



جامعة الناصر
AL-NASSER UNIVERSITY

Title

**Prevalence of *Listeria monocytogenes* in Ice Creams Sold in Mysore City
and Detection by Polymerase Chain Reaction (PCR)**

S. Ahmed Moharram, A P Charith Raj and G. R. Janardhana*

**Molecular Phytodiagnostic Lab
Department of Studies in Botany and Microbiology
University of Mysore, Manasagangotri, Mysore- 570 006, India.**

*janardhanaplantclinic@india.com

AUTHORIZED BY AL-NASSER UNIVERSITY'S RESEARCH OFFICE
جميع حقوق النشر محفوظة لمكتب البحوث والنشر بجامعة الناصر

* Corresponding Author

Abstract

Listeria monocytogenes is one of the most important food-borne pathogens causing illness in humans and animals. In this study we are reporting the incidence of *L. monocytogenes* in ice-cream samples collected and analyzed from different ice cream Parlors of Mysore City. Eighty ice cream samples were analyzed for the *Listeria monocytogenes* by culture method followed by Polymerase Chain Reaction(PCR) detection. Three samples (5%) out of 60 unbranded samples were positive for *L. monocytogenes*, none in other 20 branded samples of ice creams. To replace the lengthy, laborious and often costly, conventional, microbiological, biochemical methods, we have employed PCR technique for the quick and specific detection of *L. monocytogenes* using *iap* gene primers encode for invasive associated proteins (p60) common to all *Listeria Spp*. PCR technique makes diagnosis of *L. monocytogenes* in ice creams samples much easier and more accurate.

Keywords: Incidence, *L. monocytogenes*, PCR detection, Ice cream, Mysore

INTRODUCTION

Listeria monocytogenes is one of the most important food-borne pathogens causing an estimated 2500 cases of meningitis sepsis, encephalitis and premature fetal death (De Boer *et al.*, 1999). Although the incidence of listeriosis is low, 26.8% mortality rate makes *L. monocytogenes* important human pathogen (Jinneman *et al.*, 2001). Several major outbreaks of listeriosis have been associated with consumption of contaminated food (Bille, 1990). It was considered to be associated with invasive bacteria only in certain target organs and was merely associated with gastrointestinal symptoms (Slutsker and Schuchat, 1999). However, recent reports of a new non invasive form of listeriosis that causes febrile gastroenteritis clearly indicates that persons with no predisposing conditions may also be affected (Aureli *et al.*, 2000). Many different kinds of methods have been evaluated for detecting and enumerating *L. monocytogenes* in food samples including culturing on selective media, biochemical identification, enzyme-linked immune sorbent assay (Bansal, 1996), DNA probes and dot ELISA. (Chaudhari, 2001). Culturing methods are simple but laborious, time-consuming, insensitive and are not suitable for giving the results in the time required by the food industry (De Boer *et al.*,

1999). Antibodies-based methods suffer from low specificity resulting in false – positive results (Ben Embarek, 1994) because most commercially developed antibodies are not species specific. Molecular biology method based on Polymerase Chain Reaction (PCR) assay has several advantages like high specificity, sensitivity, rapidity and it may permit direct detection of pathogens in food and other samples without the need for isolation of pure culture (Zhou and Jiao, 2005).

Ice-creams and number of other dairy products are consumed with pleasure by general public especially children due to its contents of sweeteners, aromatic compounds and various fruits and is known to be richest in terms of content among dairy products (Saldamli and Temiz, 1988). Data on the incidence of *Listeria monocytogenes* in Indian dairy foods is limited to a few reports (Pednekar, 1997). No such work has been initiated with reference per cent incidence of *L. monocytogenes* in dairy foods and outbreaks involving food contamination. Hence, this study was conducted to determine the incidence of *L. monocytogenes* in ice cream samples sold in Mysore City by using sensitive PCR technique.

MATERIALS AND METHODS

Detection of *L. monocytogenes* by culture method

The study was conducted on 20 samples of two different known brands (A1 and A2) of ice cream (10 samples from each) and 60 Local sample (B) sold in Mysore City (India). The samples were procured from different Restaurants and Ice Cream Parlours and other

Market Outlets of Mysore City. All the samples were kept on dry ice during transportation and were stored at -20°C until analyzed (within 24h). *L. monocytogenes* was detected by enrichment method of the Food and Drug Administration (FDA) (Hitchins, 1992). Ice cream samples were thawed to room temperature and 25ml of each sample was added to 225 ml of sterile *Listeria* selective enrichment broth (Himedia, M 888). The medium was homogenized with the sample and incubated at 30°C for 24 to 48 hrs. After incubation, the enrichment broth was taken and aseptically streaked on to the selective media such as *Listeria* Oxford Medium Base and incubated for 24 to 48hrs at 36°C . After incubation, 5 - 8 suspected colonies showing black coloration with black haloes of 1-3 mm in diameter on *Listeria* Oxford Medium Base were sub cultured separately in Trypticase Soya Yeast Extract (TSYE) agar medium containing 0.6% yeast extract and incubated for 24hrs at 37°C . The colonies were checked for purity by Gram staining and comparison with known *L. monocytogenes* (MTCC-1143) culture obtained from IMTECH, Chandigarh, India. The re-isolated colonies from the TSYE agar were examined by the Henry light technique by holding the Petri-dish at 45° to a mirror which reflected a strong light; *Listeria* were identified by their transparent colonies with typical blue-green reflection. Later, the colonies were separated and then subjected to Gram staining, catalase activity, tumbling motility, β -hemolytic activity on sheep blood agar (Hi-media) and carbohydrate fermentation.

