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Prevalence of *Listeria Monocytogenes* in Freshwater Fish of Northeast India and Their Molecular Characterization by PCR

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ABSTRACT

Five different species of freshwater fish (total number sampled =1,165) were collected from various fish markets of northeast India and screened for the presence of *Listeria monocytogenes* using enrichment and selective plating methods. Polymerase Chain Reaction (PCR) methods were standardized for confirmation of *L. monocytogenes* isolates and detection of various virulence genes (*hly*A, *iap*, *plc*A and *plc*B). Out of the total samples, 27 (2.31%) *L. monocytogenes* resistant to three different antibiotics could be isolated with a prevalence rate of 5.45%, 3.67%, 1.43%, 1.38% and 0.33% in *C. batrachus*, *H. fossilis*, *A. testudinus*, *M. seenghala*, and *L. guntea*, respectively. Virulent-associated genes; h*ly*A, *iap*, *plc*A and *plc*B were detected in 40.7%, 29.6%, 40.7% and 22.2% of the 27 isolates, respectively. The prevalence of antibiotic resistant *L. monocytogenes* harbouring virulence genes in freshwater fish can contaminate other food and food products, thereby affecting human and animals directly or indirectly.

1. Introduction

Listeria monocytogenes is a ubiquitous bacterium which is frequently isolated from river water, sediment, coastal seawater, canals and lakes, ready to eat food and other food items (Farber and Peterkin 1991). Pathogenic *L. monocytogenes* causes Listeriosis, an atypical food-borne disease mostly affecting pregnant women, newborn infants and elderly immunocompromised individuals. Mortality due to Listeriosis can be as high as 30%. There are numerous reports of sporadic cases of seafood-borne Listeriosis (Facinelli *et al.,* 1989; Frederiksen 1991; Baker *et al.,* 1993; Riedo *et al.,* 1994). In New Zealand epidemic form of prenatal Listeriosis due to consumption of shellfish and raw fish was reported in 1980 (Lennon *et al.,* 1984). Several other studies reported that both fish and fish products can be contaminated with *L. monocytogenes* (Farber 1991; Fuchs and Reilly 1992; Mclauchlin and Nichols 1994). There are reports of low incidence of *Listeria* in freshwater fishes and sea foods from some parts of India (Dillon and Patel 1992; Karunasagar and Karunasagar 2000; Manoj *et al.,* 1991; Parihar *et al.,* 2008). In India, occasional cases of Listeriosis in human and animals are reported by several workers (Nagi and Verma 1967; Bhujwala *et al.,* 1973, 1974; Stephen *et al.,* 1978; Thomas *et al.,* 1981; Dhanashree *et al*., 1999). In the northeastern region of India, there are some sporadic cases of diseases in livestock due to *L. monocytogenes*, but detailed studies on *L. monocytogenes* and Listeriosis in the region is largely lacking. Since, freshwater fish and various fish products form an important part of foodstuffs of the local population, *L. monocytogenes* contaminated fish and fish products may pose a great risk to the life of the fish consumer of the region. This study was undertaken for the first time in northeast India, to investigate the prevalence of *L. monocytogenes* in some of the commercially important freshwater fish available in the region.

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2. Materials and Methods

2.1 Bacterial stains

The control strains of *L. monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), and *Rhodococcus equi* (MTCC 1135), for this study were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

2.2 Fish Samples

Freshwater fish species selected for this study were 'Magur' (*Clarius batrachus*), 'Hingi' (*Heteropneustes fossilis*), 'Tengra' (*Mystus seenghala*), 'Koi' (*Anabus testudinus*) and 'Botia' (*Lepidocephalus guntea*). These fishes, found abundantly in rivers and lakes of the region, have high economic value. A total of 1,165 fish samples, comprising 220 numbers of *C. batrachus*, 218 of *H. fossilis*, 217 of *M. seenghala*, 210 of *A. testudinus*, and 300 of *L. guntea,* were collected from different commercial fish markets of the Northeast India. The samples were collected aseptically in UV irradiated sampling bags and preserved in a cooler with ice packs and transported to the laboratory. The samples were processed and analyzed within 24 hours of collection.

