Isolation and Confirmation of *Listeria* Species from Seafood off Goa Region by Polymerase Chain Reaction

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Abstract Several food borne outbreaks have highlighted the importance of *Listeria monocytogenes* to the public health and have been recognized as an emerging, important food borne pathogen, and a causative agent of listerioses. A number of genes are involved in the manifestation of *Listeria* virulence, *hlyA* is one among them. In the present study, 111 marine fish samples including prawns, finfishes and bivalves were screened for the presence of *Listeria* species. The isolates were characterized biochemically and further *L. monocytogenes* were confirmed by polymerase chain reaction (PCR) technique using the *hlyA* gene as a tool to differentiate between *L. monocytogenes* and other non-pathogenic *Listeria* species. Out of 111 samples 5 (4.5%) samples were positive for *L. monocytogenes*. Among the three different types of samples bivalves were found to have maximum percent (12.5) of *L. monocytogenes* followed by prawns (3.84) and finfishes (2.9). Among all the 111 samples, 15 (13.51%) samples were positive for other *Listeria* species. It was observed that *Listeria* occurrence is more in shellfishes than in fin fishes. All the isolates were sensitive towards five different antibiotics in sequence ciprofloxacin > sulphafurazole > norfloxacin > ampicillin and gentamicin.

Keywords *Listeria monocytogenes* · Isolation · Seafood · PCR · *Hly A* gene

Listeriosis is an important bacterial infection caused by a gram positive facultative anaerobe, non-spore-forming, rod-shaped bacteria, and intracellular pathogen *Listeria monocytogenes*. *L. monocytogenes* has been recognized as an important opportunistic human pathogen since 1929 and as a food borne pathogen since 1981. Its public health significance lies in its ubiquitous nature that is manifested in its host range, which includes 40 mammals, 20 birds, crustaceans, ticks and fishes [1]. Multiple key virulence factors, such as internalins (encoded by *inlA* and *inlB*), hemolysin (*hlyA*), phosphatidylinositol-specific phospholipase C (PI-PLC, *plcA*), phosphatidylcholine-specific phospholipase C (PC-PLC, *plcB*) and actin polymerization protein (*actA*) are important in *L. monocytogenes* pathogenesis [2]. Although *L. monocytogenes* is a non sporulating bacterium, it is resistant to different environmental conditions, including acid pH, high NaCl concentration, microaerophilia and refrigeration temperatures [3] with mortality rates on an average approaching 30%, *L. monocytogenes* far exceeds other common foodborne pathogens, such as *Salmonella enteritidis* [4, 5]. *L. monocytogenes* can be transferred to human beings mainly through food contaminated with this organism. Hazard analysis critical control point (HACCP) approach has been suggested to control *Listeria* in the food supply [6, 7]. Because of the high fatality associated with this organism, U.S. regulatory agencies have established a ‘zero tolerance’ for ready to eat foods.

Several reports indicate that fish and fishery products can be frequently contaminated with *L. monocytogenes* as this organism has been isolated from fish and fishery

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products from different parts of the world [8–11]. In India, a few studies have been conducted for the detection and confirmation of *L. monocytogenes* from sea foods mainly bivalves and finfishes. Especially in Goa region there are no published reports on *L. monocytogenes* studies in sea foods. Goa being coastal area fish consumption is more and sea food forms the main diet for Goans. Fish can act as a major vector of *L. monocytogenes* and other pathogenic microbes to the local people. This brings the need for the screening of *L. monocytogenes* in seafoods in order to prevent the food poisoning and also to prevent the economic loss for different food industries. The rapid and reliable detection of *L. monocytogenes* has been suggested to be ideally based on the detection of virulence markers of *Listeria* species by molecular techniques [12]. PCR is a technique which possesses rapidity, sensitivity, and specificity and could therefore be employed to facilitate rapid diagnosis of *L. monocytogenes* contamination. Thus, it is considered that DNA extraction and PCR detection could become a viable alternative to the conventional methods for detection of *L. monocytogenes*. The present investigation deals with the isolation and confirmation of *L. monocytogenes* from sea foods by rapid, reliable and simple PCR technique.

