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Isolation and Characterization of *Listeria* Species from Raw and Processed Meats

S.P. Doijad, V. Vaidya[1], S. Garg[1], S. Kalekar, J. Rodrigues, D. D'Costa, S.N. Bhosle[1] and S.B. Barbuddhe*

ICAR Research Complex for Goa, Ela, Old Goa 403 402

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ABSTRACT

A total of 109 meat and meat product samples (50 beef, 52 pork sausages and 7 pork) were examined for the presence of *Listeria* species. Isolates were confirmed by biochemical characterization and haemolysis on blood agar. A total of 25 (22.94%) isolates were recovered. Of these, 7 (6.42%) were *L. monocytogenes*, while one (0.9%) was *L. ivanovii*. Other isolates were *L. seeligeri* (10), *L. innocua* (4), *L. welshimeri* (2) and *L. grayi* (1). All the isolates were further subjected to a PCR assay for detection of the *hlyA* gene, the *hlyA* gene was detected in 7 isolates. Multiplex-PCR serotyping assay revealed three of the strains belonging to serovar group 4b, 4d, 4e, and four to serovar group 1/2a, 3a. The prevalence of *L. monocytogenes* in raw meat, particularly serogroup 4b, 4d, 4e in present study indicates a potential threat to public health.

Keywords: *Listeria,* raw meat, PCR, processed meats

Introduction

Listeria species are Gram-positive organisms, ubiquitously distributed in the environment. The genus *Listeria* contains six species; of which *Listeria monocytogenes* is the only species of public health significance (Faber and Peterkin, 1991; Swaminathan *et al*., 2001). *L. monocytogenes* can cause serious diseases such as septicemia, meningitis, meningoencephalitis in immunocompromised individuals, newborns and the elderly, and abortion and stillbirth in pregnant women (Vazquez-Boland *et al*., 2001). With the increase in consumption of manufactured ready-to-eat foods, *L. monocytogenes* has been recognized as an important opportunistic human foodborne pathogen (Liu, 2006).

***Corresponding author:** barbuddhesb@yahoo.com

[1]Department of Microbiology, Goa University, Taleigaon Plateau, Goa - 403 206

A wide variety of meats have been reported to be contaminated with *L. monocytogenes* (Faber and Peterkin, 1991) such as ground beef, pork sausages (Lee and McClain, 1987), chicken meat (Schwartz *et al*., 1988). Meat products have been incriminated in foodborne outbreaks of listeriosis (Jacquet *et al*., 1995; Goulet *et al*., 1998). Raw food from animal sources may not necessarily be the origin of *L. monocytogenes*, the bacteria may contaminate the food product while being processed. The slaughtering environment and handling practices change from place to place. Although faeces and skin of slaughtered animals are considered to be sources of *L. monocytogenes* contamination, the slaughter house environment is more important factor to be considered. About 4-7% hand surfaces of the food handlers and 16% of processing tables were found to carry *L. monocytogenes* (Kerr *et al*., 1993; Jeyasekaran *et al*., 2000). Therefore, the chances of cross contamination increases. As per the requirements of the US-FDA, *L.*

monocytogenes should be absent in ready to eat foods (Fusch and Reilly, 1992).

In India, only a few surveys have been conducted to assess the presence of *Listeria* spp. in raw meats (Barbuddhe *et al*., 2000; Barbuddhe *et al*., 2002). Thus, the purpose of the present study was to generate information on the incidence of *Listeria* species in raw meat (beef, and pork) and processed meat (sausages) from west coast region of India and characterize the isolates by PCR based serotyping.

Materials and Methods

Samples of raw and processed meats were obtained from the local vendors in Goa, India. A total of 109 samples comprising 50 beef, 7 pork and 52 pork-sausages were collected in UV sterilized polyethylene bags. Samples were transported under chilled condition to laboratory, stored in refrigerated conditions and processed within 24 h.

Isolation of *Listeria* from the meat sample was attempted as per USDA method. The samples (weighing approx. 5 gm) were inoculated into 45 ml of University of Vermont medium (UVM)-1 with supplement of acriflavin (12 mg/l) and incubated for enrichment of *Listeria* at 37^{0}C for 18-24 h (Donelly and Baigent, 1986). Further enrichment of *Listeria* was carried by inoculating into 0.1 ml of enriched UVM-1 inoculum from 10 ml of UVM-2 (supplemented with acriflavin @ 25 mg/l. Inoculated UVM-2 broth was incubated for 24-36 h at 37^{0}C. A loopful inoculum from UVM-2 was streaked directly on PALCAM agar for selective isolation of listerial colonies. The inoculated agar plates were incubated at 37^{0}C for 48 h.