2.3 Isolation and identification of L. monocytogenes

For isolation of *L. monocytogenes*, method described by Al-Zoreky and Sandine, (1990) was followed with minor modifications. Listeria enrichment broth base (CM0863, Oxoid, Basingstoke, Hampshire, UK) and Listeria selective agar base (CM 0856, Oxoid, Basingstoke, Hampshire, UK) were used for enrichment and selective plating of *L. monocytogenes*. Every 500ml of *Listeria* enrichment broth base was added with 2ml of sterile distilled water containing 10 mg nalidixic acid and 6 mg acriflavine as inhibitory agents (Oxoid, Basingstoke, Hampshire, UK). Whereas, 500ml of *Listeria* selective agar base was supplemented with 5ml of 70% ethanol containing 200 mg cycloheximide, 1mg cefotetan, 10mg colistin sulphate, 95 mg fosfomycin and 2.5mg acriflavine (Oxoid, Basingstoke, Hampshire, UK). The intestines, gills, and flesh of the freshwater fish were separated aseptically and cut into small pieces. These were then added (1: 9 ratio) to *Listeria* enrichment broth and mixed properly by vigorous shaking and incubated at 37°C for 24 to 48 hours. The enriched broth cultures were streaked onto *Listeria* selective agar plates which were then incubated at 37°C for 48 hours followed by incubation at 4°C for 12 to 24 hours. All black colonies with sunken centers were streaked onto Nutrient agar plates (HiMedia Laboratories, Mumbai, India) and incubated at 37°C for 24 hours. The single colonies obtained were subjected to *L. monocytogenes* confirmation tests using the criteria of Seeliger and Jones (1986). Morphologically typical colonies were verified by Gram's staining, catalase and oxidase reactions, tumbling motility at 20-25°C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *S. aureus* and *R. equi*, haemolysis test on 5% sheep blood agar, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl-d-mannoside).

2.4 Antimicrobial susceptibility test

The antimicrobial susceptibility test of the pure isolates of *L. monocytogenes* was carried out on Mueller Hinton Agar (HiMedia Laboratories, Mumbai, India) plates by following the Kirby – Baeur Disc Diffusion method (Bauer *et al.,* 1966**)** and guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007)**.** The antibiotics used in this study are tetracycline (10mcg), ciprofloxacin (5mcg), chloramphenicol (30mcg), ampicillin (10mcg), metronidazole (4mcg), erythromycin (15mcg), nitrofurantoin (300mcg), streptomycin (10mcg), and gentamicin (10mcg) (HiMedia Laboratories, Mumbai, India). The plates were incubated at 37°C for 24 hours and the results were interpreted according to the antibiotic manufacturer's guidelines.

2.5 Detection of 16S rRNA and virulence genes

The genomic DNA was extracted from Brain heart infusion broth culture (overnight culture at 37°C) by Genomic DNA isolation Kit 2021Bt (BioServe Biotechnology Pvt. Ltd, India). Polymerase chain reaction (PCR) was used for the detection of *L. monocytogenes* specific 16S rRNA gene in all the isolates. Duplex PCR method was employed for the amplification of virulence genes; *hly*A (ListeriolysinO) and *iap* (invasive associated proteins) (Furrer *et al.,* 1991); whereas, two PCR methods was standardized to amplify *plc*A (phosphatidylinositol specific phospholipase C) and *plc*B (phospholipase C / (or) lecithinase C) genes (Nishibori *et al.,* 1995). The published oligonucleotide primers (Table 1) used for amplifications of these genes was synthesized by MetaBion GmbH (Deutschland, Germany). Three microlitres (3µl) of extracted DNA from each sample was amplified in a 25µl reaction mixture consisting of 12.5µl of 2X Master Mix (0.05 units/µl Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP, dTTP.) (MBI Fermentas Life Science, Hanover, USA), 0.125 µl (0.5µM) each of forward and reverse oligonucleotide primers and 9.25µl of nuclease free water. PCR condition for amplification of 16S rRNA was initial denaturation at 90°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation

at 72°C for 1 minute, and final elongation at 72°C for 5 minutes (Wiedmann *et al.,* 1993). For the detection of *hly*A and *iap* genes a duplex PCR condition (Furrer *et al.,* 1991) was followed where initial denaturation at 94°C for 4 minutes was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, elongation at 72°C for 1 minute and final elongation at 72°C for 10 minutes. For the amplification of *plc*A and *plc*B genes, the PCR condition was initial denaturation at 94°C for 3 minutes followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes. The amplifications were carried out in a thermal iCycler (Biorad Laboratories, USA). The PCR amplicons (5µl each) were resolved by electrophoresis (1 hour and 30 minutes at 70V) in 1.5% agarose gel in 1X TAE buffer (Bangalore Genei Pvt. Ltd., Bangalore, India) containing 0.5µg/ml of ethidium bromide (Sambrook *et al*., 1989). The amplified DNA fragments were visualized and recorded by UV transilluminator Gel Logic 100 Imaging system (Kodak, USA).

3. Results

3.1 Prevalence and antimicrobial susceptibility of the isolates

Characteristic colonies on LSA agar, which are Gram-positive, pleomorphic, non-spore forming coccobaccili that are catalase positive and oxidase negative, showing tumbling motility at 20-25°C, producing acids from rhamnose, α-methyl-d-mannoside and positive haemolsin and CAMP test were confirmed as *L. monocytogenes*. Twenty seven (27) *L. monocytogenes* were isolated from the total 1,165 freshwater fish samples with an overall prevalent rate of 2.31%. The isolation rate was 5.45% in *C. batrachus*, 3.67% in *H. fossilis*, 1.43% in *A. testudinus*, 1.38% in *M. seenghala* and 0.33% in *L. guntea*, (Table 2). Out of these 27 isolates, 12 (1.03%) *L. monocytogenes* could be isolated from intestines followed by 10 (0.85%) from gills and 5 (0.43%) from flesh samples of freshwater fish. All the isolates show susceptibility to ciprofloxacin, chloramphenicol, erythromycin, nitrofurantoin, streptomycin, and gentamicin while 51.86% were susceptible to each of tetracycline and ampicillin. Each of only 8 isolates (29.62%) show resistance to a maximum of 3 antibiotics, while 10 (37.03%) and 7 (25.92%) isolates were resistant to 2 and 1 antibiotic respectively.

Table 1.

Target gene	Primer sequences	Product size(bp)	References
*hly*A	Forward 5' -CGGAGGTTCCGCAAAAGATG-3' Reverse 5'-CCTCCAGAGTGATCGATGTT-3'	234	Furrer *et al*., 1991
Iap	Forward 5'-ACAAGCTGCACCTGTTGCAG-3' Reverse 5'-TGACAGCGTGTGTAGTAGCA-3'	131	Furrer *et al*., 1991
*plc*A	Forward 5'-GGAAGTCCATGATTAGTATGCC-3' Reverse 5'-GCTGCAGCATTAAACAACAAAG-3'	91	Nishibori *et al*., 1995 Nishibori *et al*., 1995
*plc*B	Forward 5'-GCAAGTGTTCTAGTCTTTCCG-3' Reverse 5'-ACCTGCCAAAGTTTGCTGTGA-3'	795	Wiedmann *et al*., 1993
16S rRNA	Forward 5'-GGACCGGGGCTAATACCGAATGATAA-3' Reverse 5'-TTCATGTAGGCGAGTTGCAGCCTA-3'	1200	

Table 2.

