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ORIGINAL ARTICLE

Isolation and characterization of *Listeria monocytogenes* from tropical seafood of Kerala, India

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Abstract *Listeria monocytogenes*, which is an intracellular pathogen, causes various illnesses in human as well as in animals. The pathogenicity of this organism depends upon the presence of different virulence genes. A total of 324 tropical seafood and fishery environmental samples were screened for *L. monocytogenes*. The incidence of the human pathogenic species *L. monocytogenes* was 1.2 % of the samples. *Listeria* spp. was 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 tropical seafood and environmental samples of Kerala. *Listeria monocytogenes* and *L. innocua* isolates were confirmed by multiplex PCR. *L. monocytogenes* isolates from the four positive samples showed phosphatidylinositol-specific phospholipase C reaction on Chromocult® *Listeria* selective agar. Molecular characterization of *L. monocytogenes* isolates for virulence genes revealed the presence of β-hemolysin (*hly*), *plcA*, *actA*, metalloprotease (*mpl*), *iap* and *prfA* genes in all the isolates recovered from the positive samples.

Keywords *Listeria monocytogenes* · Tropical · Seafood · Virulence gene

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Introduction

Listeria monocytogenes is the major human pathogen among eight species of *Listeria*. This organism causes abortion, encephalitis, gastroenteritis, arthritis, conjunctivitis, etc. in human (Vázquez-Boland et al. 2001). Apart from *L. monocytogenes*, *L. ivanovii* has also been reported to cause human infection in some cases (Guillet et al. 2010). *Listeria monocytogenes* also causes clinical diseases in animals. In ruminants, listeriosis is characterized as encephalitis showing typical 'circling' symptoms, third trimester abortion, stillbirth, conjunctivitis, etc. (Hoelzer et al. 2012). This pathogen is widely distributed in nature and is generally transmitted to human through contaminated food and water. Being an intracellular pathogen, it can multiply even inside different phagocytic cells like macrophages (Kuhn et al. 1988). Biofilm formation on food-contact surfaces by this pathogen is an indication of severe public health hazards (Zameer et al. 2010). *Listeria monocytogenes* is one of very few pathogenic organisms which can grow at refrigerated temperatures. Thus, the storage of food at low temperatures cannot prevent the growth of this pathogen (Junttila et al. 1988).

Listeriosis in human is very often associated with very high mortality which may be as high as 30 % (Griffiths 1989). In USA, approximately 1,591 cases of listeriosis in human have been reported in a year and it contributes to about 0.1 % of total food-borne illnesses, but is responsible for 2.6 % of hospitalizations and 18.87 % of the deaths caused by food-borne illnesses (Scallan et al. 2011). Both

the outbreaks and sporadic human listeriosis is caused by transmission of this pathogen through contaminated food and the involvement of a wide variety of foods has been reported throughout the world (Linnan et al. 1988; Ericsson et al. 1997). In USA, the recent multistate outbreak of *L. monocytogenes* apparently through consumption of contaminated cantaloupe has drawn worldwide attention. A total of 146 persons were infected and 33 deaths were reported in this outbreak in which multiple states of USA were affected (CDC 2012). In 2010, there was an incidence of another outbreak of listeriosis in Texas, USA, apparently through consumption of celery (CIDRAP 2010). Considering its immense pathogenic potentiality and high mortality rate, different regulatory agencies including USFDA have imposed zero tolerance for this pathogen in ready-to-eat (RTE) food products (Hitchins 1998).

The virulence of this pathogen is mediated by different virulence genes, i.e. β-hemolysin (*hly*), phosphatidylinositol-specific phospholipase C (*plcA*), *prfA*, *iap*, *actA*, and metalloprotease (*mpl*) (Domann et al. 1991; Rawool et al. 2007). *prfA* gene is known as a positive regulatory factor, which controls the expression of other virulence genes (Chakraborty et al. 1992). Although India has not experienced any major outbreak of listeriosis, sporadic cases in human have been reported and *L. monocytogenes* has been previously isolated from different food products including seafood (Malik et al. 2002; Dhanashree et al. 2003). Clinical cases of listeriosis in human caused by *L. monocytogenes* have also been reported from Delhi and the Karnataka area of India (Bhujwala et al. 1973; Dhanashree et al. 2003). Therefore, regular screening and constant surveillance of food products including seafood for the presence of this pathogen are required.

