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Detection of *Listeria monocytogenes* in fish samples by PCR assay

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ABSTRACT

A total of 60 samples (25 each of fish samples and fish surface swabs and 10 dried salt fish samples) collected from various markets of Hyderabad were subjected to cultural and PCR methods for the presence of Listeria monocytogenes. Primers derived from hlyA and iap genes were used for the detection of Listeriolysin O and p60 respectively. Four samples (2 fish samples, 2 fish surface swabs) were positive by PCR assay, whereas only two samples (one each of fish samples and fish surface swabs) were positive by cultural methods for presence of Listeria monocytogenes. The PCR assay detected hlyA gene of L.monocytogenes in only one fish sample. The presence of this organism in seafood is a great public health concern. The results of this study suggest that PCR could be an excellent tool for detection of Listeria monocytogenes in sea foods as well as in livestock foods.

Keywords: Fish samples, *Listeria monocytogenes*, Cultural methods, PCR assay.

INTRODUCTION

Since, 1981, *Listeria monocytogenes* has been recognized as an important food borne pathogen. [1] reported that L. *monocytogenes* is an important food borne pathogen to both humans as well as animals. This organism primarily affects pregnant women, neonates, the immunocompromised and the elderly people; showing major symptoms like septicemia, meningoencephalitis and abortion [2]. The average mortality of *L. monocytogenes* (30%) far exceeds the other common food borne pathogens such as *Salmonella enteritidis* (with a mortality of 0.38%), *Campylobacter* species (0.02-0.1%) and *Vibrio* species (0.005-0.01%) in terms of disease severity [3]. [4] isolated *L.monocytogenes* from 15% of uncooked seafood samples.

Listeria monocytogenes has several important virulence markers. Among them, Listeriolysin O (LLO) is one of the important marker encoded by *hlyA* gene and is essential for disruption of phagocytic vacuole and release of bacteria into cytoplasm. [5] designed primers targeting *hlyA* gene of *Listeria monocytogenes* which yielded 456 bp products. Another important marker is p60, encoded by *iap* gene which plays a vital role in intestinal invasion. This gene is indispensable for species-specific identification of *Listeria monocytogenes* [5]. Primers targeting *iap* gene of *L.monocytogenes* yielding a 131 bp product, was reported [5].

The European Commission and the International Commission on Microbiological Specification for foods (ICMSF) recommended that the counts of *Listeria monocytogenes* should be less than 100 cfu/g in ready-to-eat products at the time of consumption [6]. However, [7 and 8] recommended zero tolerance for *Listeria monocytogenes* in ready-to-eat foods in US. The outbreaks of *L.monocytogenes* have been associated with seafood products [9, 10 and 11]. Seafood exporting tropical countries are most concerned about the frequent incidences of product detention by importing countries due to screening of shipments for this pathogen. Little information is available on the incidence of L.monocytogenes in different types of fish on sale in markets of Hyderabad.

This work has been undertaken to detect the presence of *Listeria monocytogenes* in both wet and salted fish using cultural and PCR methods. The detection of a single virulence associated gene is not sufficient to identify *L.monocytogenes* [12], hence both genes (*iap* and hlyA) were targeted individually in this study. Primers targeting *iap* gene of *L.monocytogenes* yielding a 131 bp product, was reported by [5].

MATERIALS AND METHODS

A total of 60 samples (25 each of fish samples and fish surface swabs and 10 dried salt fish samples) collected from various markets of Hyderabad were subjected to cultural and PCR methods for the presence of *Listeria monocytogenes.* About l0 gm fish were ground with a sterile mortar and pestle and swab samples were inoculated into 90ml Listeria Enrichment broth (LEB) and incubated at 37^{o}C for 24hrs. The enriched inoculum from the broth was streaked on to PALCAM agar plates and incubated at 37^{0}C for 24h for isolation of *L.monocytogenes.* Green colour colonies surrounded by a black zone on PALCAM agar plates were collected for further confirmation by biochemical tests like IMViC tests, nitrate test, urease test, motility test, CAMP, haemolysis and sugar fermentation tests (lactose, sacharose, dextrose positive).