Samples (Total Number)	Number (percentage) of isolates from			
	Meat	Intestine	Gill	**Total**
Clarius batrachus (220)	1 (0.45%)	5 (2.3%)	6 (2.7%)	12 (5.45%)
Heteropneustes fossilis (218)	2 (0.9%)	3 (1.4%)	3 (1.4%)	8 (3.67%)
Mystus seenghala (217)	1 (0.46%)	1 (0.46%)	1 (0.46%)	3 (1.38%)
Anabus testudineus (210)	0	3 (1.43%)	0	3 (1.43%)
Lepidocephalus guntea (300)	1 (0.33%)	0	0	1 (0.33%)
Total = 1,165	**5 (0.43%)**	**12 (1.03%)**	**10 (0.85%)**	**27 (2.31%)**

3.2 Detection of virulence genes in L. monocytogenes

In all the 27 isolates, *L. monocytogenes* specific 16S rRNA gene was detected through PCR (Table 3, Figure 1). Virulence associated genes; *hly*A (234bp) was detected in 40.7%, *iap* (131bp) in 29.6%, *plc*A (91bp) in 40.7% and *plc*B (795bp) in 22.2% of the isolates (Table 3, Figure 2, Figure 3, Figure 4). All the four virulence genes were present in 4 and 2 of the *L. monocytogenes* isolated from fish intestines and gills, respectively, whereas, one isolate from fish flesh carried the *hly*A, *iap* and *plc*A virulence genes (Table 3).

4. Discussion

In this study, we found 27 (2.31%) of samples positive for *L. monocytogenes* and the prevalent rate was 5.45% in *C. batrachus*, 3.67% in *H. fossilis*, 1.38% in *M. seenghala*, 1.43% in *A. testudineus* and 0.33% in *L. guntea*. *L. monocytogenes* could be frequently isolated from intestines, gills and meat samples of freshwater fish. 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. Dillon and Patel (1992); Fuchs and Reilly, (1992) reported very low to 50% prevalence rate of *L. monocytogenes* in fish and fish products from temperate areas. Studies by Parihar, *et al.* (2008) also reported higher (9%) occurrence rate of *L. monocytogenes* from tropical seafood in India. Other related studies by Hasan, (2005) and Vaz-Velho *et al.* (2001) found higher prevalence rate. In India, earlier studies reported incidence of *L. monocytogenes* to be 1.5% in freshwater fish and 3.3-13.8% in shellfish in India (Dhanashree *et al.,* 1999; Jeyasekaran *et al*., 1996). Other studies have also found the prevalence of *L. monocytogenes* in raw fish to be quite low, ranging from 0% to 1% (Autio *et al.,* 1999; Johansson *et al.,* 1999) to 10% (Jemmi and Keusch 1994). Our study reports for the first time the prevalence of antibiotic resistant *L. monocytogenes* in freshwater fish in northeastern part of India. The fish might have been contaminated with these resistant *L. monocytogenes* from the natural environment or through water sources. This study establishes the presence of potentially pathogenic *L. monocytogenes* in freshwater fish in northeast India as evident from the detection of various virulent genes in some of the isolates. These virulent isolates could play a major role in food-borne Listeriosis in the region. Study by Gellin, *et al.* (1991) found that the mechanism of pathogenicity of *L. monocytogenes* is usually associated with haemolysin production encoded by *hly*A gene. Cooray *et al.* (1994) also reported that *prf*A and *hly*A genes unique to *L. monocytogenes* are usually associated with virulence and the determination of any one of these genes is sufficient for confirmation of *L. monocytogenes*. They also suggested that amplification of multiple virulent genes in a single reaction mixture is a better way of large-scale survey aiming at detecting virulent strain of *L. monocytogenes*. *L. monocytogenes* is an inhabitant of natural environment and fish

Table 3.