Pinpoint grayish-green colonies surrounded by black zone of esculin hydrolysis were presumed as *Listeria*. These colonies were further purified on PALCAM agar and stored in refrigerated conditions in BHI broth. All the isolates were checked for haemolysis on sheep blood agar and were subjected to various biochemical tests including lecithinase activity in order to identify the isolates.

The genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India) and were subjected to PCR amplification for detection of the *hlyA* gene (Rawool *et al*., 2007) and *Imo0737, Imo1118, Orf 2819,* and *prs* genes for serotyping (Doumith *et al*., 2004). The primers used for the detection of above mentioned genes are listed in Table 1.

PCR amplification of the *hly*A gene was set in 25 µl reaction volume. The reaction was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The completed reaction mixture was subjected to an initial denaturation at 95^{o}C for 2 min, followed by 35 cycles each of 15 sec denaturation at 95^{o}C, 30 sec annealing at 60^{o}C and 90 sec extension at 72^{o}C. It was followed by final extension of 10 min at 72^{o}C and held at 4^{o}C.

Multiplex-PCR amplification of serovar genes was set in 50 µl reaction mixtures containing 2 mmoles of each primer. The primers *Imo0737*, *Imo1118, ORF2819* and *prs* were used. Multiplex-PCR was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 30 sec, 54^{o}C for 75 sec, and 72^{o}C for 75 sec; and one final extension was carried out at 72^{o}C for 7 min. Samples were kept at 4^{o}C until agarose gel electrophoresis.

The resultant PCR products were further analysed by agarose gel (0.8%) electrophoresis to resolve the amplified DNA fragment of the target gene. The bands of amplified DNA in the gel were visualized by a UV transilluminator and digitally recorded by gel documentation system.

Results and Discussion

Listeria species were isolated from 25 out of 109 samples of fresh and processed meats. Sixteen of 50 beef, 1 of 7 pork samples and 8 of 52 sausage samples were positive for *Listeria* (Table 2).

These 25 listerial isolates, when characterized biochemically, revealed presence of *L. monocytogenes* (7), *L. ivanovii* (1), *L. seeligeri* (10), *L. innocua* (4), *L. welshimeri* (2) and *L. grayi* (1). Thus, approximately 8% of samples were found positive for the presence of pathogenic *Listeria*. As the sampling size of pork was low, the study will not be able to give exact incidence.

Eighteen isolates showed hemolytic activity on sheep blood agar. Lecithinase activity was found in twelve isolates.

All the isolates were subjected to PCR detection for the presence of *hlyA* gene. Among these, seven isolates identified as *L. monocytogenes* showed presence of this gene (Table 3). Serotyping of *L. monocytogenes* is important in epidemiological studies for investigation of foodborne outbreaks (i.e., comparing clinical and food isolates), and in the food-processing environment, to identify the source of contamination. Isolates identified as *L. monocytogenes* and found to contain *hly*A gene were further subjected for serotyping by PCR amplification of *lmo0737, lmo1118,* ORF2819 and *prs* genes (Fig. 1). Serotyping of seven isolates of *L. monocytogenes* by PCR revealed three isolates belonging to serovar group 4b, 4d, 4e, while remaining four were from serovar group 1/2a,3a.

Listeria monocytogenes has been demonstrated in wide variety of foods. Raw and processed meats, soft cheese, raw milk, hot dogs, seafood and fresh vegetables have been linked to both sporadic cases and outbreaks of listeriosis (AFSSA, 2000; FICT, 2002). High risk of contamination of beef has been explained as major reason for the presence of *Listeria* which is further supported by an ideal nutritive environment for attachment and growth of bacteria compared to other meat products (Glass and Doyle, 1989). The prevalence of *L. monocytogenes* in the meat samples was observed to be 6.49%, which is comparable with earlier reports of 6% in buffalo meat samples (Brahmabhatt and Anjaria, 1993 and 6.2% in raw beef (Farber and Peterkin, 1991). However, the prevalence observed in this study was lower than reported prevalence of 11% (Wung *et al*., 1992) and 28% (Skovgaard and Morgen, 1988). Similarly, *Listeria* spp. has been isolated from 25% and 29% of samples from local and Internet markets, respectively in USA (Pao and Ettinger, 2009). About 5% samples of raw beef were contaminated with *L. monocytogenes*. High prevalence of *Listeria* species was also recorded in beef in Ethiopia (Mengesha *et al*., 2009). The differences in the methods of detection, including factors such as the source of the sample, the method used and differences in host and environmental factors may contribute to the differences in occurrence of the pathogen.

