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Isolation and PCR identification of *Listeria* spp. from fishes of Cochin area

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Listeria spp, a Gram positive and non-spore forming bacteria, are widely distributed in nature and different food products. Among *Listeria* spp., only *Listeria monocytogenes* is considered as potential human pathogen. In this study, we have screened fish samples procured from local markets of Cochin for the presence of *Listeria* spp. including *L. monocytogenes*. 25.7 % of the samples tested were found to harbour *Listeria* spp. The incidence rate of *Listeria* spp. in finfish, prawn and clam were found to be 26.8 %, 10 % and 37.5 %, respectively. But *L. monocytogenes* has not been detected in any of the fish samples tested. *Listeria innocua* was found to be the most prevalent species of *Listeria*. The isolates were characterized by *Listeria* genus specific PCR as well as *L. monocytogenes, L. innocua* & *L. ivanovii* species specific PCR. All of our *L. innocua* isolates have been confirmed by both genus and species specific PCR. For confirmation of species of *Listeria*, PCR has been shown to be an effective tool.

Key words: PCR, *Listeria* spp., *Listeria monocytogenes, Listeria innocua.*

The genus *Listeria* includes six species viz. *L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L. seeligeri and L. welshimeri.* Among these, only *L. monocytogenes* is considered as potential human pathogen, causing both outbreaks and sporadic infections leading to various illness *viz.* abortion in pregnant women, meningitis, febrile gastroenteritis, septicemia, conjunctivitis etc. (Va´zquez-Boland *et.al.*, 2001). The ability of *Listeria* spp. to grow at refrigerated temperatures and its resistance to many commonly used sanitizing agents are of great concern in public health point of view (Schuchat *et.al.*, 1991). Several food borne outbreaks of listeriosis have been reported in United States, Canada, Germany and Australia (Fleming *et.al.*, 1985; James *et.al.*, 1985). The reports of outbreaks of listeriosis due to consumption of seafood are also occasionally available (Ericsson *et.al.*, 1997; Farber *et.al.*, 2000). In India, there is no report of major listeriosis outbreak due to consumption of seafood, but *Listeria* spp. has occasionally been isolated from seafood and fishery environment (Fuchs & Surendran, 1989; Jeyasekaran *et.al.*, 2003). Polymerase chain reaction (PCR) is considered as an easy identification tool for listeria both upto genus and species level (Border *et.al.*, 1990; Bubert *et.al.*, 1999).

This study describes the incidence of *Listeria* spp. in fish, shrimp and clam from local fish markets of Cochin and evaluation of polymerase chain reaction for subsequent identification of *Listeria* isolates.

Materials and Methods

Fish, shrimp and clam samples were procured from different local markets of Cochin. A total of 74 fish, shrimp and clam samples were screened for the period of 15 months from December, 2003 to February, 2005.

Modified USDA-FSIS method (Carnevale & Johnston, 1989) as adopted by Surendran *et.al.* (2003) with little modification was followed for isolation of *Listeria* spp. Briefly, 25 g of sample was macerated in 225 ml of UVM-I broth and was incubated at room temperature for 24 hours. 1 ml from primary enrichment was inoculated into 10 ml of Frazer's broth and was incubated at 37°C for 48 hours. Loopful of culture from both 24 and 48 hours old Frazer's broth was streaked

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on preset *Listeria* selective agar (Oxford formulation). Plates were incubated at 37°C for 48 hours. Suspected *Listeria* colonies were picked up from the plate, purified on TSA plate and streaked on TSA slants. At least 5 colonies were picked up from each plate.

Further characterization and identification of the isolates were done by Gram's staining, test for tumbling motility, hemolysis test on 5% sheep blood agar, CAMP test and different biochemical tests as described in Lovett, (1987, 1988).

Following overnight growth in BHI broth, crude preparation of template DNA from bacterial culture for PCR was done by the method as described in Fitter *et.al.*, 1992 with slight modification. Here, heating for bacterial cell lysis was performed on boiling water bath instead of microwave oven. After boiling, the suspension of lysed bacterial cells was frozen immediately at -70°C. Freezing stage was included because production of ice crystals during freezing aids in lysis of bacterial cell membrane and subsequent release of DNA. Before use as PCR template, it was thawed and centrifuged at 4000 X g to pellet the debris. 5 µl of the supernatant was used as template in the PCR reaction.