Standard cultures of *L. monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. One hundred and eleven marine fish samples, bivalves (16), prawns (26) and finfishes (69) were collected from local fish markets of Goa, West coast of India. The samples were collected in UV sterilized polyethylene sachets at the places of collection, and processed immediately (within 5 h of collection) for microbial analysis.

Isolation of *Listeria* was attempted as per the USDA method described by McClain and Lee [13] after some modifications. Grey green colonies with black sunken centers on Polymixin Acriflavin Lithium chloride Cefotaxime Esculin Mannitol (PALCAM) agar, of about 0.5 mm diameter surrounded by a diffused black zone of aesculin hydrolysis after 24 h of incubation at $37 \pm 1^\circ\text{C}$ were considered to be of *Listeria* and were further treated for the Gram's staining and motility test and also were subjected to biochemical tests i.e. methyl red, Voges–Proskauer (MR, VP), Rhamnose, Mannitol, Xylose and catalase test, Christie Atkins Munch Petersen (CAMP) test which is specific for *Listeria* species was performed with 5% sheep blood agar using the cultures of *Staphylococcus aureus* MTCC 1144 and *Rhodococcus equi* MTCC 1135. The *Listeria* isolates confirmed by above tests were further analysed by PCR [14] technique for the detection of *L. monocytogenes*.

Oligonucleotide primers used in the study were synthesized by Sigma–Aldrich, St. Louis, MO. The sequences of oligonucleotide primers used were 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3'—Forward and 5'-GCA ACG TAT CCT CCA GAG TGA TCG-3'—Reverse for the *hlyA* gene.

DNA template for PCR was prepared by simple boiling and snaps chilling method [15]. Briefly, a loopful of cells grown overnight in BHI broth at 37°C . The obtained culture was centrifuged in a microfuge at 6000 rpm for 10 min. The recovered pellet was resuspended in 100 μl of sterilized DNAs and RNAs free milliQ water, followed by heating it in boiling water bath for 10 min and then snap chilling in crushed ice and used for the PCR reaction mixture.

The PCR was set for 50 μl reaction volume containing 5.0 μl of 10X PCR buffer (consisting of 100 mM Tris–HCl, pH 8.3; 500 mM KCl; 15 mM MgCl_2 and 0.01% gelatin), 0.2 mM dNTP mix, 2 mM MgCl_2 and 10.1 μM of forward and reverse primer of each set, 1 unit of Taq DNA Polymerase, 5 μl of cell lysate and sterilized milliQ water to make up the reaction volume. The reaction was performed in Gradient Thermocycler (Thermohybrid, UK) with a preheated lid. PCR product (15 μl) after mixing with 2 μl bromophenol blue dye was analysed by gel electrophoresis by using 1.5% agarose gel containing 3 μl ethidium bromide. A reaction mix without DNA and one with 1 ng of *L. monocytogenes* MTCC 1143 total DNA were included as controls. All the isolates were also tested for antibiotics (ampicillin, ciprofloxacin, norfloxacin, sulphafurazole, and gentamycin) sensitivity by standard disc diffusion method [16]. The results were interpreted as per the NCCLS criteria [17] (data not shown).

Out of 111 samples screened 23 samples showing black colonies with centered halo on PALCAM agar due to its ability of aesculin hydrolysis [18].

Out of these the 20 catalase positive isolates showed tumbling motility at $25 \pm 1^\circ\text{C}$ were subjected to biochemical and CAMP test (Table 1) as these two tests help to differentiate between the *Listeria* species. *Listeria* species utilize dextrose, esculin, and maltose, and some species utilize mannitol, rhamnose, and xylose with production of acid. An isolate utilizing mannitol with acid production is *L. grayi*. *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* produce hemolysis on sheep blood agar. Of the three, only *L. monocytogenes* fails to utilize xylose and is positive for rhamnose utilization. The difficulty in differentiating *L. ivanovii* from *L. seeligeri* can be resolved by the CAMP test. *L. seeligeri* shows enhanced hemolysis at the *S. aureus* streak. *L. ivanovii* shows enhanced hemolysis at the *R. equi* streak. Of the non-hemolytic species, *L. innocua* may provide the same rhamnose-xylose reactions as *L. monocytogenes* but it is negative for the CAMP