Listeria monocytogenes isolates were grouped by multiplex-PCR serotyping. As serotypes 3a, 3b, 4d and 4e are relatively rare in foods (Doumith *et al*., 2004), the isolates belonging to 1/2a, 3a and 4b, 4d, 4e were presumably serotypes 1/2a and 4b, respectively. Our data showed that most of the isolates belonged to serogroup 4b, which is considered as major cause for human listeriosis (Liu, 2006). The observation indicates the potential of meat products to serve as vehicles of transmission of virulent *L. monocytogenes*.

The prevalence of *L. monocytogenes* generally increases from the farm to the manufacturing plants and this is mainly due to cross-contamination. Pork and processed pork products, such as deli meats, have been implicated in *Listeria* outbreaks (Jacquet *et al*., 1995; Goulet *et al*., 1998) during the past decade. The organism is present ubiquitously, thus food in the processing industries are easily contaminated (AFSSA, 2000).

L. monocytogenes is killed by cooking. Raw and/or semi-raw meat such as graved or smoked, are not consumed in India. However, *L. monocytogenes* in raw meats may pose a health risk in kitchen, if it contaminates cooked food or other ready-to-eat food. Considering outbreaks

Table 1. Details of primers used in present study for the amplification of virulence and serotype marker in *Listeria* species.

Gene target	Primer sequences (5'-3')	Product size (bp)	Serovar specificity
hlyA	For: 5'GCAGTTGCAAGCGCTTGGAGTGAA3'	456	Not applicable
	Rev: 5'GCAACGTATCCTCCAGAGTGATCG3'		
Imo0737	For: 5'AGGGCTTCAAGGACTTACCC3'	619	*L.monocytogenes*
	Rev: 5'ACGATTTCTGCTTGCCATTC3'		serovars1/2a,1/2c,3a,3c
Imo1118	For: 5'AGGGGTCTTAAATCCTGGAA3'	906	*L.monocytogenes* serovars
	Rev: 5'CGGCTTGTTCGGCATACTTA3'		1/2c,3c
ORF2819	For: 5'AGCAAAATGCCAAAACTCGT3'	471	*L.monocytogenes*
	Rev: 5'CATCACTAAAGCCTCCCATTG3'		Serovars 4b,4d,4e
prs	For: 5'GCTGAAGAGATTGCGAAAGAAG3'	370	All *Listeria* species
	Rev: 5'CAAAGAAACCTTGGATTTGCGG3'		

Table 2. Isolation of *Listeria* species from raw and processed meats.

Type of samples	No. of samples	No. isolated	LM[a]	LIV[b]	*Listeria* spp.
Beef	50	16	04	01	12
Sausages	52	08	03	00	04
Pork	07	01	00	00	01

[a]LM- *Listeria monocytogenes,* [b]LIV- *Listeria ivanovii*

Table 3. Characterization of the *Listeria* isolates.

Isolate	Sample	Species identified	*hlyA*	Serogroup
VS019	Sausage	*L. monocytogenes*	+	4b, 4d, 4e
VB029	Beef	*L. monocytogenes*	+	4b, 4d, 4e
VB032	Beef	*L. ivanovii*	-	
VS066	Sausage	*L. monocytogenes*	+	4b, 4d, 4e
VS067	Sausage	*L. monocytogenes*	+	1/2a, 1/2c, 3a, 3c
VS068	Sausage	*L. monocytogenes*	+	1/2a, 1/2c, 3a, 3c
VS071	Beef	*L. monocytogenes*	+	1/2a, 1/2c, 3a, 3c
VB080	Beef	*L. monocytogenes*	+	1/2a, 1/2c, 3a, 3c

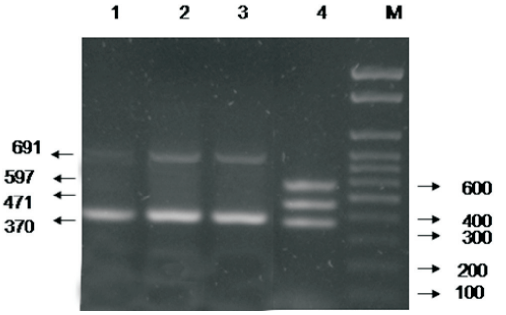

Fig. 1. Serotype profile of *Listeria* species by multiplex-PCR serotyping. Lane M : marker, Lanes 1–3: *L. monocytogenes* isolates, Lane 4: *L. monocytogenes*, 4b (NCTC 11994)

of listeriosis associated with different foods, avoidance of consumption of insufficiently cooked meats by at-risk populations is recommended.

The present study indicates the prevalence of *L. monocytogenes* in raw meat, which may be a potential threat to public health. In addition, the observation that most of the *L. monocytogenes* strains belongs to serotype 4b is also a potential public health concern, as serotype 4b has been the serotype most frequently associated with human listeriosis.

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