The primers used in this study and the reaction condition of each PCR have been shown in Table 1. All PCR assays were performed in 25 µl of reaction mixtures consisting of 5 µl of lysate as template, 10mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP (Finnzyme, Finland), 1 U *Taq* DNA polymerase (Bangalore Genei, India) and 10 pmole of respective primers. In all cases, an initial denaturation at 95°C for 3 minutes and a final extension at 72°C for 5 minutes were employed. 30 cycles of amplification was used in all the PCR reactions. The PCR assays were performed in a thermocycler (Mastercycler, Eppendorf, Germany). Nested PCR was performed for detection of *L. monocytogenes*. First step PCR was performed using primers LM1/LM2 and 0.5 µl of the product of first step PCR was used as template in nested PCR using the primer pair LL5/LL6.

The PCR products were analyzed in 1.5% agarose gel prepared in 1X TAE buffer (40mM Tris-acatate and 1mM EDTA, pH 8.0) containing ethidium bromide (0.3 µg/ml). Electrophoresis was carried out at 7 volts/cm for 1 hour. The agarose gel was photographed by an UV gel documentation system (Alpha Innotech corporation, USA).

Table 1. Primers used for PCR amplification of *Listeria* spp.

Primer pairs and reference	Sequences	Specificity	PCR Cycling condition used in this study
U1/LII (Border *et.al.*, 1990)	UI (CAGCMGCCGCGGTAATWC) and LII (CTCCATAAAGGTGACCCT)	*Listeria* genus	94°C for 45 seconds 55°C for 1 minute 72°C for 1 minute
LM1/LM2 (Border *et.al.*, 1990)	LM1 (CCTAAGACGCCAATCGAA) and LM2 (AAGCGCTTGCAACTGCTC)	*L. monocytogenes*	94°C for 45 seconds 50°C for 1 minute 72°C for 1 minute
LL5/LL6 (Thomas *et.al.*, 1991; Herman *et.al.*, 1995)	LL5 (AACCTATCCAGGTGCTC) and LL6 (CTGTAAGCCATTTCGTC)	*L. monocytogenes*	94°C for 45 seconds 55°C for 45 seconds 72°C for 45 seconds
lin0464F/ lin0464R (Liu *et.al.*, 2003)	lin0464F (CGCATTTATCGCCAAAACTC) and lin0464R (TCGTGACATAGACGCGATTG)	*L. innocua*	94°C for 30 seconds 60°C for 45 seconds 72°C for 45 seconds
liv22-228F/ liv22-228R (Liu *et.al.*, 2004)	liv22-228F (CGAATTCCTTATTCACTTGAGC) and liv22-228R (GGTGCTGCGAACTTAACTCA)	*L. ivanovii*	94°C for 30 seconds 60°C for 30 seconds 72°C for 30 seconds

Results and Discussion

Out of 74 seafood samples tested, the presence of *Listeria* spp. was detected in 19 samples. A total of 20 isolates were recovered from the samples. Out of 20 isolates, 16 have been confirmed as *Listeria innocua.* Other 4 isolates have been identified as *Listeria grayi* subsp. *murrayi* based on biochemical tests. None of the fish or shrimp samples were found to harbour *L. monocytogenes,* as all the isolates were found non-hemolytic on blood agar. The incidence of *Listeria* spp. in finfish, prawn and clam has been shown in Table 2.

The PCR assays were standardized by including standard reference strains along with the isolates. The reference strains included in the study were *L. monocytogenes* NCTC 11994, *L. monocytogenes* MTCC 657, *L. monocytogenes* NCAFB-PKS-1, *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. grayi* ATCC 19120, *Jonesia denitrificans* ATCC 14870, *Bacillus cereus* NCIM 2106, *Bacillus subtilis* NCIM 2545, *Salmonella* Dublin ATCC 15480, *Staphylococcus aureus* NCIM 2079 and *Escherichia coli* NCIM 2068. In *Listeria* genus specific PCR using primers UI and LII, all the *Listeria* isolates yielded expected PCR products of 938 bp size in agarose gel electrophoresis. This PCR assay was performed at different annealing temperatures i.e. 50°C, 53°C, 55°C, 57°C and 60°C. It has

Table 2. Incidence of *Listeria* spp. in Finfish, Prawn and Clam samples as detected by biochemical methods

Sample	Number screened	*Listeria* spp.	*L. monocytogenes*
Fin fish	56	15 (26.8%)	ND
Prawn	10	1 (10%)	ND
Clam	8	3 (37.5)	ND
Total	74	19 (25.7%)	ND

ND= Not detected

Table 3. Different isolates of *Listeria* spp.