Table 1 Biochemical and CAMP test for the presumed (23) isolates

Sr. no.	Fish samples	MR	VP	Nitrate	Rhamnose	Xylose	Mannitol	Catalase	Gram character	Motility	CAMP	Morphology
1	<i>Rostreliger Kanagureti</i>	+	+	–	+	–	+	–	+	–	–	Cocci
2	<i>Gerrus oblongus</i>	+	+	–	+	–	–	+	+	+	–	Cocco bacilli
3	<i>Caranx melampygus</i>	+	+	–	+	–	–	+	+	+	–	Rods
4	<i>Caranx Melampygus</i>	+	+	–	+	–	–	+	+	+	+	Rods
5	<i>Etroplus suratensis</i>	+	+	–	+	–	+	–	+	–	–	Cocci
6	<i>Opisthopterus tartoor</i>	+	+	–	+	–	–	+	+	+	–	Rods
7	<i>Gerrus oblongus</i>	+	+	+	+	–	–	–	+	–	–	Cocci
8	<i>Engraulis hamiltonii</i>	+	+	–	+	+	–	+	+	+	–	Cocci
9	<i>Cynoglossus macrolepidotus</i>	+	+	–	+	–	–	+	+	+	–	Rods
10	<i>Sunetta species</i>	+	+	–	+	–	–	+	+	+	+	Rods
11	<i>Meretrix meretrix</i>	+	+	–	+	–	–	+	+	+	+	Rods
12	<i>Caranx boops</i>	+	+	–	+	–	+	+	+	+	–	Cocci
13	<i>Equula dussumieri</i>	–	+	–	–	+	–	+	+	+	–	Cocci
14	<i>Mercenaria species</i>	+	+	–	+	–	–	+	+	+	–	Rods
15	<i>Engraulis commersonianus</i>	+	+	–	+	–	–	+	+	+	+	Rods
16	<i>Fenneropenaeus indicus</i>	+	+	–	+	–	–	+	+	+	–	Cocco bacilli
17	<i>Gerrus lucids</i>	+	+	–	+	–	–	+	+	+	–	Rods
18	<i>Engraulis hamiltonii</i>	+	+	–	+	–	+	+	+	+	–	Cocci
19	<i>Vellorita cyprinoids</i>	+	+	–	+	–	–	+	+	+	–	Rods
20	<i>Gerruas oblongus</i>	+	+	+	–	–	–	+	+	+	–	Rods
21	<i>Sunetta species</i>	+	–	+	+	–	+	+	+	+	–	Cocci
22	<i>Vellorita cyprinoids</i>	+	+	–	+	–	–	+	+	+	–	Rods
23	<i>Penaeus indicus</i>	+	+	–	+	–	–	+	+	+	+	Cocco bacilli
24	Positive control <i>L. monocytogenes</i> MTCC 1143	+	+	–	+	–	–	+	+	+	+	Rods
25	Negative control (blank)	–	–	–	–	–	–	–	–	–	–	Cocci

VP Voges–Proskauer test, CAMP Christie Atkins Munch–Peterson test, + positive test, – negative test, MR methyl red test

test [18]. *L. innocua* sometimes gives negative results for utilization of rhamnose. A *L. welshimeri* isolate that is rhamnose-negative may be confused with a weakly hemolytic *L. seeligeri* isolate unless resolved by the CAMP test. Positive control (*L. monocytogenes* MTCC 1143) showed methyl red, Voges–Proskauer, rhamnose and catalase tests positive after 24 h incubation at $28 \pm 1^\circ\text{C}$, whereas xylose, mannitol and nitrate tests were negative. Negative control was kept without the culture which did not show any change in the medium after 24 h also. Out of 20 catalase positive isolates 15 were showing same biochemical reactions as that of positive control and among these 15 isolates, 5 were showing the Christie Atkins Munch–Peterson (CAMP) test positive with *S. aureus* at $28 \pm 1^\circ\text{C}$ after 24 h incubation. Isolates which were showing positive CAMP test towards the *S. aureus* MTCC 1144 strain with intense β haemolysis and negative CAMP test with *R. equi* MTCC 1135 were considered to be *L. monocytogenes*. *L. seeligeri* is also CAMP-positive to the *S. aureus* strain and CAMP-negative to *R. equi*. All the 5 CAMP positive cultures were subjected

