ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Prevalence Of Listeria Monocytogenes In Various Food From Andhra Pradesh, India.

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ABSTRACT

Present study aim to know the prevalence of Listeria monocytogenes from various food sources divided into three categories i.e. Dairy products, street food and refrigerated ready to eat food. Total of 1008 sample was analyzed. The organism isolation was done by USDA method and further screened by biochemical assays such as gram straining, catalase activity, motility, hemolysis and CAMP test. Finally conformation of Listeria sps was done by prs gene and L. monocytogenes was confirmed by 5 virulence genes likely, hly A, iap, act A, prf A, plc A. from the study 321 Listeria isolates was identified among this 128 were pathogenic L. monocytogenes.

Keywords: Listeria monocytogenes, Bacteriological study of food, Prevalence of Listeria, Listeria virulence

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ISSN: 0975-8585

INTRODUCTION

Processed food and street vended food has currently became the major public health concern, governments and scientists need to focus to raise public awareness. Taking these factors into consideration this study was undertaken to examine the microbial quality of various processed, street vended food consumed in large amount. The present study aims the screening of Listeria monocytogenes, a deadly organisms occurs in all types of environment, mainly in food grown in contaminated environment, poorly processed or stored food, dairy and dairy associated products. As per health concern of public, the microbiological quality of vended food becomes important, as food can act as a major vector in transmission of food born infections and intoxications. Unhygienic food contains pathogens like Salmonella enterica, Escherichia coli, Mycobacterium tuberculosis, Mycobacterium paratuberculosis, Brucella abortus, Coxiella burnetii, Yersinia enterocolitica, Staphylococcus aureus, and Listeria monocytogenes etc. The risk of being affected by listeriosis, caused by L. monocytogenes, is more among the immune compromised people like pregnant women and their foetuses, children, individuals with chronic health issues, Tubor colossis & cancer-patients, diabetics and elderly people [1]. The infection causes fever, abortion, still birth, brain damage and death depending upon the individual's immunity and the nature of contamination. 30-40% of mortality rate in adults, 50% in neonates and 91% of hospitalization rate was observed by Listeria. Considering its pathogenesity and mortality rate of Listeria the Indian government has recently included this organism in food safety and regulations lists, in 2011 [2,3]. In the present study in Andhra Pradesh India, L. monocytogenes was found 32 % in the vended food. The findings of the study gave us insight for the present proposed work, the result of changed food habits. The processed food, street vended food is being consumed by the peoples of all ages, and hence quality of food need to be explored for microbial safety, before it affects people.

MATERIALS AND METHODS

Study Area and Sample Collection

Present study is conducted for two years (from January 2013 to December 2015), a total of 1003 various samples divided into three categories i.e., Dairy Products, Street Food and Refrigerated ready to eat food were collected from the supermarkets and small vendors of Costal Andhra Pradesh, India. The details of the samples were given in Table 1. The area of study included various cities of costal Andhra Pradesh likely; Visakhapatnam, Eluru, Vijayawada, Guntur, Chilakaluripet, Ongole and Tirupati. These samples were transferred to the laboratory keeping in ice-box (4°C) and stored at 0^0C until to assess for L. monocytogenes.

Table 1: Detailed description of Sample

Dairy Products(n=300)	Street Food (n=253)	Refrigerated ready to eat food(n=450)
1. Milk (n=50)	1. Fruit juices (n=40)	1. Sausages (n=50)
a. Raw Milk from vendors (n=30)	2. Chinese cheese (n=20)	2. Fruit jams (n=50)
b. Pasteurized milk from stores (n=20)	3. Pani Pori water (n=65)	3. Milk shakes (n=50)
2. Yogurt and butter milk (n=50)	4. Sweet corn (n=34)	4. Smoothies (n=50)
3. Butter (n=50)	5. Flavored water (n=56)	5. Squashes (n=50)
4. Cheese (n=50)	6. Chopped fruits (n=38)	6. Fruit salads (n=50)
5. Paneer(n=50)		7. Pastries (n=50)
6. Milk sweets (n=50)		8. Ice creams (n=50)
		9. Donuts (n=50)