Listeria isolates	Date of Isolation	Sample	Identified as
NCAFB-L-SD-1	28-4-2004	Mackerel (*Rastrelliger kanagurta*)	*L. innocua*
NCAFB-L-SD-2	28-4-2004	Oil Sardine (*Sardinella longiceps*)	*L. innocua*
NCAFB-L-SD-3	3-5-2004	Oil Sardine (*Sardinella longiceps*)	*L. innocua*
NCAFB-L-SD-4	3-5-2004	Mackerel (*Rastrelliger kanagurta*)	*L. innocua*
NCAFB-L-SD-5	8-6-2004	Mackerel (*Rastrelliger kanagurta*)	*L. innocua*
NCAFB-L-SD-6	8-6-2004	Black clam (*Villorita cyprinoides*)	*L. grayi* subsp. *murrayi*
NCAFB-L-SD-7	10-8-2004	Oil Sardine (*Sardinella longiceps*)	*L. innocua*
NCAFB-L-SD-8	10-8-2004	Kadalkarrop (*Epinephelus melanostigma*)	*L. innocua*
NCAFB-L-SD-9	24-8-2004	Mackerel (*Rastrelliger kanagurta*)	*L. innocua*
NCAFB-L-SD-10	13-9-2004	Small prawn (*Metapenaeus dobsoni*)	*L. innocua*
NCAFB-L-SD-11	13-9-2004	Small prawn (*Metapenaeus dobsoni*)	*L. grayi* subsp. *murrayi*
NCAFB-L-SD-12	13-9-2004	Kilimeen (*Nemipterus japonicus*)	*L. innocua*
NCAFB-L-SD-13	13-12-2004	Velameen (*Lethrinus frenatus*)	*L. innocua*
NCAFB-L-SD-14	20-12-2004	Poomeen (*Chanos chanos*)	*L. innocua*
NCAFB-L-SD-15	20-12-2004	Kannaayila (*Decapterus* spp.)	*L. innocua*
NCAFB-L-SD-16	20-12-2004	Velameen (*Lethrinus frenatus*)	*L. innocua*
NCAFB-L-SD-17	5-1-2005	Black Clam (*Villorita cyprinoides*)	*L. grayi* subsp. *murrayi*
NCAFB-L-SD-18	24-1-2005	Black clam (*Villorita cyprinoides*)	*L. grayi* subsp. *murrayi*
NCAFB-L-SD-19	16-2-2005	Pallimeen (*Otolithes* spp).	*L. innocua*
NCAFB-L-SD-20	16-2-2005	Seer fish (*Scomberomorus commerson*)	*L. innocua*

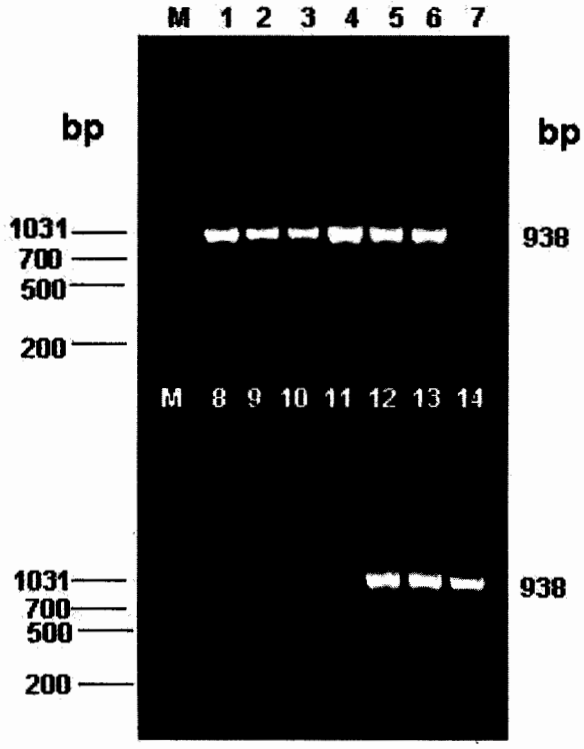

Fig. 1. *Listeria* genus specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *L. monocytogenes* NCTC 11994, Lane 2: *L. monocytogenes* MTCC 657, Lane 3: *L. monocytogenes* NCAFB-PKS-1, Lane 4: *L. innocua* ATCC 33090, Lane 5: *L. ivanovii* ATCC 19119, Lane 6: *L. grayi* ATCC 19120, Lane 7: *Jonesia denitrificans* ATCC 14870, Lane 8: *Bacillus cereus* NCIM 2106, Lane 9: *Staphylococcus aureus* NCIM 2079, Lane 10: *Salmonella* Dublin ATCC 15480, Lane 11: *Escherichia coli* NCIM 2068, Lane 12: *L. innocua* NCAFB-L-SD-1, Lane 13: *L. innocua* NCAFB-L-SD-2, Lane 14: *L. innocua* NCAFB-L-SD-3.

