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Short communication

Genotypic characterization of *Listeria* spp. isolated from fresh water fish

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Abstract

A total of 200 samples (muscles and viscera, 100 of each) of fresh water fish, walking catfish (*Clarias batrachus*) were screened for *Listeria* spp. All the samples were subjected to a two-step enrichment followed by plating on selective media. Confirmation of the isolates was on the basis of biochemical characters, haemolysis on blood agar and Christie, Atkins, Munch Petersen test. A total of 39 isolates of *Listeria* spp. were recovered. Of these 26 (67%), 8 (21%), 3 (8%) and 2 (5%) were *Listeria monocytogenes*, *Listeria seeligeri*, *Listeria grayi* and *Listeria welshimeri*, respectively. The isolates were subjected to a PCR assay for detection of the virulence-associated genes individually or together. The *plcA*, *actA*, *hlyA* and *iap* genes were detected in six strains, three genes (*actA*, *hlyA* and *iap*) in nine strains, the *plcA*, *hlyA* and *iap* in our strain, the *hlyA* and *iap* were in three strains, *actA* and *hlyA* in four strains, *plcA* and *hlyA* in our strain and *hlyA* in two strains. The *hlyA* and *iap* were also detected in *L. seeligeri*.

Keywords: Fresh water fish; *Listeria* spp.; Isolation; PCR

1. Introduction

Listeriosis is an important bacterial disease caused by *Listeria monocytogenes*. Although, *L. monocytogenes* has been recognized as a human pathogen way back in 1929, its recognition as a food-borne pathogen in 1981 (Ben Embarek, 1994) is relatively new. *L. monocytogenes* is a well-known cause of abortion, encephalitis and septicemia in man and animals. *L. monocytogenes* can cause both invasive and non-invasive infections. Invasive listeriosis is a severe disease mainly associated with a specific risk group of people and the case fatality rate is high, whereas fairly mild non-invasive infections can also occur in healthy people (Crum, 2002).

L. monocytogenes is considered to be a ubiquitous organism occurring in both terrestrial and aquatic habitats. This organism has been isolated from fish and fishery products from different parts of the world. In India, only a few studies have been conducted to assess the presence of *Listeria* spp. in seafood (Karunasagar et al., 1992; Jayasekaran et al., 1996). No data is available on prevalence of *Listeria* spp. among fresh water fishes, which are consumed widely. The objective of the present study was to study prevalence of *Listeria* spp. in fresh water fish and their genotypic characterization.

2. Materials and methods

2.1. Bacteria

The strains of *L. monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135) used in the study were obtained from the Institute of Microbial Technology, Chandigarh, India. *L. monocytogenes* 1/2a (NCTC 7973), *L. monocytogenes* 1/2b (NCTC 10887), *Listeria ivanovii* (NCTC 11846), *Listeria innocua* (NCTC 11288), *Listeria seeligeri* (NCTC 11856), *Listeria grayi* (NCTC 10812) and *Listeria welshimeri* (NCTC 11857) were included in the standardization of the PCR assay.

2.2. Samples

Samples of fresh water fish were obtained from the fish market of Nagpur, Central India. A total of 200 samples (muscles and viscera, 100 of each) of fresh water fish, walking catfish

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(*Clarias batrachus*) were collected in UV sterilized polyethylene bags and transported in chilled condition to laboratory and processed within 24 h of collection.

2.3. Isolation of Listeria spp.

Isolation of *Listeria* from the fish samples was attempted as per the US Department of Agriculture (USDA) method described by Donnelly and Baigent (1986) after making necessary modifications. The samples (muscles and viscera 5 g each) were trichurated under sterile conditions and inoculated into 45 ml of University of Vermont Medium (UVM-1, containing 12 mg of acriflavin hydrochloride) and incubated at 30 °C for 18–24 h. Enriched inoculum (0.1 ml) from UVM-1 was then transferred to 10 ml of UVM-2 (containing 25 mg of acriflavin hydrochloride) and incubated again for 24–36 h at 30 °C.

