VETERINARY SCIENCE RESEARCH JOURNAL
olume **8** | Issue 1&2 | April & October, 2017 | 20-25

DOI : 10.15740/HAS/VSRJ/8.1and2/20-25
Visit us - *www.researchjournal.co.in*
e ISSN-2230-942X

RESEARCH ARTICLE

Critical control points of *Listeria* species in two major fish catchment areas of Kerala, India

K. VRINDA MENON[1], B. SUNIL[1], C. LATHA[1] AND ASHOK KUMAR

Members of the Research Forum

Associate Author :

[1]Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy, THRISSUR (KERALA) INDIA

AUTHOR FOR CORRESPONDENCE :
ASHOK KUMAR
Indian Veterinary Research Institute, Izatnagar, BARIELLY (U.P.) INDIA

Abstract : The present investigation was undertaken to study the occurrence of *Listeria* species in seafoods and to determine the critical control points of the organism in the fish catchment areas of two coastal districts of Kerala, India *viz.*, Kozhikode and Kollam. The occurrence of *L. monocytogenes* in seafoods and fish catchment areas in the present study was 1.45 per cent and 0.63 per cent, respectively. The isolates of *L. monocytogenes* were found to possess all virulence gene *viz., iap, hly*A, *act*A, *prf*A, *plc*A *and inl*A. The occurrence of *L. innocua* was found to be more with detection at the level of 28.92 per cent and 17.50 per cent from samples of seafoods and fish catchment areas, respectively. The assessment of critical control points in fish catchment areas revealed that boat deck, ice, containers and hands of workers were the potential source of contamination of raw seafoods.

Key words : *Listeria monocytogenes, L. innocua,* Kerala, Critical control points, Seafoods

How to cite this paper : Menon, K. Vrinda, Sunil, B., Latha, C. and Kumar, Ashok (2017). Critical control points of *Listeria* species in two major fish catchment areas of Kerala, India. *Vet. Sci. Res. J.*, **8**(1&2) : 20-25, **DOI : 10.15740/HAS/VSRJ/8.1and2/20-25**.

Paper History : Received : 15.02.2017; Revised : 03.09.2017; Accepted : 17.09.2017

INTRODUCTION

Seafoods are an excellent source of high quality protein and unsaturated fatty acids. Freshly harvested fishes and shell fishes contain a diverse natural microflora and the near neutral pH and non-protein nitrogen in their tissues makes it an ideal medium for the growth of bacteria. The pathogenic microbes are usually not found on fish captured from open waters but contamination usually occurs through other external sources *viz.*, ice, soiled surfaces, containers and contamination from other human and avian sources. Due to globalisation and growth of international trade in seafoods, the improvement in their quality and safety is paramount for exporting countries to ensure good economic returns. India is the second largest producer of fish in the world contributing to 5.43 per cent of global fish production. The total fish production during 2013-14 was 9.56 million tonnes with 3.4 MT from marine sector (Report, GoI, 2014). Kerala has a total marine capture of 6.71 lakh tonnes. The proliferation of pathogens through seafoods is a major hazard, which has prompted the emergence of strict regulations by the importing countries as it affects international

seafood trade (Jami *et al*., 2014).

Listeria species are considered ubiquitous organisms and it's halophilic nature makes it a one of the major pathogen which can contaminate sea waters *Listeria monocytogenes* is one of the most important etiological agent of serious foodborne disease outbreaks associated with seafoods. *L. monocytogenes* is mostly associated with disease in man and animals whereas *L ivanovii* is predominantly a pathogen of animals. *L. innocua* has also been recently been associated with meningitis in immunosuppressed individual (Favaro *et al.*, 2014). Listeriosis can result in case fatality rate which ranges from 15.0 to 30.0 per cent with the highest hospitalisation rates (90.5%) amongst known food-borne pathogens (CDCP, 2000). The present study mainly focuses on the occurrence of *Listeria* spp. in seafoods in the major fish catchment areas *viz.,* Neendakara (Kollam) and Puthiyappa (Kozhikode) in Kerala and identification of critical control points to control this pathogen in seafoods.