The present study has been undertaken to assess the occurrence of this pathogen in the tropical seafood and fisheries environment of Kerala, India, and also to screen the presence of different virulence genes in *L. monocytogenes* isolates recovered from the samples.

Materials and methods

Samples

A total of 324 samples of seafood comprising of 255 seafood and 69 fisheries environment samples were screened for the presence of *L. monocytogenes* during the period from March 2005 to March 2006 and from November 2007 to December 2011 (Table 1). The samples were procured from fish hatcheries, fish landing centers, and retail outlets located at Ernakulum, Alappuzha, and Kottayam and Kollam districts of Kerala, India.

Standard cultures used in this study

Listeria monocytogenes ATCC 19115 and *L. innocua* ATCC 330390 were procured from the American type culture collection, USA. *Listeria monocytogenes* MTCC 657 was procured from the Microbial type culture collection, Institute of Microbial Technology, Chandigarh. *Listeria monocytogenes* NCTC 11994 was maintained in the Microbiology, Fermentation and Biotechnology Division. *Staphylococcus aureus* NCIM 5022 was procured from the National Collection of Industrial Microorganisms, National Chemical laboratory, Pune. All the type cultures were maintained on Tryptic soya agar slants at 4 °C with intermittent subculture.

Isolation and identification of the organism

The screening of the samples for the presence of *Listeria* spp. was carried out using the ISO 11290-I method mentioned in Scotter et al. (2001). Twenty five grams of sample was taken in 225 ml of half-Fraser's broth, macerated in a stomacher blender (Seward), and incubated at 30 °C for 24 h. Then, 0.1 ml was transferred to Fraser's broth and was incubated at 37 °C for 48 h. Plating from both half-Fraser's broth and Fraser's broth was done on *Listeria* selective agar (Oxford formulation) and PALCAM agar plates. Oxford and PALCAM plates were incubated at 30 and 37 °C, respectively, for 48 h. The typical colonies from *Listeria* selective agar (Oxford and PALCAM) were isolated, purified again on corresponding agar media (Oxford or PALCAM), and stored on Tryptic soya agar slants at 4 °C. Identification of the isolates was done by standard biochemical tests, restricted β-hemolysis on sheep blood agar, and the CAMP test as mentioned in Hitchins (1998). *Listeria monocytogenes* isolates were further confirmed by spot inoculation on Chromocult® *Listeria* selective agar (Merck, Germany) for phosphatidylinositol-specific phospholipase C activity. The plates were incubated at 37 °C for 48 h and were examined for typical blue-green colonies of *L. monocytogenes* with an opaque halo surrounding the colony.

Serogrouping of the *L. monocytogenes* strains and isolates

Listeria monocytogenes isolates recovered from seafoods in this study, two freshwater fish isolates from Goa, India, *L. monocytogenes* ATCC 19115 and *L. monocytogenes* MTCC 657, were employed in this study. They were serogrouped using Bacto *Listeria* Type 1 and Type 4 antisera (Difco, USA) by the slide agglutination test as per the manufacturer's instruction. Two *L. monocytogenes* isolates from Goa were kindly donated by Dr. S.B. Barbuddhe, Senior Scientist, ICAR Research Complex, Goa, India.

Table 1 *Listeria* spp. in the fish and fisheries environment of tropical area of Kerala, India, based on types of samples

Type of samples	Number of samples screened (*n*)	Positive for *Listeria* spp.	Positive for *L. monocytogenes*	Positive for *L. innocua*
Marine finfish	140	42 (30 %)	2 (1.4 %)	41 (29.3 %)
Freshwater finfish	33	12 (36.4 %)	1 (3 %)	12 (36.4 %)
Prawn	28	7 (25 %)	0	7(25 %)
Clams and mussels	31	7 (22.6 %)	0	7 (22.6 %)
Fishery environments	69	20 (29 %)	1 (1.4 %)	19 (27.5 %)
Crab	23	7 (30.4 %)	0	7 (30.4 %)
Total	324	95 (29.3 %)	4 (1.2 %)	93 (28.7 %)
Listeria spp. in fresh, frozen and dry fish samples				
Fresh fish	198	64 (32.3 %)	3 (1.26 %)	63 (28.9 %)
Dry fish	20	1 (5 %)	0	1 (5 %)
Frozen fish	37	10 (27.1 %)	0	10 (25.6 %)
Total	255	75 (29.4 %)	3 (1.2 %)	74 (29 %)
Listeria spp. in fishery environment				
Ice	40	12 (30 %)	1 (2.5 %)	11 (27.5 %)
Mud and sand	22	8 (36.4 %)	0	8 (36.4 %)
Seawater	5	0	0	0
Water hyacinth	2	0	0	0
Total	69	20 (29 %)	1 (1.4 %)	19 (27.5 %)

