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Note

Prevalence and characterisation of *Listeria* spp. from seafood

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ABSTRACT

Listeria monocytogenes, the causative organism of listeriosis, is primarily transmitted to humans through contaminated food. We examined the prevalence of *L. monocytogenes* isolates from fishery products marketed in Goa, India. A total of 221 raw seafood samples were examined for the presence of *Listeria* species following ISO 11290 protocol. Thirty seven (16.74%) samples were positive for *Listeria* species. Out of these, isolates from 4 (1.8%) samples were confirmed as *L. monocytogenes*, the remaining 33 isolates were *Listeria innocua*. All the isolates were subjected to PCR for the *hlyA* gene, which was detected in all the *L. monocytogenes* isolates. The isolates were also found to express phosphatidyl-inositol specific phospholipase C activity on ALOA agar. Multiplex PCR-based clonal typing revealed three *L. monocytogenes* isolates to be of serovar group 1/2a, 1/2c, 3a, 3c while the remaining isolates belonged to serovar group 1/2b, 3b 4b, 4d and 4e. The isolates were grouped into two *Asc*I and *Apa*I PFGE (pulsed field gel electrophoresis) profiles. Prevalence of *L. monocytogenes* in fresh seafood is of significance as it may contaminate and persist in the processing environment.

Keywords: *Listeria monocytogenes*, PCR, PFGE, Pulsed field gel electrophoresis, Seafood, Serotyping

Listeria is Gram-positive, non-spore forming, facultative anaerobic and psychrotrophic bacteria that are widely distributed in nature. The genus *Listeria* has ten species *viz., L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi,* as well as four newly identified species reported in 2009, *L. marthii, L. rocourtiae* (Graves *et al*., 2010; Leclercq *et al*., 2010) and in 2012 *L. weihenstephanensis* and *L. fleischmanii* (Bertsch *et al*., 2013; Halter *et al*., 2013). *L. monocytogenes* is widely recognised as a principal human pathogen that causes serious diseases, such as septicemia, meningitis, meningoencephalitis in immunocompromised individuals, newborns and the elderly; and abortion and stillbirth in pregnant women (Vázquez-Boland *et al*., 2001). Although rare, listeriosis manifests as a severe illness with an exceptionally high level of mortality (20–40%), particularly of those who are most vulnerable, for both epidemic and sporadic cases. More recently, an increasing number of cases have been associated with febrile gastroenteritis (Barbuddhe and Chakraborty, 2009). *Listeria* spp. are indigenous to the marine and estuarine environments (Reilly and Kaferstein, 1997).

L. monocytogenes and other *Listeria* species, isolated from a variety of raw and processed foods like milk and milk products, meat and meat products, vegetables or salads, seafood and seafood products as well as from ready-to-eat foods, have been responsible for several epidemics and sporadic outbreaks (Kalorey *et al*., 2008; Kathariou, 2002). There are 13 recognised serotypes of *L. monocytogenes*, out of which only 3 serotypes *i.e*., 1/2a, 1/2b and 4b are largely involved in causing human listeriosis (Ramaswamy *et al*., 2007). Because of the importance of *L. monocytogenes* strain characterisation for epidemiological investigations, a number of discriminatory subtyping methods have been described (Djordjevic *et al*., 2002; Liu *et al*., 2006). However, most of these methods are not always convenient for routine use in clinical laboratories and require special instruments and skilled personnel. Multiplex-PCR (MPCR) serogrouping is currently used by several public and private laboratories for the characterisation of *L. monocytogenes* (Doumith *et al*., 2004).

In India, *L. monocytogenes* has been isolated from various foods (Barbuddhe *et al*., 2002; Jallewar *et al*., 2007; Parihar *et al*., 2008). The organism has been isolated from fish and fishery products from different parts and interestingly the incidence rate reported from tropical fish is rather low (Das *et al*., 2013). The objective of this study was phenotypic and genotypic characterisation of *Listeria* spp. isolated from seafoods.

