RESEARCH ARTICLE

Molecular Characterization of *L. monocytogenes* Isolated From Milk and Milk Products

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Abstract

A total of 194 (121 bovine milk samples and 73 milk products) were screened for *Listeria monocytogenes*. The isolation was attempted by two-step enrichment procedure in UVM-1 and UVM- 2 broths followed by plating on DRIA. The isolates were confirmed by biochemical test and *in-vitro* PI-PLC pathogenicity test. The *L. monocytogenes* isolates from milk and milk products were also tested for virulence-associated genes *plcA, hlyA, actA,* and *iap* by multiplex polymerase chain reaction (PCR). Prevalence of *L. monocytogenes* found to be 15.70% and 10.95% in milk and milk products, respectively. The PCR profile of *L. monocytogenes* isolates revealed variation in the presence of the virulence-associated genes namely *plcA, hlyA, actA,* and *iap*. Four *L. monocytogene* isolates from milk samples and two isolates from milk products revealed PI-PLC activity. *L. monocytogenes* isolates were sensitive to ampicillin, penicillin, ciprofloxacin and vancomycin.

Keywords : Antibiotic sensitivity test, *L. monocytogenes*, Milk & Milk products, PCR.

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Introduction

Listeriosis is one of the important bacterial zoonotic infections (Baylegen *et al.,* 2004). Out of17 recognized species in the genus *Listeria, L. monocytogenes* and *L. ivanovii* were found pathogenic. *L. monocytogene* is recognized worldwide as one of the most important food-borne pathogens of concern for the food industries. *L. monocytogenes* has been isolated from various foodstuffs including milk (Barbuddhe *et al.,*2002).

Multiple key virulence factors such as hemolysin A (*hlyA*), phosphatidylinositol phospholipase C (*plcA*), actin polymerization protein (*actA*), and invasive associated protein (*iap*) are important in *L. monocytogenes* pathogenesis (Furre r*et al.,* 1991). Therefore, detection of just one virulence-associated gene by PCR is not always sufficient to identify *L. monocytogenes* (Nishibori *et al.,* 1995).

With the advent of PCR, it has become possible to identify virulence marker genes of *Listeria monocytogenes* and to carry out its detailed molecular characterization. The available literature on the status of food borne listeriosis is very limited in public health sector in India. Hence the present study was planned to assess prevalence of Listeria *monocytogenes* from milk and milk products, antibiogram and genotypic characterization of the *L. monocytogenes* strains with reference to virulence marker genes (*hlyA, actA, iap and plcA*).

Materials and Methods

Isolation and Characterization of *Listeria* spp.

A total of 194 (121 Bovine milk samples and 73 milk products) were collected from various bovine farms and local shops in Nagpur. All samples were collected in sterilized vial and quickly transported to the laboratory under chilled condition and stored at 4°C until used for isolation.

Isolation of *Listeria* from the milk samples was carried out as per the US Department of Agriculture (USDA) method described by McClain and Lee (1988) after making necessary modifications.

The milk products were ground into a paste using a sterile homogenizer. The resulting homogenate was used for cultural examination. One gram of each homogenate was first dispensed in a test tube containing 9 mL of sterile UVM-1 broth containing 15 mg of acriflavine hydrochloride and was incubated at 30°C. Enriched inoculum (0.1-mL) from UVM-1 was then transferred to 10 mL of UVM-2 and incubated again for 24 hours at 30°C. The enriched inoculum from UVM-2 was streaked directly on DRIA. The inoculated petri dishes were incubated at 30°C for 48 hours. The listeria was further

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confirmed by biochemical test and PI-PLC pathogenicity test.

Characterization of the Isolates

The smears prepared from typical colonies of listeriae were stained by standard Gram's staining method in practice. The Gram- positive coccobacilli were subculture on Brain Heart Infusion agar and stored at 4°C. The isolates in BHI broth exhibiting characteristic tumbling motility at 20–25°C were confirmed biochemically and evaluated for their *in-vitro* pathogenicity.