2.4. Identification of Listeria spp.

The enriched inoculum from UVM-2 was streaked directly on Dominguez–Rodriguez isolation agar (DRIA) and ALOA (Agar *Listeria*, according to Ottaviani and Agosti, Himedia Labs, Mumbai, India). The inoculated plates were incubated at 30 °C for 48 h. The greenish-yellow glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffused black zone of aesculin hydrolysis were considered to be of listeriae. On ALOA, light blue colonies showing a halo formation were considered of *Listeria* spp. The presumptive colonies of *Listeria* spp. were further subjected to biochemical tests (catalase, oxidase, methyl red, Voges–Proskauer's and nitrate reduction test) and sugar fermentation (rhamnose, xylose, mannitol and α-methyl-D-mannopyranoside) as standard procedure described by Cruikshank et al. (1975) and Cowan and Steel (1994). The isolates were further confirmed by haemolysis on blood agar and CAMP test (BIS, 1994).

Table 1
Details of primers for amplification of virulence marker associated genes of *Listeria*

Primer names	Primer sequence		Product size (bp)	Reference
plcA	Forward	5′-CTG CTT GAG CGT TCA TGT CTC ATC CCC C-3′	1484	Notermans et al. (1991)
	Reverse	5′-CAT GGG TTT CAC TCT CCT TCT AC-3′		
actA	Forward	5′-CGC CGC GGA AAT TAA AAA AAG A-3′	839	Accession No. AF103807
	Reverse	5′-ACG AAG GAA CCG GGC TGC TAG-3′		
hlyA	Forward	5′-GCA GTT GCA AGC GCT TGG AGT GAA-3′	456	Paziak-Domanska et al. (1999)
	Reverse	5′-GCA ACG TAT CCT CCA GAG TGA TCG-3′		
iap	Forward	5′-ACA AGC TGC ACC TGT TGC AG-3′	131	Furrer et al. (1991)
	Reverse	5′-TGA CAG CGT GTG TAG TAG CA-3′		

Table 2
Prevalence of *Listeria* spp. in fresh water fish samples

Species	Number of isolates recovered from		Total
	Muscles	Viscera	
L. monocytogenes	8	18	26 (13%)
L. seeligeri	5	3	8 (4%)
L. grayi	0	3	3 (1.5%)
L. welshimeri	1	1	2 (1%)
Total	14	25	39 (20%)

2.5. Polymerase chain reaction (PCR)

The PCR was standardized for the detection of virulence genes of *L. monocytogenes* as the method described (Notermans et al., 1991) with suitable modifications. In brief, the standard strain of pathogenic *L. monocytogenes* (MTCC 1143) was grown overnight in brain heart infusion broth at 37 °C. The culture (approximately 1.5 ml) was then centrifuged in a microcentrifuge at 6000 rpm for 10 min. The recovered pellet was resuspended in 100 µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 min and then snap chilled in crushed ice. The obtained lysate (2.5 µl) was used as a DNA template in the PCR reaction mixture.

For detection of the genes encoding phosphatidylinositol phospholipase C activity (*plcA*), actin polymerization protein (*actA*), haemolysin activity (*hlyA*), and p60 protein (*iap*) of *L. monocytogenes* oligonucleotide primers were synthesized from Sigma Aldrich. The details of the primer sequences are shown in Table 1.

The PCR was set for 25 µl reaction volume. Initially for the detection of individual virulent genes of *L. monocytogenes*, PCR conditions were optimized by using varying concentrations of biologicals. Based on optimization trials the reaction mixture for PCR was optimized as follows, 10× 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 0.1 µmol of forward and reverse primer of each set, 1.6 unit of Taq DNA Polymerase, 2.5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The reaction was performed in Px2 Thermal cycler (Thermo Hybaid, UK) with a preheated lid. The cycling conditions included an initial denaturation at 95 °C for 2 min followed by 35 cycles each of 15 s denaturation at 95 °C, 30 s annealing at 60 °C and 1 min 30 s extension at 72 °C. It was followed by final extension of 10 min at 72 °C and held at 4 °C. The resultant PCR products were further analyzed by agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) and visualized by a UV transilluminator (Villber Lourmat, France).

Later, a multiplex PCR was standardized for the detection of virulence genes namely, *plcA*, *actA*, *hlyA* and *iap*. The multiplex PCR was set up in 25 µl reaction volume. Based on the results of various trials, the reaction mixture was optimized as follows, 10× PCR buffer (consisting of 100 mM Tris–HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$ and 0.01% gelatin), 1 mM dNTP mix, 7.5 mM $MgCl_2$ and 10 µM forward and reverse primer of each set, 2.5 unit of Taq DNA Polymerase, 2.5 µl of cell lysate

