previous results by Gelbicova et al., (2010) [20]. WHO reported the low findings of *Listeria* species in ten (1.8%) of 549 analyzed samples. From the analyzed products (pasteurized cow's milk, ripened cheeses, ice creams, butter, and semi-hard cheeses), the most predominant source of *L. monocytogenes* was found mainly in blue-veined cheese (9/60). *L. monocytogenes* was seen in 6.3 percent of 195 soft cheese samples, 4.4 percent of 45 hard cheese samples, 2.1 percent of 1656 Gorgonzola cheese samples, and 20 percent of 10 fresh soft cheese samples [21]. This detection value is significantly lower than our value and another study that analyzed 14 cheese samples 6 samples were found in *L. monocytogenes* this finding value is higher than our value [21]. Another twenty-one (n = 21, 13.55%) *L. monocytogenes* was confirmed from 155 presumptive *L. monocytogenes* isolates recovered from dairy samples. The highest prevalence was observed in cheese (n = 12, 57.14%), while 7 (33.33%) was observed in fresh milk and 2 (9.52%) was observed in raw milk. *L. monocytogenes* was detected in 12 (18.46%) of all the dairy samples collected. Six (42.86%) samples from cheese 4 (16%) of fresh milk samples and 2 (7.69%) of raw milk samples tested positive for *L. monocytogenes* among the dairy samples analyzed. [22].

Table 1. Detection of *L. monocytogenes* in different ready–to–eat food samples

S. No.	Samples	Total number tested	Number (%) of positive samples	Number (%) of negative samples
1	Banana Milkshake	15	2(13.3%)	13(86.6%)
2	Chocolate Milkshake	10	1(10.0%)	9(90.0%)
3	Rose milk	15	0	15(100%)
4	Vanilla Milkshake	15	0	15(100%)
5	Strawberry Milkshake	10	1(10.0%)	9(90.0%)
6	Almond milk	20	5(25.0%)	15(75.0%)
7	Curd	10	3(30.0%)	7(70.0%)
8	Cheese	10	3(30.0%)	7(70.0%)
	Total	105	15(14.3%)	90(85.7%)

No = Serial number; 0 = Not Detected

Table 2. Sample wise detection of *L. monocytogenes* strains in RTE foods

S. No	Isolate no	Source	Place
1	SELM01	Banana milkshake - I	ENR
2	SELM02	Banana milkshake - I	ENR
3	SELM03	Banana milkshake - II	WBD
4	SELM04	Banana milkshake - II	WBD
5	SELM05	Chocolate milkshake - I	ENR
6	SELM06	Strawberry milkshake - II	ENR
7	SELM07	Almond milk – I	ENR
8	SELM08	Almond milk – I	ENR
9	SELM09	Almond milk – I	WBD
10	SELM10	Almond milk – I	WBD
11	SELM11	Almond milk – III	KPM
12	SELM12	Almond milk – II	ENR
13	SELM13	Almond milk – II	ENR

Continued on next page

Table 2 continued

14	SELM14	Almond milk – III	WBD
15	SELM15	Curd – II	ENR
16	SELM16	Curd - II	ENR
17	SELM17	Curd - I	KPM
18	SELM18	Curd -I	WBD
19	SELM19	Curd - I	WBD
20	SELM20	Cheese - I	WBD
21	SELM21	Cheese - I	WBD
22	SELM22	Cheese - III	WBD
23	SELM23	Cheese - III	WBD
24	SELM24	Cheese - II	KPM
25	SELM25	Cheese - II	KPM

WBD - Walajabad, KPM -Kanchipuram, ENR– Enathur

In our study, ten varieties of curd samples were considered for studying the prevalence rate of *L. monocytogenes* and the result was about 30% which was higher than the result of earlier reports [23]. This dissimilarity in the ratio of prevalence might be due to the type of cheese, use of thermal treatment, natural properties, cleaning procedures, personal hygiene, aeration, and processing circumstances.