For biochemical tests, such as catalase, oxidase and fermentation of sugars (rhamnose, xylose, mannitol and α-methyl D-mannopyranoside) were performed by routine standard procedure. The isolates were identified by comparing with standard *Listeria* spp. The Isolates were further subjected to specific tests such as hemolysis on sheep blood agar (SBA), Christie Atkins, Munch-Petersen (CAMP) test and Phospatidylinositol phospholipase C activity (PI-PLC activity) (Gorski, 2008).

Genotypic Characterization

Biochemically confirmed *L. monocytogene* isolates were subjected to multiplex PCR method using published primers described by Notermans *et al.* (1991) with suitable modifications. The genomic DNA was extracted using DNA isolation kit (Chromos Biotech, India) Oligonucleotide primers procured from Sigma Aldrich (Bangalore). Agarose gel electrophoresis was performed as per Lee *et al*., (2012) to visualized band of desired molecular weight.

Antibiotic Sensitivity of Isolates

All the *Listeria monocytogene* isolates recovered were tested for *in vitro* antibiotic sensitivity. The test was performed by employing disc diffusion method described by Bauer *et al*. (1966) using 11 different antibiotic discs procured from M/s. Hi Media Lab. Ltd. India. The diameter of zone of inhibition was measured to nearest millimeter.

Table 1: Details of antibiotics used in *in vitro* antibiogram of *L. monocytogenes*

Group	*Name of antibiotic*	*Code of antibiotic disc*	*Concentration per disc*
Aminoglycoside	Gentamicin	G	10 µg
Cephalosporins	Ceftriaxone	Ci	30 µg
Quinolone	Ciprofloxacin	Cf	5 ug
Amphiqunilon	Chloramphenicol	C	10 µg
Penicillin	Penicillin G	P	10 units
	Ampicillin	A	10 µg
Tetracycline	Oxytetracycline	o	30 µg
Quinolone	Enrofloxacin / Ciprofloxacin	Ex/ Cf	10 µg
Non-specific	Vancomycin	Va	30 µg
Macrolide	Erythromycin	E	10 µg
Sulphonamide	Sulphazidine	Sz	30 ug

Results & Discussion

Prevalence of *L. monocytogenes*

The overall prevalence of *Listeria* was (57/194; 29.38 %) in milk and milk products. Results on the prevalence study on for *Listeria spp.* and *L. monocytogenes* reveals that there was higher (41/121; 33.88%) prevalence of *Listeria spp*.in milk as compared to milk products (16/73; 21.91%), further *L. monocytogenes* were observed at 15.70 and 10.95 % in milk and milk products respectively (Table 1). The prevalence of *L. monocytogenes* reported in the present work from the bovine raw milk samples is higher than that reported by Bhilegaonkar *et al.* (1997) Kalorey *et al.* (2008) Yadav *et al.* (2010) and Warke *et al*. (2019). While 22.2% prevalence in raw milk was recorded by Kells and Gilmour (2004). The prevalence of *L. monocytogenes* in milk samples varied as per geographical areas., which might be due to adaptation of diverse isolation and enumeration techniques. Similar prevalence of *L. monocytogenes* in milk product was reported by Pini and Gilbert (1988) and it was found to be higher than that reported by Kessel *et al.* (2004).

In-vitro Pathogenicity Test: PI- PLC activity assay:

All the *L. monocytogenes* isolates were streaked on Agar Listeria Ottavani & Agosti (*ALOA*) *Medium* to assess PI-PLC activity. PI-PLC activity was observed in 4/19 *L. monocytogene* isolates of milk samples and 2/8 *L. monocytogene* isolates of milk products.

PCR Targeting Virulence-associated Genes of *L. monocytogenes*

All the four virulence-associated genes primer sets namely *plcA, actA, hlyA* and *iap* were subjected to multiplex PCR assay. Each primer set was found to be specific to the corresponding gene amplifying the DNA fragments of the expected size from standard strain of *L. monocytogenes* MTCC 1143.