to PCR test for the confirmation of *L. monocytogenes* using *hlyA* gene as target. The *hlyA* gene codes for listeriolysin, a thiol activated cytolysin which is essential for the release of the organism after initial phagocytosis and after actin-mediated transport to neighbouring cells [19] and one of the virulence factors for pathogenesis.

Based on all the above tests performed, we found the 18.01% occurrence of *Listeria*. Maximum numbers of *Listeria* isolates were found in bivalves followed by finfishes and prawns. Out of 20 *Listeria* isolates 5 (4.5%) isolates were confirmed as *L. monocytogene* since they showed the presence of *hlyA* gene when subjected to PCR assay represented by a single band of ~ 450 bp (Fig. 1) similar to that of positive control. Regarding primer specificity a primer that involve targeted gene *hlyA* was selected.

The highest incidence of *L. monocytogenes* was recorded from bivalves followed by finfishes and prawns (Table 2). All other *Listeria* isolates were found to be negative for *hlyA* gene. In present study the percentage

Fig. 1 PCR profile of the isolates showing *hlyA* (~450 bp) virulence associated gene of *L. monocytogenes*. Lane M PCR marker (300 bp to 2000 bp), Lane 1 *L. monocytogenes* MTCC1143, Lanes 2–6 isolates from the samples

Table 2 Isolation of *Listeria* species from seafood

Samples	Samples tested no.	<i>Listeria</i> spp. No. (%)	<i>L. monocytogenes</i> No. (%)	Overall %
Bivalves	16	2 (12.5)	2 (12.5)	25
Prawns	26	2 (7.69)	1 (3.84)	11.53
Finfishes	69	11 (15.94)	2 (2.9)	18.84
Overall	111	15 (13.51)	5 (4.5)	18.01

occurrence of *L. monocytogenes* is less than that reported by Jayasekaran et al. [20], as 12.1% in fresh shell fishes and 17.2% in fresh finfishes where as it is higher than the 3% occurrence in European fish [21]. Compared to fresh water fishes [20] (13%), there is low *L. monocytogenes* occurrence in marine finfishes (2.9%). Also the prevalence is more in tropical region than temperate [20, 22]. Bivalve molluscs showed high occurrence of *L. monocytogenes* (12.5%) among all three varieties of samples and compared to (7.5%) findings of Simon et al. [10]. Since bivalves are filter feeders they can accumulate more microorganisms than fishes, from waters impacted by sewage pollution etc. Compared to the 3.84% occurrence of *L. monocytogenes* in prawns, earlier studies [23] shows 44% frequency of *L. monocytogenes* in raw samples and 22% in ready to eat prawns. Overall occurrence of *L. monocytogenes* in present study was higher than the values reported in the fish squid and bivalve mollusks samples in San, Luis Argentina [24]. The antibiotic sensitivity of all the *Listeria* isolates towards five antibiotic discs was in the sequence ciprofloxacin > sulphafurazole > norfloxacin > ampicillin and gentamicin.

Isolation of *L. monocytogenes* and other *Listeria* spp. from the raw seafood indicates the potential public health hazard. Diligent enforcement of sanitary conditions of food contact surface and handling areas, and personal hygiene practices should reduce the potential contamination of fishery products by *L. monocytogenes* at the retail level. Earlier studies show the low occurrence of *L. monocytogenes* in fresh water fishes than in marine water [20, 25]. Since the climatic conditions were also different for some studies, study should be conducted in the same region in order to check the variation in occurrence of this organism both in fresh and marine fishes. The PCR method used in the present study can be used for the rapid identification of the *L. monocytogenes*, as the traditional practice of biochemical tests can be time consuming and sometimes confusing within the species.

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