Raw/fresh seafood samples (n=221) were collected from retail outlets in Goa, India, during the period January to October 2009. Samples comprised two main categories; finfishes (n = 115) and shellfishes (n=106). The samples were collected in UV sterilised polyethylene sachets, transported to the laboratory and processed for microbiological analysis.

Isolation of *Listeria* spp. from the seafood samples was attempted as per ISO 11290-1, 2. The samples (approximately 25 g each) were inoculated into 225 ml of half Fraser broth and incubated for enrichment of *Listeria* at 37^{0}C for 18-24 h. Further enrichment of *Listeria* was carried out by inoculating 10 ml of full Fraser broth supplemented with acriflavin and nalidixic acid with 0.1 ml of half Fraser broth. Inoculated full Fraser broth was incubated further for 24 h at 37 ^{0}C. A loopful of enriched full Fraser broth was streaked directly on PALCAM agar for selective isolation of listerial colonies (Scotter *et al.*, 2001a, b). The inoculated agar plates were incubated at 37 ^{0}C for 48 h.

Pinpoint grayish-green colonies surrounded by black zone of esculin hydrolysis were presumed as *Listeria*. Morphologically typical colonies were verified by Gram staining, catalase reaction, tumbling motility at 20–25^{0}C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *Staphylococcus aureus* and *R. equi*, nitrate reduction, and fermentation of sugars (rhamnose, xylose, and α-methyl-D-mannopyranoside) and hemolysis on 5% sheep blood agar (SBA). The isolates were further tested for their phospholipase C activity.

The genomic DNA of all the isolates was extracted and was subjected to PCR amplification for detection of *hlyA* gene (Notermans *et al.*, 1991). The primers employed were, forward 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3' and reverse 5'-GCA ACG TAT CCT CCA GAG TGA TCG-3' (Paziak-Domańska *et al.*, 1999). The reaction was performed in Mastercycler epGradient (Eppendorf, Germany) with a preheated lid. The reaction mixture was subjected to an initial denaturation 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. It was followed by final extension of 10 min at 72^{0}C and held at 4 ^{0}C. Standard strain of *Listeria monocytogenes* 4b MTCC 1143 (NCTC 11994), procured from IMTECH, Chandigarh was used as standard for PCR optimisation. The isolates were serotyped using commercial *Listeria* antisera (Denka Seiken, Japan), in accordance with the manufacturer's instructions.

Multiplex serotyping PCR was performed as described by Doumith *et al.* (2004) in a Master Cycler Gradient Thermocycler (Eppendorf, Germany). Pulsed field gel electrophoresis (PFGE) was performed according to the Pulse Net standardised protocol (Graves and Swaminathan, 2001). Briefly, sample plugs were prepared by mixing 240 µl of a standardised cell suspension and 60 µl of a 10 mg ml^{-1} lysozyme solution (Sigma, St. Louis, MO) Sample plugs were digested with 25 U of *Asc*I (Fermentas, MY, USA) at 37°C for 3 h or 160 to 200 U of *Apa*I (Fermentas, MY, USA) at 30°C for 5 h. Plugs were then loaded on 1% agarose gel in 0.5x TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) buffer and electrophoresed on a CHEF-DR II apparatus (Bio-Rad, USA). Gels were stained with ethidium bromide and visualised in a UV transilluminator.