RESEARCH METHODOLOGY

Collection of samples :

The areas of collection of samples were from the important harbours of the southernmost coastal state, Kerala, India off the coast of the Arabian sea. The fish including dry fish, crustacean, molluscs and samples from critical control points *viz.*, soil, sea water, ice, boat deck, fish landing centres, containers and hand wash of workers were collected from Kozhikode (Puthiyappa harbour) and Kollam (Neendakara harbour), districts in Kerala, India. A total of 160 samples of marine fish *viz.*, Sardine, Mackeral, Tuna, Fin bream, Anchovy and milk fish were collected at the harbour. The dry fish samples (25 samples each from two districts) were collected from retail outlets near the harbours which mainly included two species of fish *viz.*, tongue sole and silver belly. The crustaceans mainly collected for the detection of the organism included prawn and crab samples. A total of 80 samples were collected for detection of *Listeria* spp. The molluscs *viz.*, squid, mussels and clams (80 samples) were collected for the isolation of *Listeria* spp.

A total of 20 samples each of soil (one kg), sea water (500ml), ice (100g), boat deck (swabs), fish landing centers, fishing vessel, containers (swabs) and hand wash of workers were collected from the fish catchment areas for detection of critical control points.

All the samples were collected carefully into aseptic containers/polythene bags and stored in thermocool containers during transportation to the laboratory. Swabs collected from control points were directly transferred to the sterile enrichment broth. The samples were brought as quickly as possible to the laboratory for further processing.

Isolation and identification of the organism :

The samples of fish, crustaceans and molluscs were transferred to a stomacher bag and were homogenized in a stomacher (Smasher, AES, France) for 120 sec. For surface swabs, 25 ml of peptone water with swabs formed the initial test sample. The hand wash of workers was taken in 100 ml of 0.1 per cent peptone water.

The isolation protocol for *Listeria* spp. was a modification of USDA (McClain and Lee, 1988) and FDA (Lovett, 1988) methods. It included a two step enrichment of the sample in University of Vermount broth (UVM I) for 24 h at 37^0C followed by inoculation of one millilitre of enriched sample in 10 ml of UVM II broth for 48 h at 37^0 C. The selective plating after enrichment was done in Polymyxin- Acriflavin- Lithium Chloride- Ceftazidime- Aesculin-Mannitol (PALCAM) (HiMedia) agar.

For identification, five or more suspected colonies from PALCAM agar plates were transferred on to Trypticase Soy Agar (TSA) (HiMedia) plates containing 0.6 per cent yeast extract and incubated at 30^0 C for 24 to 48 h. The isolates were subjected to further characterization and identification by cultural, morphological and biochemical reactions as described by Barrow and Feltham (1993).

Molecular characterisation :

The *Listeria* isolates obtained from various food and processing environment samples were subjected to PCR as per the procedure described by Momtaz and Yadollah (2013) with slight modifications. Apart from the conventional

Table A : Primers used for the identification of *L. innocua*

Primer name	Length	Primer sequence (5'-3')	Size of product
Lis1B	21	TTATACGCGACCGAAGCCAAC	1025 bp
Ino2	22	ACTAGCACTCCAGTTGTTAAAC	1025 bp

methods, the virulence of the isolates was also assessed by screening them for the presence of virulence genes which encodes for phosphatidylinositol phosphatase C activity (*plcA*), regulatory activity (*prfA*), actin polymerization protein (*actA*), haemolysin activity (*hlyA*), p60 protein (*iap*) and internalin A protein (*inlA*) (Liu, 2008 and Rawool *et al.*, 2007a and b). The DNA was extracted by snap chill method. The oligonucleotide primers were custom synthesized commercially and obtained in lyophilized form. The primers (forward and reverse) used were procured from Sigma–Aldrich, St. Louis, MO. The *iap* gene encoding p60 protein was used as target which is common to all members of the genus Listeria. The comparison of *iap* gene in different species of *Listeria* indicated that there are conserved gene portions at the 5' and 3' ends, while the internal portions are species-specific. The primers targeting the specific gene sequence in *L. innocua* were selected. The sequence of primers used in the study is shown in Table A.