All the samples were subjected to PCR analysis for the presence of *L.monocytogenes* using primers specific to haemolysis gene (*hlyA*) and invasive associated protein (*iap*). The primers were listed in the table.1.

Table 1: Primers used in the present study (Ritu Arora *et.al*., 2007)

Primers	Target gene	Length	Primer sequence	Amplification product (bp)
iap-F	*iap*	20	5' ACAAGCTGCACCTGTTGCAG 3'	131
iap-R	*Iap*	20	5' TGACAGCGTGTGTAGTAGCA 3'	131
hlyA- F	*hlyA*	24	5' GCAGTTGCAAGCGCTTGGAGTGAA 3'	456
hlyA-R	*hlyA*	24	5' GCAACGTATCCTCCAGAGTGATCG 3'	456

The *Listeria monocytogenes* strain obtained from Microbial Type Culture Collection (MTCC), Chandigarh was used as known positive strain in PCR analysis. 1.5 ml of enriched broths was taken into eppendorf tubes and bacteria were pelleted by centrifugation at 6000 rpm for 5 min. To the pellet 50 µl of molecular grade water was added and incubated at 100^{0}C for 10 min. and snap chilled to release DNA. Then centrifuged at 13000 rpm for 5 min and the supernatants were used in PCR for DNA amplification.

PCR master mix used in this study contains containing 2.5 µl of 10X Taq DNA polymerase (containing 100mM Tris with p^{H} 9.0, 500mM KCl, 15mM $MgCl_2$ and 1% Triton X-100), 2.0 µl of 25mM of $MgCl_2$, 1 µl of 10mM dNTP mix, 2 µl of each forward and reverse primer (4pmol/ µl) and 0.9 U/ µl of Taq DNA polymerase which was made up to 20 µl using molecular grade water. Then, this master mix was distributed to the PCR tubes and finally 5 µl of bacterial lysate was added as template. Amplification was done following the conditions shown in Table.2.

Table 2. Cycling conditions used for two sets of primers

S.No	Step	*iap* (*L.monocytogenes*)	*hlyA* (Listeriolysin O)
1.	Initial denaturation	95^{0}C / 2 min	95^{0}C / 2 min
2.	Final denaturation	95^{0}C/15 sec	95^{0}C/15 sec
3.	Annealing	60^{0}C/30 sec	60^{0}C/30 sec
4.	Initial extension	72^{0}C/1min. 30 sec	72^{0}C/1min.30 sec
5.	Final extension	72^{0}C/10 min	72^{0}C/10 min
6.	Hold	4^{0}C	4^{0}C

The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5µg/ml) and visualized with a UV transilluminator.

RESULTS AND DISCUSSION

Green colour colonies of *L.monocytogenes* surrounded by a black zone on PALCAM agar plates were collected for further confirmation by biochemical tests. *L.monocytogenes* has given positive results for Indole, Methyl Red, VP test, and sugar fermentation tests like lactose, sacharose, dextrose and also shown ß haemolysis on blood agar plate. The colonies also shown CAMP test positive i.e. arrow head shape haemolysis with *Staphylococcus aureus.*