Out of the 221 raw seafood samples examined for the presence of *Listeria* species, 37 (16.74%) samples were positive for *Listeria* species. On the basis of biochemical characterisation, isolates from 4 (1.8%) samples were identified as *L. monocytogenes*. The remaining 33 isolates were *L. innocua* (Table 1). All the four *L. monocytogenes* isolates expressed PI-PLC activity on ALOA medium. In tropical regions, *L. monocytogenes* and other *Listeria* species have been isolated from fishery products on a regular basis since the late 1990s. Moharem *et al.* (2007) isolated *L. monocytogenes* from three (1.83%) fresh fish samples. The prevalence of *Listeria* spp. during the course of shrimp culture was investigated and *L. monocytogenes* was found to be absent from all samples (Bhaskar *et al.*, 1995, 1998). Of the samples analysed during farming, only feed (clam meat) showed the presence of *L. innocua* and *L. seeligeri* in 10% of the samples tested whereas at harvest 50% of sediments samples contained *L. seeligeri, L. innocua* and *L. murrayii* (Bhaskar *et al.*, 1998). The incidence of *L. monocytogenes* in seafood and fishery environmental samples was reported to be 1.2% of the samples from the coast of Kerala (Das *et al.*, 2013). *Listeria* spp. were detected in 32.3, 27.1, and 5% of fresh, frozen, and dry fish samples, respectively. *Listeria innocua* was found to be the most prevalent species of *Listeria* in the seafood and environmental samples of Kerala (Das *et al.*, 2013). Earlier reports also documented *Listeria* spp. other than *L. monocytogenes* to be common in tropical areas (Fuchs and Surendran, 1989; Manoj *et al.*, 1991). *L. monocytogenes* was isolated from 17 fish (7.25%) and

Table 1. Isolation of *Listeria* spp. from seafoods

Species	No. of samples	*Listeria monocytogenes*	*Listeria innocua*
Finfishes	115	2	14
Shellfishes	106	2	19
Total	221	4	33

1 shrimp (2.5%) samples while *L. innocua, L. ivanovii* and *L. seeligeri* were detected only in fish samples [2 (0.9%), 3 (1.36%) and 1 (0.45%), respectively] in Iran (Momtaz and Yadollahi, 2013). Ben Embarek (1994) reviewed the incidence of *Listeria* in seafood worldwide and found that the prevalence of *L. monocytogenes* varied from 4 to 12% in surveys from temperate areas. Nevertheless, some studies conducted after 1994 demonstrated that the prevalence of *L. monocytogenes* in tropical shrimp is equal to that in temperate shrimp, thus refuting the initial hypothesis. Jeyasekaran and Karunasagar (1996) claimed that the absence of *L. monocytogenes* in earlier reports could be due to inadequate methodology used. These authors demonstrated that both *L. monocytogenes* and other *Listeria* spp. could be found simultaneously in shrimp and other seafood tested, and this finding indicated that these species might share the same ecological niche. This is significant because the presence of *L. innocua* and other non-pathogenic species of *Listeria* serve as indicators of the presence of *L. monocytogenes*. A relatively high prevalence of *L. monocytogenes* was later reported from other tropical areas such as Brazil (8.8-18.0%) (Destro, 2000) and Malaysia (44.0%) (Arumugaswamy *et al.,* 1994).

Doumith *et al.* (2004) developed a rapid multiplex-PCR serotyping assay which separated the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c and 4b) into distinct groups This assay was employed in the present study to evaluate serotyping profile of *L. monocytogenes* isolates recovered from seafood samples. Three *L. monocytogenes* isolates were assigned to serotype 1/2a and another one to serotype 1/2b by serology. Multiplex PCR based serotyping revealed three *L. monocytogenes* isolates to be 1/2a, 1/2c, 3a, 3c group (Table 2, Fig. 1). One isolate belonged to 1/2b, 3b, 4b, 4d and 4e serogroup. Earlier, Handa *et al.* (2005) reported 1/2a to be the dominant serotype among *L. monocytogenes* isolated from seafood in Japan. Chou and Wang (2006) studied the genetic relationship between *L. monocytogenes* isolates from catfish, non-catfish seafood and humans. Most of the non-catfish seafood and catfish isolates were genetically distinct from human isolates. Serotype 4b was dominant in human isolates, whereas serotypes 3b, 1/2a, and 1/2b

Table 2. Characterisation of *Listeria monocytogenes* isolated from seafood

Isolate	Haemolysis	PI-PLC	*hly*A	Serotype	Serogroup
1	+	+	+	1/2a	1/2a, 1/2c, 3a, 3c
2	+	+	+	1/2a	1/2a, 1/2c, 3a, 3c
3	+	+	+	1/2a	1/2a, 1/2c, 3a, 3c
4	+	+	+	1/2b	1/2b, 3b, 4b, 4d, 4e