RESULTS AND DISCUSSION

Out of the 345 seafood samples screened, 2.16 per cent and 0.14 per cent of the samples were positive for *L. monocytogenes*. However, *L. innocua* was the predominant species observed and the analysis of the samples revealed that 18.91 per cent and 34.59 per cent of samples from Kozhikode and Kollam districts, respectively revealed the presence of the organism. The low prevalence of *L. monocytogenes* in fishes have been reported by Dhanashree *et al.* (2003); Panda and Garg (2003) and Moharem *et al.* (2007) who reported a prevalence of 1.3, 1.66 and 1.83 per cent, respectively. Sunil *et al.* (2013) have reported a prevalence of 0.6 per cent of *L. monocytogenes* in shrimp (Crustaceans) samples from Thrissur, Kerala which is in agreement with our study. Das *et al.* (2013) have reported a higher prevalence (5%) of *L. monocytogenes* in dry fish samples from Kerala than the present study. The presence of the organism in dry fish could be attributed to the presence of less competitive bacteria and survivability at low water activity and salt tolerance.

The maximum prevalence of *Listeria* spp. was observed in crustaceans followed by fishes and molluscs. The details of the presence of organism in each category of seafoods is shown in Table 1. The increased occurrence of *Listeria* in shellfishes may be attributed to their filter feeding habit which concentrates the micro-organisms in their tissues. This finding is in agreement with some previous studies of Soultos *et al.* (2007); Parihar *et al.* (2008); Das *et al.* (2013) and Sunil *et al.* (2013) who had reported a predominance of *L. innocua* in seafood samples procured from Greece, Goa, Cochin (Kerala) and Thrissur (Kerala), respectively. This could be explained by the fact that *L. innocua* has a shorter generation time than other *Listeria* spp. (Curiale and Lewus, 1994) and the recovery of *L. monocytogenes* using selective broth was lower when *L. innocua* was present. King *et al.* (1990) have suggested that *L. monocytogenes* and *L. innocua* share the same ecological niche and *L. innocua* could be used as an indicator for the presence of *L. monocytogenes*.

Statistical analysis using Chi-square multiple proportion test was used to know whether there was any significant difference between the occurrence of the organism in the two districts. The results have shown that the presence of

Table 1 : District wise distribution of *Listeria* spp.in seafoods

Listeria species	Fish	Dry fish	Crustaceans	Molluscs	Per cent occurrence
Kozhikode					
L. monocytogenes	1	1	0	2	2.16
L. innocua	20	1	4	6	18.19
Kollam					
L. monocytogenes	0	0	1	0	0.14
L. innocua	20	1	35	7	34.59

Listeria species in Kollam was significantly higher compared to Kozhikode ($p< 0.05$).

Out of the 160 samples screened from several fish catchment areas of the two coastal districts of Kerala, *L. monocytogenes* and *L. innocua* were isolated. The overall occurrence of the organism from two districts was found to be 17.5 per cent. The district wise distribution of *Listeria* spp. in control points in fish catchment areas is shown in Table 2.

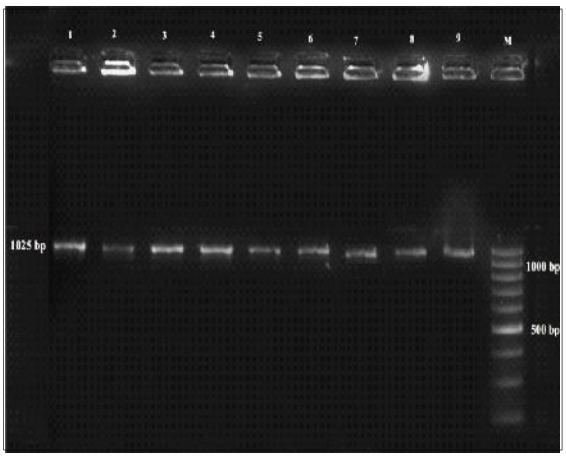
The standardized PCR with standard strain (ATCC 33090, serotype 6a) and suspected isolates allowed the amplification of virulence associated gene *iap* of *L. innocua* to their respective base pair, 1025 bp. The PCR product was represented by a single band in the corresponding region of the DNA marker ladder as shown in Fig. 1. The *Listeria monocytogenes* isolates (6) obtained from all the samples were screened by polymerase chain reaction for the presence of six virulence genes *viz*., *iap, hlyA, actA, prfA, plcA and inlA.* All the six isolates of *L. monocytogenes* obtained had shown the presence of all six virulence genes (Fig. 2).