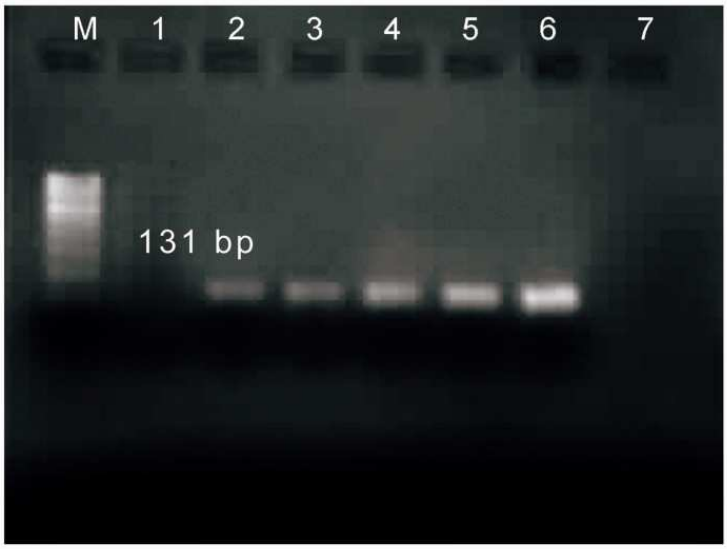

Fig. 1 : Standardization of PCR assay for detection of Listeria monocytogenes (iap)

Lane M : 100 bp DNA Ladder
Lane 2 : PCR product at an annealing temperature at 59^{0}C
Lane 3 : PCR product at an annealing temperature at 60^{0}C
Lane 4 : PCR product at an annealing temperature at 62^{0}C
Lane 5 : PCR product at an annealing temperature at 64^{0}C
Lane 6 : PCR product at an annealing temperature at 65^{0}C

Fig. 2: Standardization of PCR assay for detection of Listeriolysin O (hlyA)

Lane M : 100 bp DNA Ladder
Lane 1 : PCR product at an annealing temperature at 59^{0}C
Lane 2 : PCR product at an annealing temperature at 60^{0}C
Lane 3 : PCR product at an annealing temperature at 62^{0}C
Lane 4 : PCR product at an annealing temperature at 64^{0}C
Lane 6 : PCR product at an annealing temperature at 65^{0}C

The primers targeting specific genes i.e. *iap* and *hlyA* used in PCR assay were standardized by optimizing the annealing temperatures, primer concentration, $MgCl_2$ concentration, template volume and cycling conditions. The primer sequences for *iap* and hlyA used by [5] standardization of PCR assay in this study that allowed amplification at 131 bp for *iap* (Fig.1) and at 456 bp for *hlyA* (Fig.2) genes respectively which were in accordance with the findings of my study. These PCR products were stored at -20^0c for further use.

The results for the presence of *Listeria monocytogenes* different samples are represented in Table 3.

Table 3. Occurrence of *Listeria monocytogenes* in sea food samples

Type of sample	No. of samples	Positive results for *L.monocytogenes*	
		Cultural	PCR
Fish samples	25	1	2
Fish swab samples	25	1	2
Dried salt fish	10	--	--

Listeria monocytogenes was isolated from 8 % (2 out of 25) and 4 % (1 out of 25) of fish and fish swab samples by PCR and cultural methods respectively. [13] reported higher incidence (23.9%) of *L.monocytogenes* by PCR method than the present study (8%), whereas [14] reported very low incidence i.e. 1.83% (only 3 out of 132 fish samples). [11] also reported slightly higher incidence (6.9%) by cultural method than the present study (4%). Slightly low incidence i.e. 2.9% and 2.3% were reported by [15] and [16] respectively in raw fish. Several workers reported high incidences of this organism in seafood samples by cultural method i.e. 26% by [17]; 24% by [18]; 10.5% by [19] in Taiwan; 13% by [20] in Japan. Zero incidence of *L.monocytogenes* in fish samples in India was reported by [21 and 22]. Rapid detection of even low levels of this organism in fish can be achieved with PCR protocols described so that food safety may be ensured in addition it also helps in promoting the exports of fish and its products from our country.

In the present study, the results indicated that this organism could not be detected in dried salted fish though L.monocytogenes has been reported to survive higher concentrations and lower moisture contents [23]. This could be due to exposure of salt fish to UV radiation during sun drying where almost all the vegetative cells are destroyed.

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