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Molecular Identification of *Listeria* Species from Vegetables Marketed in Mysore, Karnataka, India

Mahadevaiah Shantha Sangeetha and Gopal Shubha*
Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysore 570 006, INDIA

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Abstract

The incidence of Listeria species in vegetables marketed in Mysore city, Karnataka, India was studied. One hundred and sixty five vegetable samples marketed were analyzed for the presence of Listeria spp. Cold enrichment procedure was used for the isolation and plating was done on two selective agars PALCAM and Oxford agars. The identification of the isolates was done by biochemical characterization and confirmation by Polymerase Chain Reaction using species and genus specific primers. Three samples were found positive for Listeria spp. and they were confirmed as L. innocua and L. seeligeri.

Keywords: *Listeria* spp., raw vegetables, radish, cabbage, spinach.

Introduction

Vegetables are the essential components of our diet. The consumption of vegetables has increased based on the awareness of health benefits of vegetables. Besides health benefits of vegetables, raw vegetables act as vehicle for human diseases due to lack of post harvesting processing. Food borne diseases caused by contaminated vegetables are unreported due to lack of disease surveillance and investigation in the developing countries. According to CDC, approximately 12% of the reported food borne-illnesses in the United States are linked with fresh fruits and produce[1]. A study released by CDC showed that by eating leafy vegetables 2.2 million people were sick annually. Raw vegetables harbour potential food borne pathogens and act as vehicle for the cause of the disease. The fresh produce may be contaminated by soil, manure, effluents, sewage, decaying vegetation, human activities, post harvest processing. *Listeria* is one of the pathogen associated with fresh produce. It can be isolated from vegetables like cabbage, lettuce, celery, radish[2]. The genus *Listeria* consists of ten species namely *Listeria monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischmannii* and *Listeria weihenstephanensis*[3-6]. Among them two are pathogenic, i.e., *Listeria monocytogenes* and *Listeria ivanovii*[7]. *L. monocytogenes* lives in soil as a saprophyte and can turn out to be an intracellular pathogen and it has wide range of host species[8]. *Listeria* is most commonly found in raw foods, soil, stream water, silage, sewage and plants. It is also found in uncooked meats, fish, uncooked vegetables, unpasteurized milks, their products and processed foods indicating the widespread presence of the pathogen in nature.

The study was undertaken to investigate the incidence of *Listeria* spp. in the fresh vegetables.

Material and Methods

Samples Used for the study: A total of 165 vegetable and green leafy vegetable samples were analyzed. The sample included amaranthus green, amaranthus red, coriander, dill, spinach, fenugreek, cabbage, curry leaves, cauliflower and radish. The samples were randomly purchased from different supermarkets of Mysore city, Karnataka, India and were analysed on the same day.

The samples were collected in UV sterilized polythene bags and brought to the laboratory and stored under refrigeration conditions until processed. The samples were thoroughly washed with sterile distilled water before processing.

Isolation of *Listeria* spp.: Cold enrichment procedure was used for the recovery and isolation of *Listeria* spp.10 g of sample was homogenized in 90 mL of Brain Heart Infusion Broth (BHI, Hi-Media Laboratories, Mumbai) using sterile Pestle and Mortar. The homogenized sample was incubated at 4°C for 48 h to six week. At weekly intervals the enrichment was plated on Oxford and PALCAM agar plates[9].

Identification of *Listeria* spp.: On PALCAM agar plates Listeria spp. appear as Grey green colonies with black sunken centres and on Oxford agar they appear as black colonies with black sunken centre. The suspected colonies were picked up and cultured on Brain Heart Infusion Agar (BHI, Hi-Media Laboratories, Mumbai). Suspected colonies were subjected to standard biochemical tests such as catalase test, motility at 25°C and 37°C, Carbohydrate fermentation and gas production (glucose, mannitol, rhamnose, xylose and α- methyl-D-mannoside), nitrate reduction, hydrolysis of esculin, methyl red test and Voges Proskauer test.

Confirmation of *Listeria* spp. by PCR.: All the *Listeria* isolates confirmed by biochemical tests were subjected to molecular identification by Polymerase Chain reaction (PCR). The isolates were grown on BHI agar plates for 24hours at 30°C. The Cell lysis was done by heat lysis method in a dry bath (Bangalore Genei Pvt. Ltd., Bangalore) for obtaining DNA. Primer pairs Lis1A and Lis1B were used for the identification of the genus *Listeria*[10]. Positive isolates were subjected to species identification by using primers Mono A and Mono B for *L. monocytogenes*, Ino 2 and Lis1B for *L. innocua*, Wel 1 and Lis 1B for *L. welshimeri*, Sel 1 and Lis1B for *L. seeligeri*, Iva1 and Lis 1B for *L. ivanovii*[11].

PCR amplification was performed in 50µl reaction mixture containing 5µl of 10X PCR buffer; 1µl of 10mM dNTP mix; 0.5µl of 10µM of each primer; 0.25µl of 5U/µl of Taq polymerase; 4µl of 25mM $MgCl_2$; 2µl DNA template; 39.75µl of distilled water. All the reagents used in PCR were purchased from Fermentas.

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

Each amplification reaction included an initial denaturation temperature of 94°C for 5 min and was completed with the final elongation step at 72°C for 8 min. Amplification conditions varied in the denaturation, annealing and elongation step with the different primer pairs as listed in table-1.