Isolation and Identification of Bacteria

On the immediate next day of sample collection, to recover the stressed organisms the samples were enriched prior to isolation. For enrichment, 20 gms of each solid sample and 5 ml of liquid samples were inoculated into 40 ml Pre Enrichment Broth (pH7.3±0.2) for 6-8 hours at room temperature, and isolation was done according to the USDA- method, which contains two enrichment process. Briefly about the process - 5 ml of this enrichment broth was transferred to 45 ml of UVM –I incubated at 37^0C for 24 hrs. After respected

incubation 0.1 ml of UVM- I to UVM-II and further incubated at 37^{0}C for 24 hrs., from this a loop full of the enriched culture was streaked in Petri plates in duplicate on selective media PALCAM® agar and the plates were incubated for 48h at 37 ºC.

The isolated colonies (Black centred round colonies) were tested for Gram staining, catalase activity, oxidase activity, motility, carbohydrate fermentation [4] Haemolysis on sheep blood agar [Himedia-M073] and CAMP tests [5]. These purified cultures were further inoculated into Brain Heart infusion (BHI) broth for molecular conformation of L. monocytogenes.

Molecular conformation

Listeria Sps was confirmed by using prs gene and further L. monocytogenes was confirmed by five virulence associated genes: hemolysin gene (hlyA), regulatory gene (prfA), Phosphatidylinositol phospholipase C gene (plcA), Actin gene (actA) and p60 gene (iap). Chromosomal DNA was extracted as per the methodology of Makino et al., [6]. Isolation method in breif, 1 mL of overnight culture (from BHI) broth was transferred to 1.5-mL centrifuged in a microcentrifuge at 10000 rpm for 10 min. The recovered pellet was resuspended in 100 µL of sterilized DNAse and RNAse-free milliQ water, heated in a boiling water bath for 10 min. and then snap chilled in crushed ice. The obtained lysate (5µL) was used as a DNA template in PCR reaction mixture.

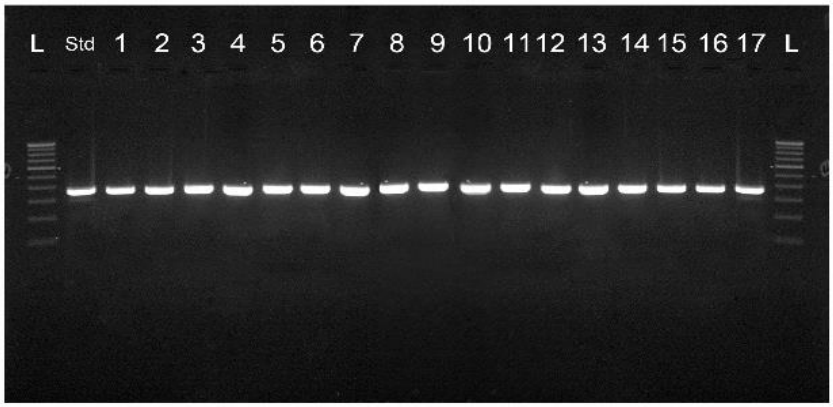
PCR condition for detection of Listeria spp.

The details of the primers sequences for amplification of Listeria spp., L. monocytogenes, prs and its virulence genes are shown in Table 2. DNA amplification was performed in a DNA thermal cycler (Eppendorf Mastercycler pro) with preheated lid. The PCR was standardized for the detection of prs gene in L. monocytogenes, DNA amplification volumes and program were studied using the method described by Bubert et al [7].The PCR program for virulence genes of L. monocytogenes with suitable modifications in Notermans et al., [8]. Initially, for the detection of individual virulent genes of L. monocytogenes, PCR conditions were optimized by using varying concentrations of biologicals. The standardized multiplex PCR protocol for 50 µL reaction mixture included 10.0 µL of 10X master mix PCR buffer (Emerald Amp® GT PCR master mix which contains dNTPs, Tag Polymerses and PCR Buffer), and 1 µmol L^{-1} forward and reverse primers of each, 5 µL of cell lysate and sterilized milliQ water to make up the reaction volume. The cycling conditions for PCR included an initial denaturation of DNA at 95^{0}C for 2 min followed by 35 cycles each of 15 s denaturation at 95^{0}C, 30 s annealing at 60^{0}C and 1 min 30 s extension at 72^{0}C, followed by a final extension of 10 min at 72^{0}C and held at 4^{0}C [9]. All the five set of primers for virulence-associated genes were amplified under the similar PCR conditions and amplification cycles in a single vail. The resultant PCR product were further analyzed by agarose gel electrophoresis stained with ethidium bromide (0.5 µg mL^{-1} and visualized by a UV transilluminator [Pic.1, 2].