Samples (Number of isolates)	**Number (percentage) of isolates with**				
	16S rRNA	***hly*A gene**	***iap* gene**	***plc*A gene**	***plc*B gene**
Fish meat (5)	5 (100%)	1(20%)	1(20%)	1(20%)	0
Fish intestine (12)	12 (100%)	6(50%)	5(41.7%)	6(50%)	4 (33.3%)
Fish gill (10)	10 (100%)	4(40%)	2(20%)	4 (40%)	2(20%)
Total isolates (27)	**27 (100%)**	**11 (40.7%)**	**8 (29.6%)**	**11 (40.7)**	**6 (22.2%)**

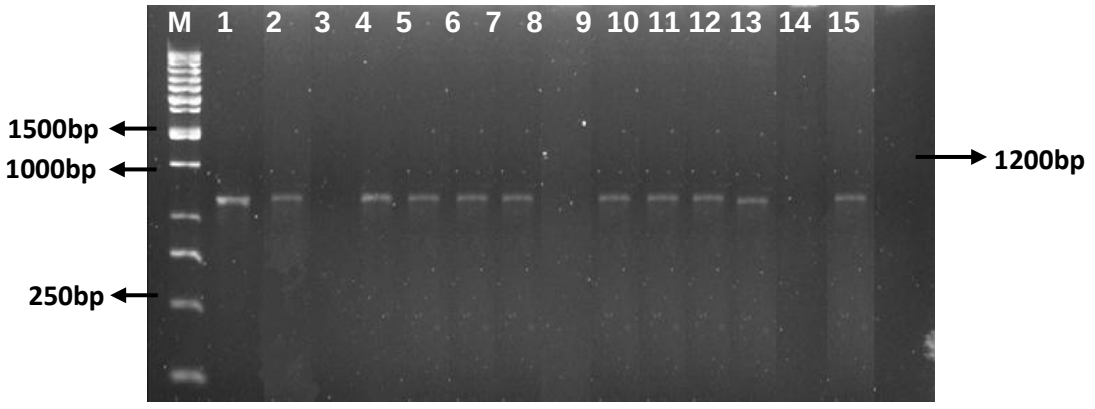

Figure 1

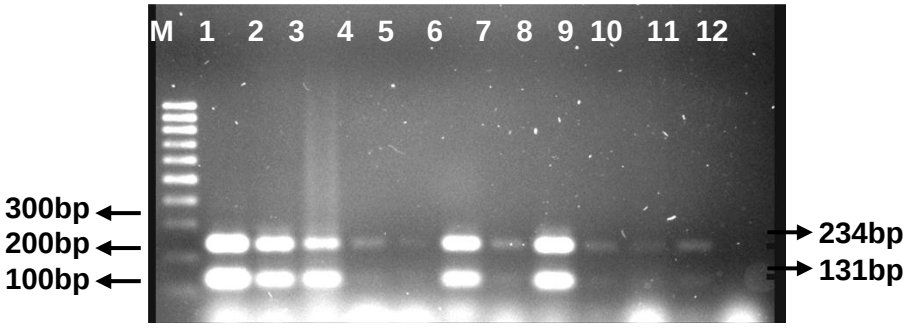

Figure 2

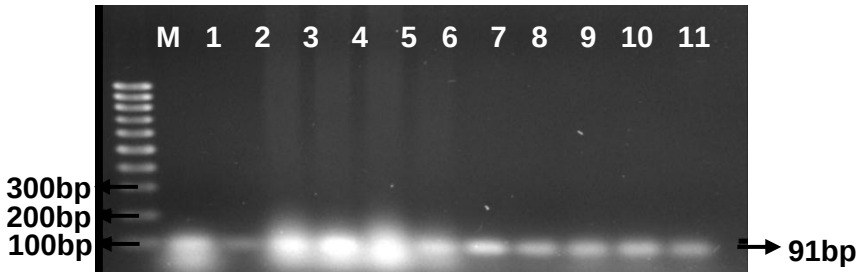

Figure 3

captured or cultivated in contaminated water may possibly carry this microorganism and thereby, fish handlers and people may get infected. Detection of antibiotic resistant *L. monocytogenes* harboring virulent factors from freshwater fish in the region is of great concern from the public health point. These virulent strains can contaminate other food items (raw/ready-to-eat) while processing and packing or during storage. So, screening and survey of various food and food products of northeast India for the presence of virulent *L. monocytogenes* is of utmost important. Through this study, we have standardized PCR based methods for rapid detection of *L. monocytogenes* in freshwater fish of northeast India. These methods can also be employed for detecting *L. monocytogenes* in other food items.

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