The statistical significance of on the occurrence of *Listeria* spp. in different critical control points was calculated by test for proportion and it was found that the occurrence of the organism in the hand wash of personnels was significantly higher ($p<0.01$) than from other sources. It was worthy to note that in hand wash samples *L. monocytogenes* was isolated. The activities in catchment area reveals that handling of seafoods by workers after collection is observed in all the steps after the fish is caught from the sea and transferred into the boat. Hence, the occurrence of *L. innocua* and *L. monocytogenes* in hand wash makes it a critical control point for *Listeria* sp. in the fish catchment area. The presence of *L monocytogenes* in worker's hands was in accordance with the result of Jeyasekaran *et al*. (2003). However, Thimothe *et al*. (2004) have reported a higher incidence of 10.4 per cent of *L. monocytogenes* from employee contact surfaces (gloves, aprons) in a smoked fish processing plant. The presence of *Listeria innocua* from hands of workers in the present study was more than that reported by Papadopoulos *et al*.

Table 2 : District wise distribution of *Listeria* spp. in control points in fish catchment areas

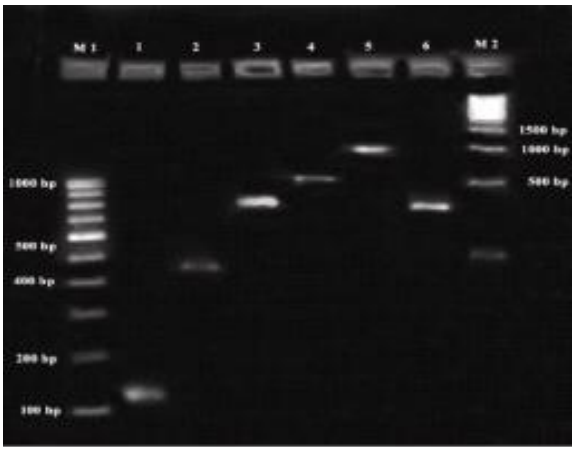
Sr. No.	District	Number of samples positive for *Listeria* spp. Li/Lm								Total
		Soil	SW	Ice	Cont	BD	FV	FLC	HW	
1.	Kozhikode	2/0	1/0	2/0	3/0	1/0	0/0	1/0	4/1	14/1
2.	Kollam	0/0	2/0	1/0	2/0	1/0	1/0	1/0	6/0	14/0
Total		2	3	3	5	2	1	2	10	29

SW (Sea Water), Cont (Container), BD (Boat deck), FV (Fishing vessel), HW(Hand wash), FLC (Fish landing center) , Li (*Listeria innocua*), Lm (*Listeria monocytogenes*) Figures in parenthesis represent per cent prevalence



Lane M : 100bp ladder**,** Lane1: BD1, Lane2 : P1, Lane 3: P2, Lane 4 :H6 and Lane 5: H10 Lane 6 : Sq38, Lane7 SW(Kol), Lane 8 : ATCC 33090, Lane 9. Soil (Koz), Lane10 : S8

Fig. 1 : PCR profile of *Listeria innocua* isolates from sea foods and fish catchment areas



Lane M : 100 bp ladder, Lane 1 : *iap* gene (131 bp), *Lane 2 : hly* A gene (456 bp*),* Lane 3 : *act* A gene (839 bp), Lane 4 : *prf* A gene (1060 bp), Lane 5 : *plc* A gene (1484 bp), *Lane 6 : Inl* A gene (~ 800 bp) Lane M : 500 bp ladder

Fig. 2 : PCR profile of *L. monocytogenes* isolate obtained with six virulence associated genes