Detection of *L. monocytogenes* by PCR

DNA from *L. monocytogenes* was extracted using CTAB (cetyltrimethylammonium bromide) buffer (Zhang *et al.*, 1998) with some modification. One ml of *Listeria*

enrichment broth was transferred to 1.5 ml microfuge tube and centrifuged at 8000 rpm for 5 min and the supernatant was discarded. CTAB buffer (800µl) at 60°C was added to the microfuge tube containing bacterial pellet and the mixture was held in water bath at 60°C for 20 min; during incubation, the mixture was briefly mixed several times. After incubation, 600µl of chloroform/octanol (24:1) was added and mixed followed by centrifugation at 3000rpm for 5 min. The supernatant was transferred to a clean microfuge tube, and an equal volume of ice-cold isopropanol was added and kept on ice bath for 2hrs. Centrifuged at 8000 rpm for 8 min. and the aqueous phase was discarded. The pellet was rinsed with 80% ethanol, air-dried and re-suspended in 50µl of distilled water. This preparation was used for PCR.

PCR Detection

Oligonucleotide primers for PCR analysis were synthesized according to the sequences developed by (Klein and Juneja, 1997). The forward primer 5' CAAACTGCTAACACAGCTAC'3 and reverse primer 5' GCACTTGAATTGCTCTTATTG '3 used for amplification of *iap* gene of *Listeria monocytogenes* which is common to all *Listeria Spp*. The expected size of amplified products was 371 bp. The primers were obtained from Bangalore GENEI. The PCR mixture include 25µl of aliquot of PCR buffer contained 24µl of PCR super mix (20pmol/µl of each primer, 10X PCR buffer, 25 mM MgCl₂, 200mM dNTPs mix, 1.5U of *Taq* DNA polymerase). DNA sample (1µl) was added to the PCR mixture. The PCR was performed using Advanced Primus 25 Thermocycler, Peqlab, Germany. The conditions were: 4 min at 94°C, followed by 35 alternating cycles of 94°C for 1 min,

primer annealing at 57°C for 1 min, and extension at 72°C for 1 min. there was a final extension at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide (1mg/ml) along with 100bp DNA ladder. The gel was analyzed using Biorad gel documentation system.

RESULTS AND DISCUSSION

The suspected *L. monocytogenes* isolates from local ice cream samples showed Gram positive bacilli, catalase activity, tumbling motility, β -hemolytic activity on blood agar (Himedia) and carbohydrate fermentation and these tests are in conformity with authentic MTCC-1143 *L. monocytogenes*. The results of the experiments are presented in Table -1 and Figure -1. Out of 80 ice cream samples analyzed, 3.7 % of samples were positive for *L. monocytogenes* both by cultural and PCR methods. Interestingly no *L. monocytogenes* detected in branded A1 and A2 ice cream samples. 5% incidence of *L. monocytogenes* detected only in unbranded local ice cream samples. Out of 60 local samples analyzed, three samples were found to be contaminated with *L. monocytogenes* as confirmed by both cultural and PCR diagnosis. The results are in correlation with the earlier reports from India and elsewhere (Pdenekar *et al.*, 1997) reported out of 48 ice cream samples screened only one sample was positive for *L. monocytogenes*. (Waker *et al.*, 2000) reported the incidence of *L. monocytogenes* in ice cream in Mumbai City. (Farber *et al.*, 1989) reported the low incidence of *L. monocytogenes* in ice cream. (Dhanashree *et al.*,

2003) reported that, dairy products in Mangalore city (India) were free from *L. monocytogenes*.

According to WHO reports on surveillance data of *L. monocytogenes*, contamination in ice cream in various countries varied from 0 - 5.5 % (Warke *et al.*, 2000). The large variation in the reported incidence rate may be partly due to differences in methods followed for detection, including the factors like procedures used, the sample size, and the source of samples. It is known that *L. monocytogenes* grows at cold temperature in milk and milk products in the presence of other psychrotrophs like *Pseudomonas* and *Flavobacterium*. Presence of even small numbers of *L. monocytogenes* in any ready to eat food such as ice cream constitutes a potential risk particularly to children, pregnant woman, immuno-compromised adults and elderly (Rocourt, 1996). In USA, *L. monocytogenes* tolerance is zero, which translates to less than one cell in 25 g of food while several other countries have adopted 100 cell/g as the tolerance level. According to the Indian specifications for ice cream, no pathogens are allowed other than coli-forms of the level 100cells/g (Warke *et al.*, 2000). The detection of *Listeria monocytogenes* in many samples of local ice creams warrants need for better quality control measures to be incorporated in the production of ice creams in Mysore City. More stringent measures need to be observed while ensuring microbiological quality from production to the final consumers.