been found that 55°C is the most suitable annealing temperature for this PCR. At 50°C annealing temperature, cross-amplification was observed with *B. cereus* NCIM 2106 and *Bacillus subtilis* NCIM 2545. In addition to these, many non-specific products were also visible when 50°C annealing temperature was used. But no cross-amplification was observed at 55°C annealing temperature. But at 60°C annealing temperature, no amplification was observed even in case of *L. ivanovii* ATCC 19119. This finding is at the variance from the finding of Border *et.al.* (1990), who performed this PCR reaction at 50°C annealing temperature and found this PCR reaction to be specific for *Listeria* genus.

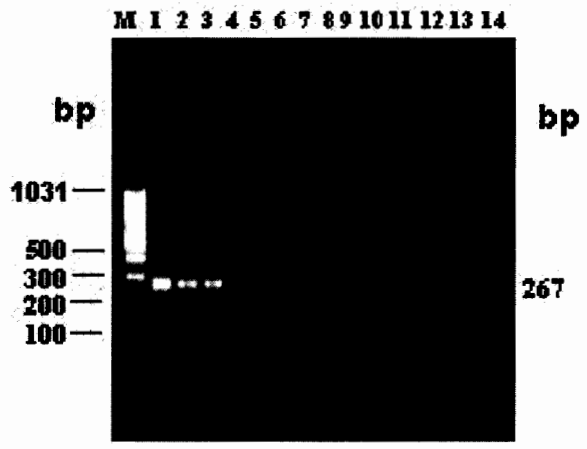

Fig. 2. *L. monocytogenes* specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *L. monocytogenes* NCTC 11994, Lane 2: *L. monocytogenes* MTCC 657, Lane 3: *L. monocytogenes* NCAFB-PKS-1, Lane 4: *L. innocua* ATCC 33090, Lane 5: *L. ivanovii* ATCC 19119, Lane 6: *L. grayi* ATCC 19120, Lane 7: *Jonesia denitrificans* ATCC 14870, Lane 8: *L. innocua* NCAFB-L-SD-1, Lane 9: *L. innocua* NCAFB-L-SD-2, Lane 10: *L. innocua* NCAFB-L-SD-3, Lane 11: *L. innocua* NCAFB-L-SD-4, Lane 12: *L. innocua* NCAFB-L-SD-5, Lane 13: *L. grayi* subsp. *murrayi* NCAFB-L-SD-6, Lane 14: *L. innocua* NCAFB-L-SD-7.

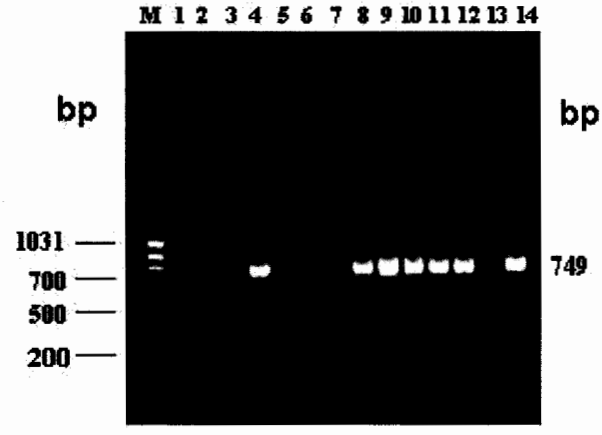

Fig. 3. *L. innocua* specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *L. monocytogenes* NCTC 11994, Lane 2: *L. monocytogenes* MTCC 657, Lane 3: *L. monocytogenes* NCAFB-PKS-1, Lane 4: *L. innocua* ATCC 33090, Lane 5: *L. ivanovii* ATCC 19119, Lane 6: *L. grayi* ATCC 19120, Lane 7: *Jonesia denitrificans* ATCC 14870, Lane 8: *L. innocua* NCAFB-L-SD-1, Lane 9: *L. innocua* NCAFB-L-SD-2, Lane 10: *L. innocua* NCAFB-L-SD-3, Lane 11: *L. innocua* NCAFB-L-SD-4, Lane 12: *L. innocua* NCAFB-L-SD-5, Lane 13: *L. grayi* subsp. *murrayi* NCAFB-L-SD-6, Lane 14: *L. innocua* NCAFB-L-SD-7.