Table 3 : Physico-chemical parameters of sandy soil from coastal areas of Kerala

Sr. No.	Region	pH	Electrical conductivity (ds/m)	Organic carbon (%)
1.	Kollam (Neendakara)	7.5	6.16	0.08
2.	Kozhikode (Harbour)	8.0	0.78	0.02

(2010) have isolated *L. innocua* from five per cent of the samples. But Soultos *et al*. (2007) did not detect *Listeria* from hand wash samples from workers of fish markets. The organisms was also isolated from 25 per cent of container swab samples in the catchment area. The sea water samples collected from these areas also revealed the presence of the organism as shown in Table 3. The source of occurrence of the organism in the ice and containers could be attributed to the fact that sea water is normally in the harbour under study, for preparation of ice and washing of containers. Hence, the presence of the organism in ice and containers could inturn contaminate raw seafoods.

A low level of *Listeria* (2 %) was observed from fish landing area on the harbour where fishes were unloaded and put on the floor. The overall prevalence of *Listeria* spp. in fish catchment areas is in agreement with the result of Jeyasekaran *et al*. (2003) who have reported a prevalence of 7.6 per cent from Mangalore coastal area. However, they could not isolate *L. innocua* from fishing vessel, boat deck and fish landing centre. Manoj *et al.* (1991) who have reported the presence of *Listeria* spp. in boat decks and containers as observed in the present investigation but they reported a very low level of isolation of *L. innocua* from fish contact surfaces from Mangalore area.

The sandy soils collected from the harbour areas also revealed a low occurrence of Listeria (2%). The low occurrence could be attributed to its physico-chemical characteristics of sandy soil as shown in Table 3.

The presence of the organism in soil from Kozhikode reveals the hardy nature of the organism as the physico-chemical characteristics are unfavourable for the survivability of the organism. The result of the analysis revealed that the soil was alkaline in nature. The electrical conductivity of soil in Kozhikode harbour was 0.78 ds/m. However, the occurrence of the organism in soil with high electrical conductivity, low organic carbon and alkaline pH may be attributed to reduction in the level of competitive bacteria which might have promoted the survivability of *Listeria* as the organism survives in adverse environmental conditions.

Hence, the critical control point which are major sources of contamination of *Listeria* include hand wash of workers and containers. But the remaining control points where the organism was detected can be considered as minor sources of contamination. The results of the study revealed that seafoods can be a potential source of contamination by *Listeria* species and strict hygienic measures in the fish catchment areas will help in controlling the organism in seafoods.

Aknowledgement:

The financial assistance provided by ICAR under the project 'Outreach programme on zoonotic diseases is gratefully acknowledged'.

LITERATURE CITED

Barrow, C.J. and Feltham, R.K.A. (1993). *Cowan and steel's manual for the identification of medical bacteria* (3rd Ed.). Cambridge press, London, 238p.

Curiale, M.S. and Lewus, C. (1994). Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. *J. Food Prot.*, **57**: 1048-1051.

Das, S., Laliths, K.V., Thampuran, N. and Surendran, P.K. (2013). Isolation and characterization of *Listeria monocytogenes* from tropical seafood of Kerala, India. *Annals Microbiol*., **63**: 1093-1098.

Dhanashree, B., Otta, S. K., Karunasagar, I. and Karunasagar, I. (2003). Typing of *Listeria monocytogenes* isolates by random amplification of polymorphic DNA. *Indian J. Med. Res.*, **117**: 19-24.

Favaro, M., Sarmati, L. Sancesario, G. and Fontana, G. (2014). First case of *Listeria innocua* meningitis in a patient on steroids

and eternecept. *JMM Case Reports*, **1**(2) : 1-5.

Jami, M., Ghanbari, M., Zunabovic, M. and Domig, K.J. (2014). *Listeria monocytogenes* in aquatic food products-a review. *Comp. Rev. Food Sci. Food Safety*., **13** : 798–813.

Jeyasekaran, G., Karunasagar, I. and Karunasagar, I. (2003). Occurrence of *Listeria* spp. in seafood handling environments. *Indian J. Fish*., **50** : 211-214.