References

- Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L. and Salmaso, S. 2000. An outbreak of febrile gastroenteritis association with corn contamination by *Listeria monocytogenes*. *New Engl J Med* 342: 1236-1241
- Bansal, N.S. 1996. Development of a polymerase chain reaction assay for the detection of *Listeria monocytogenes* in foods. *Letters in Applied Microbiol* 22: 353-356.
- Ben Embarek, P.K. 1994. Presence, detection and growth of *Listeria monocytogenes* in sea foods. A review. *Int J Food Microbiol* 23: 17-34
- Bille, J. 1990. Food - born listeriosis, Elsevier Publications, Amsterdam, p 71-74
- Chaudhari, S.P., Malik, S.V., Rekha,G.B.and Barbuddhe, S.B. 2001. Detection of anti-listeriolysin O and *Listeria monocytogenes* in experimental infected buffaloes(*Bubalus bubalis*). *Tropical Animal Health and Production* 33(4): 285-393
- Dhanashree, B., Otta, S.K., Karunasagar, I. K., Guebel, W. and Karunasagar, I. 2003. Incidence of *Listeria* Spp. In clinical and food sample in Mangalore-India. *Food Microbiol.* 20 : 447-453
- Dalton, C., Austin, C., Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M.E. and Griffin, P.M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk *New Engl J Med* 336: 100-105
- De Boer, E. and Beumer, R.R. 1999. Methodology for detection and typing of food borne microorganisms, *J Food Microbiol.* 50: 119-130
- Farber, J.M., Sanders, G.W.and Johnston, M.A. 1989. A survey of various foods for the presence of *Listeria* species. *J Food Protection* 52: 456-458

Hitchins, A.D. 1992. Bacteriological Analytical Manual (BAM), Food and Drug Administration. 7th edn. *Association of Official Analytical Chemists*, Arlington,VA

Jinneman, K.C.and Hill, W.E. 2001. *Listeria monocytogenes* lineage group classification by MAMA-PCR of the listeriolysin genes. *Curr Microbiol* 43:129-133

Klein, P.G.and Junija, K.V. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Appl Environ Microbiol* 63(11) 4441-4448

Pednekar, M.D., Kamat, A.S.and Adhikari, H.R. 1997. Incidence of *Listeria* species in milk and milk products. *Indian J Dairy Sci* 50: 1-10

Rocourt, J. 1996. Risk factors for Listeriosis. *Food Control* 7: 195-202

Saldamli, I.and Temiz, A. 1988. The investigation on quality of the ice creams of maras which sold in Ankara. *Dairy Products* 7(1):17-21

Slutsker, L.and Schuchat, A. 1999. *Listeria*, Listeriosis and food safety.2nd edn. New York, p 75-95

Warke, R., Kamat, A., Kamat, M.and Thomas, P. 2000. Incidence of pathogenic psychrotrophs in ice creams sold in retail outlets in Mumbai. *Indian Food Control* 11: 77-83.

Zhang, Y., Uyemoto, J.K.and Kirkpatrick, B.C. 1998. A small-scale procedure for extracting nucleic acid from woody plants infected with various phytopathogens for PCR assay. *J Virological Methods* 71: 45-50

Zhou, X.and Jiao, X. 2005. Polymerase chain reaction detection of *Listeria monocytogenes* using oligonucleotide primers targeting *act A* gene. *Food control* 16: 125-130

Table-1: The percent (%) incidence of *Listeria monocytogenes* in Ice cream samples as detected by cultural and PCR methods.

Brand	No. of Samples	Culture method Positive	Per cent Incidence	PCR technique Positive	Per cent Incidence
A1	10	ND	0	ND	0.0
A2	10	ND	0	ND	0.0
B	60	3	5	3	5.0
Total	80	3	5	3	3.7

*ND-Not detected

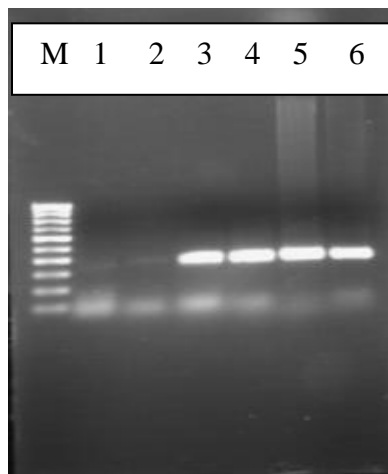


Figure-1: Agarose (1.5%) gel showing the amplified products of 371bp *iap* gene of positive isolates of *L. monocytogenes*. Lane - M-100bp DNA ladder, Lane-1 *E. coli*, Lane -2 *Salmonella typhimurium*, Lane - 3-5 *L. monocytogenes* isolates from ice cream samples, Lane-6 *L. monocytogenes* (MTCC-1143).