Preparation of bacterial lysate for multiplex PCR

All the cultures were grown in BHI broth for the preparation of lysate. Lysate from the bacteria was prepared by boiling method as done by Fitter et al. (1992) with slight modifications. The bacterial culture was centrifuged at 7,000 *g* for 10 min at 4 °C and the resultant pellet was washed once in normal saline. The pellet was suspended in 150 µl sterile distilled water and kept in boiling water bath for 10 min and frozen immediately at −30 °C. Before use as the PCR template, it was thawed and centrifuged at 4,000 *g* to pellet the debris. Five µl of the supernatant was used as template in the PCR reaction.

Multiplex PCR for differentiation of *L. monocytogenes* and *L. innocua*

Three sets of primers were used for this multiplex PCR reaction. Primers pairs UI/LII, LL5/LL6, and lin0464F/l in0464R are specific to *Listeria* genus, *L. monocytogenes*, and *L. innocua*, respectively (Table 2). Primers pairs UI/LII and LL5/LL6 are based on the 16S rRNA gene and the *hly* gene, respectively, whereas the primer pair lin0464F/lin0464R is based on a gene with unknown function in *L. innocua*. This PCR assay was carried out using the protocol of Das et al. (2010). PCR assays were started with an initial denaturation at 95 °C for 3 min followed by 30 cycles of 94 °C for 45 s (denaturation), 55˚ for 45 s (annealing), and primer extension at 72˚ for 45 s. Final extension was carried out at 72 °C for 5 min. PCR products were resolved on electrophoresis on 1.5 % agarose gel containing 0.3 µg/ml ethidium bromide in 0.5× TBE buffer at 7 V/cm for 1 h.

Screening for virulence genes in *L. monocytogenes*

Listeria monocytogenes isolates from the four positive samples of this study, as well as the standard strains, were screened separately for the presence of different virulence genes, e.g., β-hemolysin (*hly*), *plcA*, *actA*, metalloprotease (*mpl*), *iap* and *prfA* genes by PCR. Genomic DNA from all the standard strains and isolates were purified as per the method of Liu et al. 2003. The concentration and purity of the genomic DNA were estimated by taking the OD at 260 and 280 nm. For screening of *hly*, *plcA*, *actA*, *iap* and *prfA* genes, the primers as mentioned in Rawool et al. (2007) were used. The primer pair targeting the *mpl* gene has been designed based on the published sequences of *mpl* genes of *L. monocytogenes* using Primer 3 software, which is freely available online at http://frodo.wi.mit.edu/. The sequences of all the primers used in this study are shown in Table 2. For amplification of the *hly*, *plcA*, *actA*, *iap* and *prfA* genes, PCR was carried out in a 50 µl reaction mixture consisting of 10× PCR buffer (Fermentas), 2 mM $MgCl_2$, 0.2 mM dNTPs mix, 1U *Taq* DNA polymerase and 10 µM of the respective primer pair. The PCR cycling condition consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min 30 s. Final