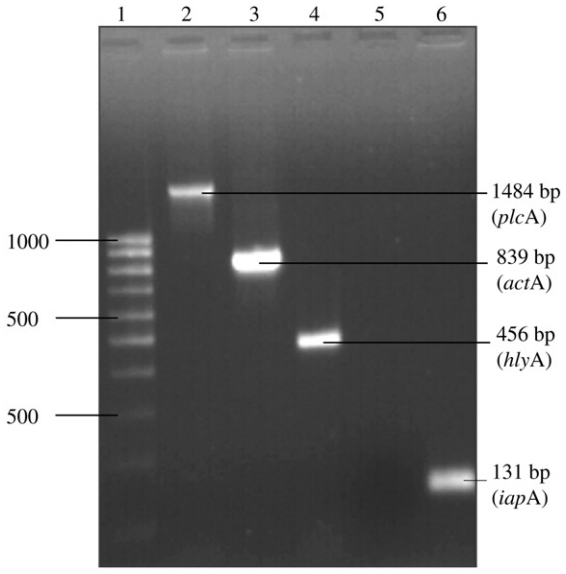

Fig. 1. Amplification of virulence genes associated with *Listeria* spp by PCR. Lane 1: 100 bp ladder. Lane 2: *plcA* gene. Lane 3: *actA* gene. Lane 4: *hlyA* gene. Lane 5: Negative control. Lane 6: *iap* gene.

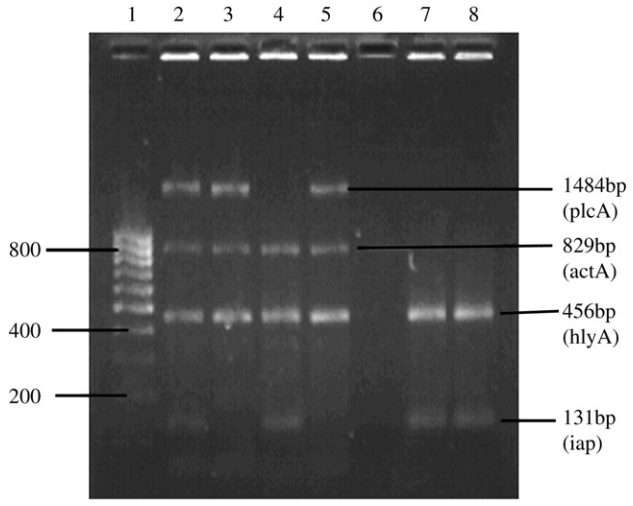

Fig. 2. Amplification of virulence genes associated with *Listeria* spp by multiplex PCR. Lane 1: 100 bp ladder. Lane 2: *L. monocytogenes* amplification of *plcA* (1484 bp), *actA* (829 bp), *hlyA* (456 bp) and *iap* (131) genes. Lanes 3 and 5: *L. monocytogenes* amplification of *plcA* (1484 bp), *actA* (829 bp) and *hlyA* (456 bp) genes. Lane 4: *L. monocytogenes* amplification of *actA* (829 bp) and *hlyA* (456 bp) and *iap* (131) genes. Lane 6: Negative control. Lanes 7 and 8: *L. seeligeri* amplification of *hlyA* (456 bp) and *iap* (131) genes.

and sterilized milliQ water to make up the reaction volume. The reaction mixture was performed in a Px2 Thermal cycler (Thermo Hybaid, UK) with a preheated lid. The cycling conditions were same as those described earlier.

3. Results and discussion

We screened fresh water fish for *Listeria* spp. employing a two-step enrichment followed by plating on selective media. *Listeria* spp. could be isolated from 39 (20%) of 200 samples. *Listeria* spp. was isolated from 13 (33%) muscle and 26 (67%) viscera samples (Table 2). Of these, 26 (67%), 8(21%), 3 (8%) and 2(5%) were *L. monocytogenes*, *L. seeligeri*, *L. grayi* and *L. welshimeri*, respectively.

In India, prevalence of *Listeria* has been reported in marine fishes. However, scanty information is available on isolation from fresh water fishes. In the present investigation 20% prevalence of *Listeria* has been recorded in fresh water fish samples. Manoj et al. (1991) reported 5.1% prevalence of *Listeria* in fish collected from an Indian market. In Turkey, *Listeria* spp. were isolated from 6.6% intestinal content of fresh water fishes (Ertas and Seker, 2005). Higher (38.66%) prevalence was recorded by Loncarevic et al. (1996) in smoked and gravad fish. In the present study, occurrence of *L. monocytogenes* was (66.67%) predominant. A similar finding has been reported earlier (Wilson, 1998; Jemmi et al., 2002). *L. monocytogenes* has also been predominantly isolated from seafoods (Nakamura et al., 2004), fish meat products (Van Coillie et al., 2004) and smoked fish (Dominguez et al., 2001). The frequency of isolation of *L. grayi* (5.29%), *L. welshimeri* and *L. seeligeri* (1.76% each) has been documented from smoked fish (Dominguez et al., 2001).