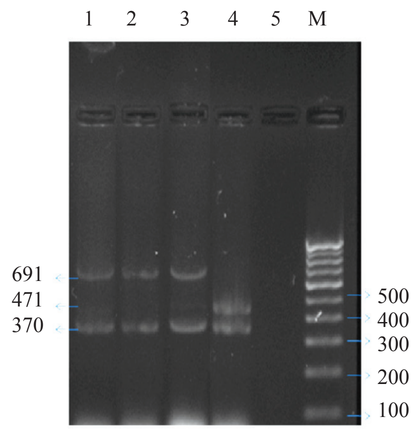
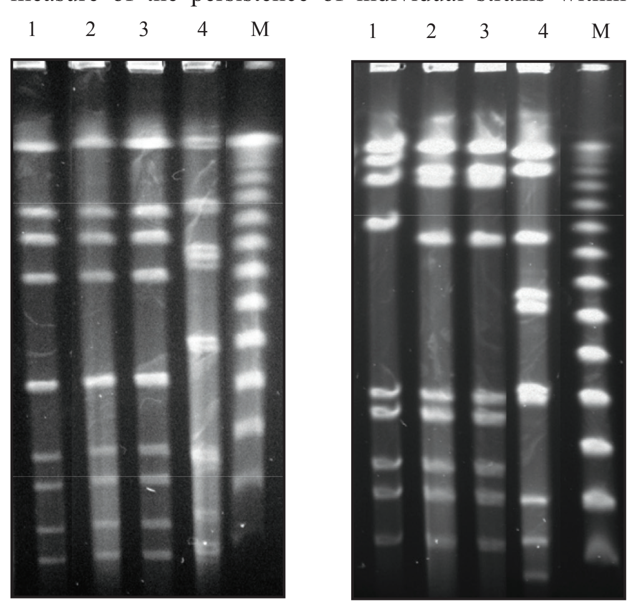


Fig. 1. Lineage/Serogroup profile of *Listeria monocytogenes* isolates by PCR-based typing. Lanes 1: *L. monocytogenes* 1/2a (NCTC 7973), Lane 2-4: *L. monocytogenes* isolates, Lane 5: Negative control, M: DNA ladder

and serotypes 4b, 1/2a and 1/2b were commonly found in catfish and non-catfish seafood, respectively.

PFGE analysis allowed discrimination among isolates of the different serotypes. The isolates were grouped into two *AscI* and *Apa*I PFGE profiles (Fig. 2a and 2b). Application of PFGE in characterisation of *L. monocytogenes* isolated from food can provide valuable information on potential sites of cross-contamination. To enable more precise identification of sites of cross-contamination, or critical control points, and to give some measure of the persistence of individual strains within



(a). *Asc*I digestion pulsotype (b). *Apa*I digestion pulsotype

Fig. 2. *Asc*I and *Apa*I enzyme restriction digestion of *L. monocytogenes* isolates obtained from raw seafood samples. Lanes 1, 2, and 3: *L. monocytogenes* 1/2a isolates; Lane 4: 4b isolates; Lane 5: PFGE marker

the processing environment, epidemiological tracking of strains over time is required.

Contamination with *L. monocytogenes* may take place long before the raw material reaches retail trade or processing factories. Potential sources of *L. monocytogenes* include contamination from water and ice, soiled surfaces and boxes, as well as contamination from human and avian sources (Parihar *et al.*, 2008). Little is known on the potential *Listeria* contamination of fish and fish products at the retail level. Products that are purchased in bulk and re-packaged prior to sale may be vulnerable to *L. monocytogenes* contamination. Despite the occurrence of *L. monocytogenes* in raw and frozen seafood, these products do not pose a threat to the majority of people as they undergo some processing before being eaten. However, they still pose risk to susceptible populations when consumed raw or lightly cooked. In addition, the possibility of cross contamination in the processing plant, kitchen or food service establishment is also of concern.

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