Table 2 Primers used in this study

Primer pair and sequences (5′–3′)	Specificity	References	Product size (bp)
UI (CAGCMGCCGCGGTAATWC) and LII (CTCCATAAAGGTGACCCT)	*Listeria* genus	Border et al. 1990	938
LL5 (AACCTATCCAGGTGCTC) and LL6 (CTGTAAGCCATTTCGTC)	*L. monocytogenes*	Herman et al. 1995	267
lin0464F (CGCATTTATCGCCAAAACTC) and lin0464R (TCGTGACATAGACGCGATTG)	*L. innocua*	Liu et al. 2003	749
Forward (CTGCTTGAGCGTTCATGTCTCATCCCCC) and Reverse (CATGGGTTTCACTCTCCTTCTAC)	*plcA* gene *of L. monocytogenes*	Notermans et al. 1991; Rawool et al. 2007	1,484
Forward (CTGTTGGAGCTCTTCTTGGTGAAGCAATCG) and Reverse (AGCAACCTCGGTACCATATACTAACTC)	*prfA* gene of *L. monocytogenes*	Notermans et al. 1991; Rawool et al. 2007	1,060
Forward (GCAGTTGCAAGCGCTTGGAGTGAA) and Reverse (GCAACGTATCCTCCAGAGTGATCG)	*hlyA* gene *of L. monocytogenes*	Paziak-Domanska et al. 1999; Rawool et al. 2007	456
Forward (ACAAGCTGCACCTGTTGCAG) and Reverse (TGACAGCGTGTGTAGTAGCA)	*iap* gene of *L. monocytogenes*	Furrer et al. 1991; Rawool et al. 2007	131
Forward (CGCCGCGGAAATTAAAAAAAGA) and Reverse (ACGAAGGAACCGGGCTGCTAG)	*actA* gene of *L. monocytogenes*	Rawool et al. 2007	839
Forward (TGGTAATAGCTTTTCAGGCTCA) and Reverse (CTTTAAGGCGCGGAAGTAAAG)	*mpl* gene of *L. monocytogenes*	Designed for this study	1,376

extension was done at 72 °C for 5 min. The PCR reaction mixture for detection of *mpl* was same as in case of other virulence genes except the $MgCl_2$ concentration, which was 1.5 mM. For the amplification of the *mpl* gene, the same cycling condition was used except for the annealing temperature, which was 59 °C. PCR products were resolved as described previously.

Results

Isolation of *Listeria* spp.

Among 324 samples, *Listeria* spp. were recovered from 95 samples (occurrence rate 29.3 %) and *L. monocytogenes* was detected in 4 samples, i.e. 2 marine fish samples, 1 freshwater fish and 1 ice sample (Table 1). However, *L. innocua* was found to be the most prevalent species of *Listeria* present in the tropical water of Kerala with an incidence rate of 28.7 %. The incidence of *L. monocytogenes* was found to be very low, i.e. only 1.2 % of total samples tested. Among fresh, dry, and frozen samples, the lowest occurrence of *Listeria* spp. was found in the dry fish (5 %). In fresh and frozen fish samples, the occurrence of *Listeria* spp. was found to be 32.3 and 27.1 %, respectively. Among fisheries environmental samples, 29 % of the samples (20 out of 69) were found to harbor *Listeria* spp. and *L. monocytogenes* was detected in one sample (ice).

Listeria monocytogenes isolates were identified by standard biochemical tests and were confirmed in Chromcult® *Listeria* selective agar. All the *L. monocytogenes* type cultures and isolates produced blue-green colonies with opaque halos, whereas *L. innocua* colonies yielded blue-green colonies but without an opaque halo.

Serogrouping of *L. monocytogenes*

Listeria monocytogenes isolates from two marine fish and freshwater fish were found to be belong to serogroup 1, whereas ice isolates belong to serogroup 4. On the other hand, both the freshwater fish isolates from Goa belong to serogroup 4. *Listeria monocytogenes* ATCC 19115 and MTCC 657 also belong to serogroup 4.

Multiplex PCR

In multiplex PCR assay, all the *L. monocytogenes* type cultures and isolates from four positive samples yielded 267 and 938 bp PCR products, whereas *L. innocua* isolates yielded 938 and 749 bp PCR products (Fig. 1). *Staphylococcus*

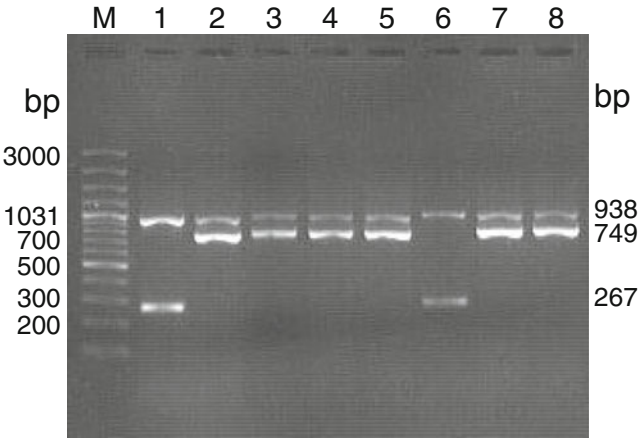

Fig. 1 Identification of isolates by multiplex PCR. *Lane M* GeneruleR™ DNA ladder plus, *lane 1 L. monocytogenes* NCTC 11994, *lanes 2–5 L. innocua* isolates, *lane 6 L. monocytogenes* isolates from fish, *lanes 7–8 L. innocua* isolates