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

Table-1
Primer pairs, their PCR parameters for the amplication steps and amplicon size[10,11]

SI.No	Primer name	Primer sequence (5´-3´)	Denaturation	Annealing	Extension	Cycles	Amplicon size	Species identified
1	Lis 1B Lis1B	(TTATACGCGACCGAAGCCAA) (ATGAATATGAAAAAAGCAA)	94°C for 45 s	55°C for 60 sec	72°C for 60 sec	30	1.6 Kb	All *Listeria* species
2	Mono A Mono B	(CAAACTGCTAACACAGCTACT) (GCACTTGAATTGCTCTTATTG)	94°C for 45 sec	55°C for 60 sec	72°C for 60 sec	30	~0.4 Kb	*L. monocytogenes*
3	Ino2 Lis1B	(ACTAGCACTCCAGTTGTTAAAC) (ATGAATATGAAAAAAGCAA)	94°C for 45 sec	62°C for 60 sec	72°C for 45 sec	30	~0.87 Kb	*L. innocua*
4	Iva 1 Lis1B	(CTACTCAAGCGCAAGCGGCAC) (ATGAATATGAAAAAAGCAA)	95°C for 30 sec	62°C for 30 sec	72°C for 90 sec	30	1.1 Kb	*L. ivanovii*
5	Wel 1 Lis1B	(CCCTACTGCTCCAAAAGCAGCG) (ATGAATATGAAAAAAGCAA)	95°C for 30 sec	62°C for 30 sec	72°C for 90 sec	30	1.05 Kb	*L. welshimeri*
6	Sel 1 Lis1B	(TACACAAGCGGCTCCTGCTCAAC) (ATGAATATGAAAAAAGCAA)	95°C for 30 sec	62°C for 30 sec	72°C for 90 sec	30	1.1 Kb	*L. seeligeri*

Table -2
Incidence of *Listeria* spp. in vegetables

SI.No	Type of Vegetables	No. of Samples	*L.monocytogenes*	*L.seeligeri*	*L.welshimeri*	*L.innocua*	*L.ivanovii*
1	Amaranthus (Red)	15	-	-	-	-	-
2	Coriander	15	-	-	-	-	-
3	Mint	15	-	-	-	-	-
4	Dill	15	-	-	-	-	-
5	Spinach	15	-	-	-	+	-
6	Fenugreek	15	-	-	-	-	-
7	Cabbage	15	-	+	-	-	-
8	Amaranthus (Green)	15	-	-	-	-	-
9	Curry leaves	15	-	-	-	-	-
10	Cauliflower	15	-	-	-	-	-
11	Raddish	15	-	-	-	+	-

Results and Discussion

The organism *Listeria* has been isolated from fresh vegetables and ready to eat vegetables from different parts of the world. In India, only a few surveys have been conducted to assess the presence of *Listeria* in fresh produce and its presence was reported from Dhanashree *et al*[9]. In the study undertaken, out of 165 samples tested, three samples were found positive for *Listeria* spp. The isolates were confirmed as *L. innocua* and *L. seeligeri*. Two isolates of *L. innocua* were found in spinach, radish and one isolate of *L. seeligeri* from cabbage. (table-2, figure-1).

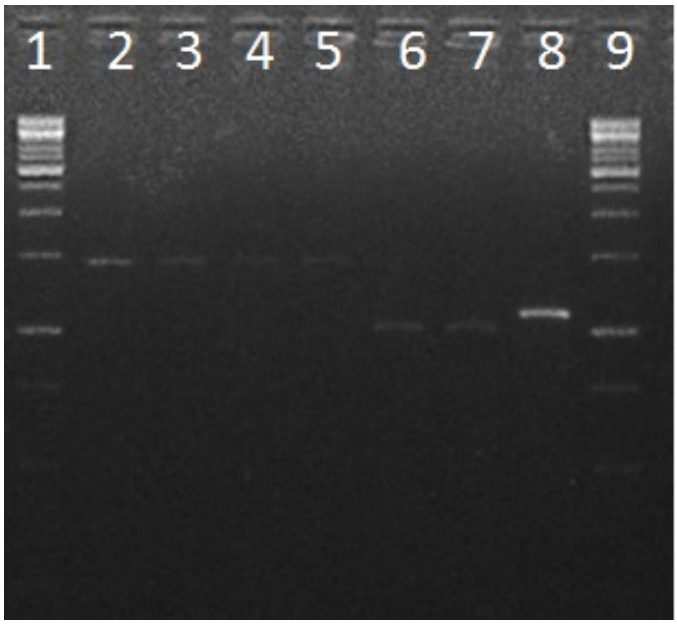

Figure-1
Identification of *Listeria* spp. using the genus specific and species specific primer pairs

M: 1 Kb Marker Lane 1 - Control *L. monocytogenes* EGD-e; Lane 2 - 4 – Isolates tested with the primer pair Lis1A and Lis1B; Lane 6-8 *Listeria* species confirmed with species specific primers.

L. innocua has been reported to be present in 10% of palak leaves and 30% coriander leaves[9] which correlate our observation. Cabbage acts as vehicle for outbreaks of listeriosis caused by *L. monocytogenes*[12,13]. Many reports discuss the presence of *L. monocytogenes* in leafy vegetables[14-17].

L. monocytogenes has been isolated from strawberries (10%), parsley (5%) and potatoes (15%) [18]. Lin *et al*[19] reported that among 63 salad vegetables tested one was contaminated with *L. monocytogenes*. Heisick *et al*[20] found that Broccoli, Carrots, cauliflower, tomatoes were negative for *L. monocytogenes*, cabbage, cucumber, lettuce, mushrooms, potatoes, radishes were positive for *L. monocytogenes*. 33.3% of *Listeria* spp. and 22.5% of *L. monocytogenes* was isolated from raw vegetables; more frequency was in Japanese Parsley and Yardlong bean[21].

Conclusion

In the present study, incidence of *Listeria* spp. was found to be 1.81%. The results and the data suggest that *Listeria* spp. is present in vegetables. As *Listeria* can be killed by cooking; eating raw vegetables without processing should be avoided as this may lead to listeriosis.

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