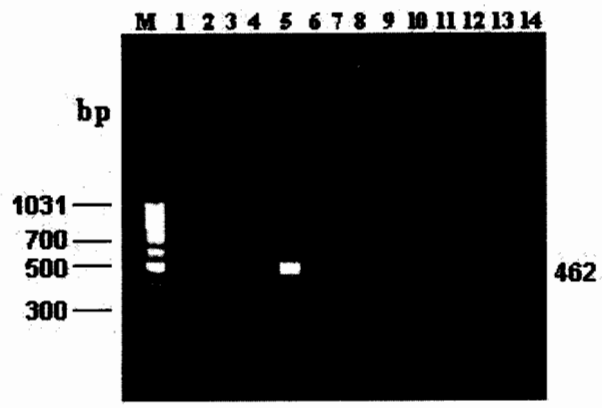

Fig. 4. *L. ivanovii* specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *L. monocytogenes* NCTC 11994, Lane 2: *L. monocytogenes* MTCC 657, Lane 3: *L. monocytogenes* NCAFB-PKS-1, Lane 4: *L. innocua* ATCC 33090, Lane 5: *L. ivanovii* ATCC 19119, Lane 6: *L. grayi* ATCC 19120, Lane 7: *Jonesia denitrificans* ATCC 14870, Lane 8: *L. innocua* NCAFB-L-SD-1, Lane 9: *L. innocua* NCAFB-L-SD-2, Lane 10: *L. innocua* NCAFB-L-SD-3, Lane 11: *L. innocua* NCAFB-L-SD-4, Lane 12: *L. innocua* NCAFB-L-SD-5, Lane 13: *L. grayi* subsp. *murrayi* NCAFB-L-SD-6, Lane 14: *L. innocua* NCAFB-L-SD-7.

Nested PCR for *L. monocytogenes* using primer pairs LM1/LM2 and LL5/LL6 yielded 267 bp amplified product only in case of *L. monocytogenes* NCTC 11994, *L. monocytogenes* MTCC 657 and *L. monocytogenes* NCAFB-PKS-1. None of our isolates was found to be positive in this nested PCR. 16 isolates, which were identified as *L. innocua* by biochemical tests and *L. innocua* ATCC 33090 yielded 749 bp amplicon in *L. innocua* specific PCR reaction using primers lin0464F/lin0464R. Primer pair liv22-228F/ liv22-228R, which is *L. ivanovii* specific, amplified specific product of nearly 465 bp size only in case of *L. ivanovii* ATCC 19119. All the other strains tested including our isolates didn't yield any amplified product by this set of primer. All the PCR results have been illustrated in Figs. 1,2,3 & 4. Reactions of all the isolates have not been shown in the figures.

Among *Listeria* spp., only *Listeria monocytogenes* is considered as pathogenic to human being (Schuchat *et.al.*, 1991). *L. ivanovii*, which causes abortion in sheep, has not been shown to cause any disease in human being. The presence of *L. monocytogenes* in seafood is of great concern both in public health and export point of view. USFDA and European Union (EU) have imposed zero tolerance level for *L. monocytogenes* in food. In the current study, it was found that 25.7% of fish, prawn and clam samples were positive for *Listeria* spp. But *L. monocytogenes* was absent in all the samples. The *L. monocytogenes* species specific nested PCR results of the study supports this observation. All of these *Listeria* isolates were also negative to β-hemolysis. This study also reveals that the most prevalent species of *Listeria* in the fish samples of Cochin area is *L. innocua*. Confirmation of *L. innocua* has been done both by biochemical tests and PCR. *L. innocua* is considered as non-pathogenic species of *Listeria*. But *L. monocytogenes* and *L. innocua* share a common ecological niche. Hence the presence of *L. innocua* can be considered as an indicator organism for an environment, which favours the presence of *L. monocytogenes* (Cited in Bubert *et.al.*, 1999). This finding necessitates that frequent screening of fish samples for the presence of *L. monocytogenes* have to be implemented.

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