King, W., Raposa, S.M., Warshaw, J.E., Johnson, A.R., Lane, D., Klinger, J.D. and Halbert, D.N. (1990). *A calorimetric assay for the detection of Listeria using nucleic acid probes*. In: Miller, A.J., Smith, J.L. and Somkuty, G.A. (eds.), *Foodborne listeriosis* pp. 117-124.

Liu, D. (2008). *Epidemiology*. In: Liu D, Editor. Handbook of Listeria monocytogenes Boca Raton: CRC Press. 27–59pp.

Lovett, J. (1988). Isolation and enumeration of *Listeria monocytogenes*. *Food Technol.*, **42**: 172-175.

Manoj, Y.B., Rosalind, G.M., Karunasagar, I. and Karunasagar, I. (1991). *Listeria* spp. in fish and fish handling areas, Manglore, India. *Asian Fish. Sci*., **4**: 119-122.

McClain, D. and Lee, W.H. (1988). Development of USDA-FSIS for isolation of *Listeria monocytogenes* from raw meat and poultry. *J. Assoc. Off. Anal. Chem.*, **71** : 660- 663.

Moharem, A.S., Raj, A.P.C. and Janardhanan, G.R. (2007). Incidence of *Listeria* species in seafood products of Mysore, India. *J. Food Safety*, **27**: 362-372.

Momtaz, H. and Yadollah, S. (2013). Molecular characterisation of *Listeria* monocytogenes isolated from fresh seafood samples in Iran. *Momtaz & Yadollahi Diagnostic Path.*, **8**:149.

Panda, A.K. and Garg, S.R. (2003). Prevalence of *Listeria* in foods of animal origin. *Indian J Anim. Sci*., **73**: 967-966.

Papadopoulos , T., Abrahim, A., Sergelidis, D., Kirkoudis, I. and Bitchava, K. (2010). Prevalence of *Listeria* spp. in freshwater fish (*Oncorhynchus mykiss* and *Carassius gibelio*) and the environment of fish markets in Northern Greece. *J. Hellenic Vet. Med. Soc*., **61**: 15-22.

Parihar, V.S., Barbuddhe, S.B., Danielsson-Tham, M.L. and Tham, W. (2008). Isolation and characterization of *Listeria* species from tropical seafoods. *Food Control*., **19** : 566–569.

Rawool, D. B., Malik, S. V. S., Barbuddhe, S. B., Shakuntala, I. and Aurora, R. (2007a). A multiplex PCR for detection of virulence associated genes in Listeria monocytogenes. *Internet. J. Food Safety*, **9** : 56–62.

Rawool, D. B., Malik, S.V. S., Shakuntala, I., Sahare, A. M. and Barbuddhe, S. B. (2007b). Detection of multiple virulence-associated genes in Listeria monocytogenes isolated from bovine mastitis cases. *Internat. J. Food Microbiol.*, **113**: 201–207.

Soultos, N., Abrahim, A., Papageorgiou, K. and Steris, V. (2007). Incidence of *Listeria* spp. in fish and environment of fish markets in Northern Greece. *Food Control*., **18**: 554-557.

Sunil, B., Latha, C., Ajaykumar, V.J., Vrindamenon, K. and Kumar, A. (2013). Occurrence of *Listeria* organisms in the shrimp samples from a fishing harbor in Kerala, India. [abstract]. In: *Compendium, International Symposium on Problems of Listeriosis*; 19-22 September, 2013, Goa, India. p. 167. Abstract No. P/EPI/04.

Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N. and Wiedmann, M. (2004). Tracking of *Listeria monocytogenes* in smoked fish processing plants. *J. Food Prot.*, **67** : 328-341.

WEBLIOGRAPHY :

Center for Disease Control and Prevention (2000). URL-*http://cdc.gov/nczed/divisions/dfbmd/diseases/listeriosis.*

Handbook of Fisheries Statistics (2014). Department of animal husbandry fisheries and dairying. Ministry of agriculture government of India, New Delhi. *http://dahd.nic.in.*