In present study, *L. monocytogenes* strain isolated from milk and milk products were analyzed for the presence of virulence associated genes employing multiplex PCR (Plate 1). Different combinations of genes were detected in different isolates. The choice of the target gene is of utmost importance for the detection of *L. monocytogenes* by PCR. (Barbuddhe *et al.,* 2008). *L. monocytogenes* recovered from milk products exhibited virulence genes in different combinations.The order of combination of *L. monocytogenes* was *actA, hlyA, iap* (63.15%); *hlyA, iap* (15.78%); *plcA, hlyA, iap* (15.78%) and *plcA, actA, hlyA, iap* (5.26 %).

Out of 8 isolates (10.95%) of *L. monocytogenes* from milk products, two virulence -associated gene patterns comprising two isolates (25%) of *plcA, actA. hlyA, iap* and 6 isolates (75%) of *actA, hly A, iap* were obtained. Gunasena *et al.*

Table 2: Prevalence of *L. monocytogenes* in milk and milk products

Sr. No.	*Source*	*No.of samples investigated*	*No. of Listeriae isolated*	*No. of L. monocytogenes isolated*
1	Milk	121	41 (33.88 %)	19/121 (15.70 %)
2	Milk products	73	16 (21.91 %)	8/73 (10.95 %)
3	Total	194	57 (29.38 %)	27/194 (47.36 %)

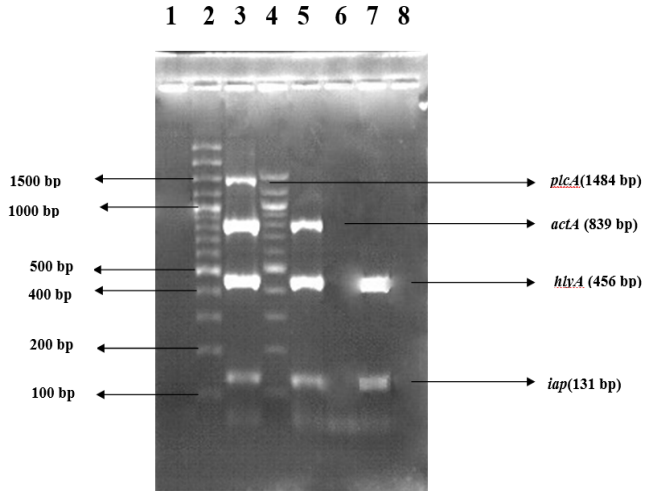

Plate 1: Virulence-associated gene patterns in *L. monocytogenes* of milk and milk products by multiplex PCR.
Lane1: Blank
Lane2: 100bp plus DNA ladder
Lane3: Four-gene combination (*plcA, actA, hlyA* and *iap)*
Lane 4: 100 bp DNA ladder
Lane 5: three-gene combination (*actA, hlyA,* and *iap)*
Lane 6: Blank
Lane 7: two-gene combination (*hlyA and iap*)

(1995) reported that contaminated processed milk products pose the greatest threat from *L. monocytogenes* because they are consumed without further treatment.

Antibiotic Sensitivity Testing

L. monocytogenes (27) were tested for their susceptibility towards the commonly prescribed antibiotics. All isolates were sensitive towards, gentamicin, erythromycin, oxytetracycline, ampicillin, doxycycline and ciprofloxacin and showed intermediate resistance towards the chloramphenicol, penicillin, and vancomycin. All isolates of *L. monocytogene* showed resistance to sulpazidine.

The antibiotic resistance of the pathogen is asignificant public health concern. Recentreports suggest the evolution of *L. monocytogenes* towards antibiotic resistance (Charpentier and Courvalin 1999; Altuntas., 2012; Soni *et al*., 2013) Antibiotic resistance patterns of *L. monocytogenes* in food and environmental sources may change with geographical area (Yan *et al.,* 2010). General consensus is that ampicillin or penicillin alone or in combination with gentamicin is the treatment of choice for listeriosis (Jones and McGowan, 1995). Hansen *et al.,* (2005) suggested that the acquired antimicrobial resistance in clinical strains is rare.

Conclusion

The present study showed that milk and milk products are potent source of *listeriae* and *L. monocytogenes* indicating threat to the public health.

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ANNOUNCEMENT: SVSBT-NS-2022

IX Annual Convention and National Seminar of SVSBT

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