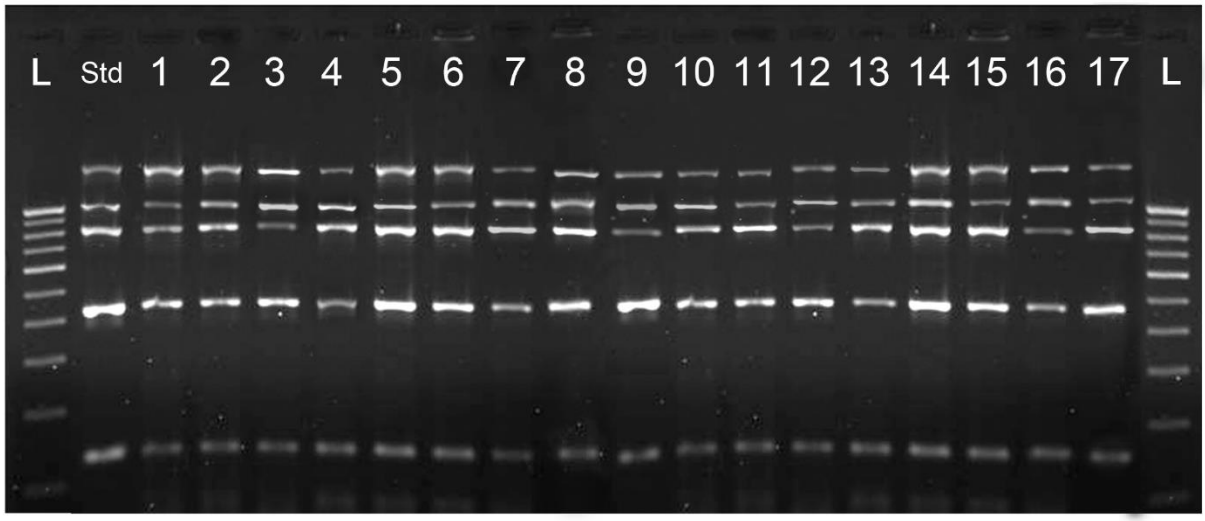
Table 2: Primers for amplification of Listeria spp. and virulence associated genes of L. monocytogenes

Primer name	Primer sequence (5'-3')	Size of product (bp)	Target	References
Prs	5'- GCTGAAGAGATTGCGAAAGAAG-3' 5'- CAAAGAAACCTTGGATTTGCGG -3'	370	Listeria spp.	[10]
hlyA	5'- GCAGTTGCAAGCGCTTGGAGTGAA-3' 5'- GCAACGTATCCTCCAGAGTGATCG-3'	456	L. monocytogenes	[11]
Iap	5'- ACAAGCTGCACCTGTTGCAG-3' 5'- TGACAGCGTGTGTAGTAGCA-3'	131	L. monocytogenes	[12]
actA	5'- CGCCGCGGAAATTAAAAAAAGA - 3' 5'- ACGAAGGAACCGGGCTGCTAG-3'	839	L. monocytogenes	[13]
prfA	5'- CTGTTGGAGCTCTTCTTGGTGAAGCAATCG-3' 5'- AGCAACCTCGGTACCATATACTAACTC-3'	1060	L. monocytogenes	[14]
plcA	5'- CTGCTTGAGCGTTCATGTCTCCATCCCCC-3' 5'- CATGGGTTTCACTCTCCTTCTAC-3'	1484	L. monocytogenes	[14]

ISSN: 0975-8585



Picture1: The isolates which amplified for prs gene L- 1000bp ladder, Std- MTCC-1143(L. monnocytogenes), 1-5: isolates from dairy products, 6-13: isolates from street vended food, 14-17: RTE food