aureus NCIM 5022 (used as negative control) and the isolates, which were not confirmed as *Listeria* spp. did not yield any amplified product (not shown in the image).

Virulence genes in *L. monocytogenes*

Listeria monocytogenes standard strains and isolates were screened for the presence of different virulence genes. All the isolates tested showed the presence of 456, 1,484, 131, 1,060 and 1,376 bp amplified products in *hlyA*, *plcA*, *iap*, *prfA* and *mpl* gene-specific PCR, respectively. *actA* gene-specific PCR using primers yielded 839 bp PCR products in all the strains tested except *L. monocytogenes* MTCC 657.

Discussion

The ability to grow in different food products at low temperatures and cause different types of human illnesses render *L. monocytogenes* a potential public health hazard in food industry. This pathogen is ubiquitous in nature and is more resistant to different sanitizing agents including quaternary ammonium compounds (Mereghetti et al. 2000). The presence of *L. monocytogenes* in food is sourced from raw materials as well as food processing environments, which include different food contact surfaces on which there is the possibility of the formation of biofilm by this pathogen. Being an important and emerging food-borne pathogen, it requires special attention with special reference to its prompt detection and control of growth in food. In this study, it has been observed that, in the tropical water of India, the incidence of *L. monocytogenes* was 1.2 % in fish and environmental samples. *Listeria monocytogenes* isolates of both serogroups 1 and 4 have been detected in the present study. The most prevalent species of *Listeria* was *L. innocua*, as the incidence has been found to be 28.7 %. These findings are in agreement with those of Fuchs and Surendran (1989), who observed that 33.3 % of the seafood samples were positive to *L. innocua*. In a study on the incidence of *Listeria* spp. in fishes of Mysore, India, it was found that 37.8 % of the samples were positive to *Listeria* spp., whereas *L. monocytogenes* was detected in 1.83 % of the samples (Moharem et al. 2007). However, Jeyasekaran et al. (2003) observed that 7.6 % of the fisheries environmental samples of Mangalore, India, were positive for both *Listeria* spp. and *L. monocytogenes*. *Listeria innocua* and *L. monocytogenes* share a common ecological niche and *L. innocua* is sometimes considered as an indicator organism for *L. monocytogenes* (Bubert et al. 1999). Thus, the frequent screening of seafood samples for the presence of *L. monocytogenes* is of utmost importance.

Different virulence genes, i.e. β-hemolysin, phosphatidylinnositol-specific phospholipase C, *iap, actA*, metaloprotease, and *prf A* gene play important roles in the virulence of *L. monocytogenes*. All the virulence factors act synergistically in intracellular survival and pathogenicity of *L. monocytogenes* (Vázquez-Boland et al. 2001). *Listeria monocytogenes* isolates from all the four positive samples as well as two freshwater fish isolates from Goa showed the presence of *mpl*, *hly*, *plcA*, *actA*, *prfA* and *iap* genes, which are responsible for key steps of *L. monocytogenes* cellular proliferation. The presence of these virulence genes is indicative of probable virulence.

The present study indicates that *L. monocytogenes* harboring key virulence genes is present in seafood and the fisheries environment of Kerala, India. Thus, this psychrotrophic pathogen requires constant monitoring and surveillance in fish and fisheries products for effective control.

Conclusion

In this study, *L. monocytogenes* was isolated in 4 out of 324 samples showing the low occurrence rate (1.2 %). But a comparatively high occurrence rate was found in the case of *L. innocua* (28.7 %). All the virulence genes screened were detected in the *L. monocytogenes* isolates recovered from the positive samples. Isolates of both serogroups 1 and 4 were detected in the tropical water of Kerala. Thus, it can be concluded that constant monitoring and surveillance for the presence of this psychrotrophic pathogen in Indian seafood are required.

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