In the current study, *L. monocytogenes* was identified in other RTE foods like Almond milk 25% (n = 25), Banana milkshakes 13.33% (n = 15), chocolate and strawberry milkshakes 10 % (n= 10). Alternatively, it should be highlighted that *L. monocytogenes* was not detected in vanilla milkshakes and rose milkshakes (n= 10) of the RTE food array, because of the composition of the food material and various test methods used.

3.2 Antimicrobial resistance profile of *L. monocytogenes* of RTE foods

The antimicrobial susceptibility of 25 isolates against 12 antibiotics was examined using the Kirby-Bauer method. A susceptibility test was assigned through the disk diffusion method using CLSI guidelines value and the results are summarized in Table 3. The antimicrobial susceptibility curve of isolated strains: among these 24% of isolates from RTE foods showed resistance and 76% of isolates were sensitive to penicillin - G. Amoxicillin resistance was found in about 24% of the isolates, and 76 percent of isolates were sensitive. 36% of isolates exhibited resistance to Carbenicillin antibiotics, and 64% of isolates showed sensitivity. In this study, penicillin- G exhibited resistance to most of the isolates from RTE foods. These results resemble an earlier report by Sanalibaba et al., (2018) [14]. The previous study by Olaniyan et al reported a large proportion of the isolates were susceptible to Amoxycillin in contrast to the present finding [24]. In addition, *L. monocytogenes* is susceptible to beta-lactams, and the standard antibiotic treatment for human listeriosis combines penicillin/ampicillin with an aminoglycoside (gentamicin) [14]. Methicillin resistance was found in nearly 52% of isolates, and in Azithromycin antibiotics, 28% of isolates showed resistance.

Of the total isolates, 40% exhibited resistance to clindamycin. 44% of the isolate showed resistance to Lincomycin and 80% of isolates exhibited sensitivity to the Vancomycin antibiotic. The susceptibility pattern of *L. monocytogenes* was also observed to several antibiotics including amoxicillin, vancomycin, methicillin, clindamycin, and lincomycin. Our findings showed similar to those of Abdeen et al., (2021) [10]. Who reported susceptibility patterns against amoxicillin and vancomycin. Several, reports have showed that *L. monocytogenes* from dairy foods were sensitive to vancomycin [25].

Table 3. Antibiotic-resistant profile of *L. monocytogenes* isolates from different ready–to–eat foods

S. No.	Antibiotics	Resistant Number	%
1	P	6	24
2	AMX	6	24
3	CB	9	36
4	MET	13	52
5	AZM	7	28
6	CD	10	40
7	L	11	44
8	VA	5	20
9	RF	13	52
10	RO	9	36

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Table 3 continued

11	TEI	14	56
12	LZ	6	24

(1. Penicillin G - P (10 unit), 2. Amoxicillin – AMX (10 mcg), 3. Carbenicillin - CB (100 mcg), 4. Methicillin – MET (5 mcg), 5. Azithromycin – AZM (15 mcg), 6. Clindamycin -CD (2 mcg), 7. Lincomycin -L (2 mcg), 8. Vancomycin – VA (30 mcg), 9. Rifampicin –RIF (30 mcg), 10. Roxithromycin – RO (15 mcg), 11. Teicoplanin -TEI (30 mcg), and 12. Linezolid -LZ (30 mcg))

Subsequently, 52% of isolates exhibited resistance to Rifampicin, and 36% of isolates showed resistance to Roxithromycin antibiotics. 56% of isolates found resistance to Teicoplanin antibiotics and 24% resistant against linezolid. In detail 6 isolates exhibited resistance to 1 antibiotic, 7 isolates showed resistance to 2 antibiotics and 1 isolate showed resistance to many antibiotics Figure 1. However, from the latest reports, *L. monocytogenes* showed 100% susceptibility to most antibiotics, which emphasized the importance of continuous monitoring of antimicrobial susceptibility patterns and their effects on public health [10]. The levels of resistance differ from the strains and the antibiotics used in humans, and animals and also by the geographical differences [14].