Picture2: The isolates which amplified five virulence associated genes L- 1000bp ladder, Std- MTCC-1143(L. monnocytogenes), 1-5: isolates from dairy products, 6-13: isolates from street vended food, 14-17: RTE food Banding pattern from top to down side-- plc A: 1484 bp, prf A: 1060 bp, act A: 839 bp, hly A: 456 bp and iap: 131 bp

RESULTS AND DISCUSSION

In the present study a wide range food was taken for screening of Listeria sps and the methods of culturing was specific to species. Specific method in brief - UVM I & II contains different concentrations of Acriflavin and Nalidixic acid to inhibit the growth of remaining organisms. Biochemical analysis gives primary insight of organism from sugar fermentation and motility. PCR amplification of specific genes conforms the listeria sps and virulence genes establish insight on pathogenic sps. This study includes a total of 1003 sample collected from various places of Andhra Pradesh and were studied for presence of Listeria spp. A total of three twenty one samples (32%) were positive for Listeria spp. Also, 128 samples (12.76%) were positive for the L. monocytogenes. The detailed sample sources for L. monocytogenes along with percentages were given in table 3. The standardized PCR allowed amplification of virulence associated genes of L. monocytogenes plcA, prfA, actA, hlyA and iap to their respective base pairs, 1484 bp, 1060 bp, 839 bp, 456 bp and 131 bp, and allowed visualization of each virulence associated gene, each gene represented by a single band in the corresponding region of the DNA ladder. The primers used in the PCR were specific to the target genes and all the five genes were detected in standard strains of L. monocytogenes, whereas 5 virulence genes was detected in 34 isolates, 4 genes in 34 isolates, 3 genes in 35 isolates, 2 genes in 20 isolates and 1 gene in 5 isolates were observed.

ISSN: 0975-8585

Table 3: Detailed data of L.monocytogenes isolates from various sources

Sample Details (n=1003)	Listeria sps prs	L. monocytogenes Total no. of Positives for virulence genes[a]
Dairy Products(n=300)	**76**	**38**
1. Milk (n=50)	-	-
a. Raw Milk from vendors (n=30)	-	-
b. Pasteurized milk from stores (n=20)	-	-
2. Yogurt and butter milk (n=50)	-	-
3. Butter (n=50)	-	-
4. Cheese (n=50)	42	26
5. Paneer(n=50)	20	06
6. Milk sweets (n=50)	14	06
Street Food (n=253)	**116**	**52**
1. Fruit juices (n=40)	17	11
2. Chinese cheese (n=20)	12	10
3. Pani Pori water (n=65)	42	16
4. Sweet corn (n=34)	10	01
5. Flavored water (n=56)	31	14
6. Chopped fruits (n=38)	04	-
Refrigerated ready to eat food(n=450)	**129**	**38**
1. Sausages (n=50)	15	01
2. Fruit jams (n=50)	09	-
3. Milk shakes (n=50)	14	05
4. Smoothies (n=50)	16	02
5. Squashes (n=50)	09	03
6. Fruit salads (n=50)	04	01
7. Pastries (n=50)	21	11
8. Ice creams (n=50)	24	12
9. Donuts (n=50)	17	03

[a] amplified at least one virulence associated gene in multiplex PCR

Prevalence of Listeria spp. and L. Monocytogenes

This study clearly indicated considerable levels of contamination in dairy products, street vended food and refrigerated food of Andhra Pradesh. Many of these contaminants have been found to be pathogenic and the occurrence of Listeria sps in the studied samples paralleled with several other studies conducted in various parts of the world.

The contamination of the studied food vended in Andhra Pradesh can be attributed to a number of factors such as hygienic condition of food handlers, unclean surroundings and utensils, contaminated water, improper handling and processing of food material and lack of proper storage as well as unhygienic display of food. The qualitative analysis of the samples revealed a wide range of Listeria contamination. These findings demonstrate that the food vended is a potential hazard for human health which needs to be addressed. Health education provision to the vendors and enforcing them to implementation of appropriate hygienic practices

would improve vended food quality. Regular monitoring of the food processing environment is suggested as this will help improving their quality and will also make the general public aware of the microbiological status of the vended food.

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