Out of 39 *Listeria* isolates recovered 34 were haemolytic on sheep blood agar and 26 isolates were CAMP positive with *S. aureus* and were characterized as *L. monocytogenes*. Isolates biochemically characterized as *L. seeligeri* were CAMP negative with *S. aureus* and *R. equi*.

In the present study, a PCR was standardized for the detection of individual virulence-associated genes of *L. monocytogenes* namely, *plcA*, *actA*, *hlyA* and *iap*. The primer sets for these genes allowed amplification of 1484 bp, 839 bp, 456 bp and

Table 3
Frequency of virulence-associated genes in *Listeria* spp. isolates recovered from fresh water fish

Species	No. of isolates	Amplified PCR products of virulence-associated genes detected in *Listeria* spp. isolates			
		plcA (1484 bp)	*actA* (839 bp)	*hlyA* (456 bp)	*iap* (131 bp)
L. monocytogenes	6	+	+	+	+
L. monocytogenes	9	−	+	+	+
L. monocytogenes	1	+	−	+	+
L. monocytogenes	1	+	−	+	−
L. monocytogenes	4	−	+	+	−
L. monocytogenes	3	−	−	+	+
L. monocytogenes	2	−	−	+	−
L. seeligeri	4	−	−	+	+
L. seeligeri	1	−	−	+	−
L. seeligeri	1	−	−	−	+

131 bp PCR products, respectively, each represented by a single band (Fig. 1).

Among the strains of *L. monocytogenes*, the *plcA*, *actA*, *hlyA* and *iap* genes were detected in six strains, three genes (*actA*, *hlyA* and *iap*) in nine strains, the *plcA*, *hlyA*and *iap* in a strain, the *hlyA* and *iap* were in three strains, *actA* and *hlyA* in four strains, *plcA* and *hlyA* in a strain and *hlyA* in two strains. The *hlyA* and *iap* genes were also detected in *L. seeligeri* (Table 3).

The primer for the *actA* gene employed in the study could detect the gene in standard strains of *L. monocytogenes* 4b (MTCC 1143) by amplifying the DNA fragment of 839 bp in PCR. The *iap* gene encoding for p60 homologous proteins is synthesized by all *Listeria* spp., but in each species there are specific differences in an amino acid sequence that can be used for identification purposes in PCR and immunological assays (Vazquez-Boland et al., 2001). However, in the present study the same primer could amplify the *iap* gene from both *L. monocytogenes* and *L. seeligeri*. Overall, the present findings commensurate with the published work for detection of *hlyA* gene (Paziak-Domanska et al., 1999), *plcA* gene (Notermans et al., 1991), *iap* gene (Furrer et al., 1991) and *actA* gene (Suarez et al., 2001) with respective sets of primers. The amplification of haemolysin gene (*hlyA*) in *L. seeligeri* genome could be explained on the basis of homology at both the protein (86–91%) and in the nucleotide (76–78%) of gene sequence of LLO and seeligerilysin O (Mengaud et al., 1988).

Multiplex PCR with four virulence-associated genes primer sets namely *plcA*, *actA*, *hlyA* and *iap* amplified the DNA fragments of the expected size (Fig. 2). None of the PCR products of virulence-associated genes was observed in DNA samples prepared from *Listeria* spp. other than *L. monocytogenes* and *L. seeligeri* (Table 3).

In the present study, by PCR analysis variable intact genes were detected in different isolates. It is not clear whether it is because of the defect or mutation of the particular region. Therefore, detection of one virulence-associated gene by PCR is not always sufficient to identify *L. monocytogenes* (Nishibori et al., 1995). The detection of multiple virulence-associated genes and the expression of virulence marker genes by *in-vitro* assay may be performed for differentiation of pathogenic *L. monocytogenes* from other *Listeria* species.

The present study indicated the prevalence of *L. monocytogenes* in fresh water fish, which may possess a potential threat to public health. Further studies are in progress to ascertain the sources of *Listeria* spp. in fresh water fishes.

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