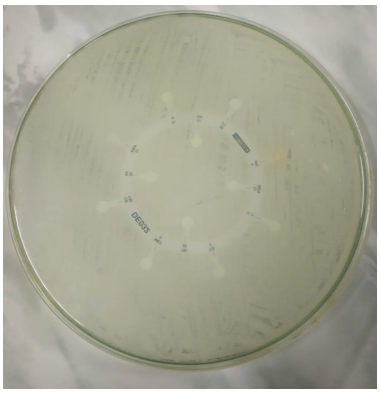

Fig 1. Antibacterial resistance of *L. monocytogenes* (SELM23)strain isolated from RTE foods. (1. Penicillin G - P (10unit), 2. Amoxicillin – AMX (10 mcg), 3. Carbenicillin - CB (100 mcg), 4. Methicillin – MET (5 mcg), 5. Azithromycin – AZM (15 mcg), 6. Clindamycin -CD (2 mcg), 7. Lincomycin -L (2 mcg), 8.Vancomycin – VA (30 mcg), 9. Rifampicin –RIF (30 mcg), 10. Roxithromycin – RO(15 mcg), 11. Teicoplanin -TEI (30 mcg), and 12. Linezolid -LZ (30 mcg))

3.3 Sequencing analysis of *L. monocytogenes*

Genetic comparison of *L. monocytogenes* isolated from RTE foods was analyzed using 16s rRNA sequence and compared with global strains. The partial sequence data were deposited to GenBank and the accession number is OM004047. The phylogenetic analysis of the study revealed that the *L. monocytogenes* strain (OM004047) isolated from this study was closely clustered with other *L. monocytogenes* strains (accession numbers MW13628, MT124478, MT534278, OK001790, MT509640). Also, our species also clustered with other *Listeria spp. L. welshimeri* (strain OK001784), *L. innoca* (strain MW242696, OK001783, MW242699) in the phylogenetic tree inFigure 2.

From this sequencing result, it has been evidenced that the species isolated from RTE food was the *Listeria* species. In the present study *L. monocytogenes* (strain SELM23) showed resistance to the majority of the antibiotics selected and used for 16s rRNA sequencing. The 16s rRNA gene was used to determine genetic homology by the partial sequencing among *L. monocytogenes* isolated from almond milk, cheese, curd, and a variety of milkshake samples. The phylogenetic tree of L. monocytogenes strains from many global clones conformed to a high grade of nucleotide similarity. The UPGMA approach was used to describe the evolutionary antiquity of *L. monocytogenes* strains [18]. In 500 bootstrap replications, the maximum composite likelihood approach was employed [16]. and the percentage of duplicate trees in which connected taxa were clustered together [26]. The present findings corroborate with other earlier studies [10,14].

The phylogenetic tree grouped clustered was compared with our *L. monocytogenes* strain (OM004047) with some other *L. monocytogenes* strains (accession numbers MW13628, MT124478, MT534278, OK001790, MT509640) and other species of *Listeria* strains (accession numbers MT124497, MT534278, OK001790). In contrast, a cluster was detected in the phylogenetic tree, further *Listeria* species with *L. welshimeri* (strain OK001784), *L. innoca* (strain MW242696, OK001783, MW242699). These findings were supported by studies by Abdeen et al., (2021) [9].

Fig 2. Dendrogram based on UPG MA analysis of SELM23 profile obtained for *L. monocytogenes*

4 Conclusion

In India, no clear documentation regarding the epidemics of human listeriosis was studied, the current study showed about a 14.3% prevalence of *L. monocytogenes* in ready-to-eat food in the Kanchipuram District. The increasing occurrences of multi-drug resistance throughout India have been the main concern for the last few years. The *L. monocytogenes* of this study were resistant against at least three antibiotic classes, and their multidrug resistance patterns may implement an idea about the cure to which the people of India may be exposed. An outbreak of listeriosis, a food-borne disease against which public awareness is insufficient, could have a significant impression on the country's public health. Accordingly, these studies will help find out a sustainable solution to eliminating multidrug-resistant *L. monocytogenes* from RTE foods in India.

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