

Isolation and Molecular Characterization of *Listeria Monocytogenes* from Raw Milk

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Abstract— Altogether 100 raw milk samples were collected aseptically from different dairy farms of Meghalaya and Assam of India and screened for *Listeria* species. All the isolates were characterized by biochemical tests and were subjected to polymerase chain reaction (PCR) assay for the detection of 16SrRNA, hlyA and iap genes. Out of all samples analyzed 4 samples were found to be positive for *L.monocytogenes*. The application of molecular characterization has been made for more sensitive and quick isolation and to determine the virulent species of the strains.

Keywords: Bovines, *Listeria Monocytogenes*, Raw Milk, PCR

I. INTRODUCTION

L. monocytogenes is regularly implicated in human and animal diseases. It principally affects pregnant women, their fetuses or newborn children, and the individual of weakened immune system. Some disease incidence occurs in apparently healthy people also. Listeriosis is usually sporadic, but large outbreaks related to consumption of contaminated foods have been reported [1].

There are many reports of outbreaks of food born infection from consumption of raw or partially pasteurized milk and their products. Many pathogenic organisms have been incriminated in these outbreaks. Different strains of *Listeria* especially *L. monocytogenes* has been involved in numerous outbreaks of listeriosis occurring through consumption of raw milk and milk products [2], [3], [4]. Within the last two decades various reports show that *Listeria* spp. has been found in milk and dairy products [5]. Moreover, several outbreaks of listeriosis were proven to be associated with the consumption of unpasteurized milk and the nearly 30% overall mortality rate of these outbreaks [6]. Therefore, it is very important to isolate and detect *Listeria* spp. from raw milk to admonish people about the risks of unpasteurized milk consumption.

II. MATERIALS AND METHODS

A. Samples

A total of 100 milk samples, 85 of cows and 15 of buffaloes were collected from different private farm of Meghalaya and Assam. All the samples about 100ml of each were collected aseptically, transported to the laboratory under chilled conditions and processed for microbiological analysis.

B. Isolation of *L.monocytogenes*

Approximately 1 ml from each of the 100 milk samples were directly inoculated into 9 ml. of University of Vermont Medium-1 (UVM-1) (Oxoid, Basingstoke, Hampshire, UK) with the *Listeria* selective enrichment supplement, UVMI, (Oxoid, Basingstoke, Hampshire, UK) and incubated overnight at 37°C. The inoculums from enriched UVM-1 was streaked on to *Listeria* selective agar base (Oxoid, Basingstoke, Hampshire, UK), supplemented with *Listeria* selective supplement, Oxford formulation (Oxoid, Basingstoke, Hampshire, UK). Plates were incubated at 37°C for 48 h. On LSA only the colonies of round button shaped with blackish-grayish colored were suspected to be *L.monocytogenes* were allowed for pure culture on nutrient agar and incubated at 37°C for 24 h.

C. Confirmatory Methods

1) Biochemical tests

The suspected colonies of *Listeria* were sub cultured on 5% sheep blood agar. Morphologically typical colonies were verified by Gram's staining, oxidase and catalase reaction, tumbling motility at 20–25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl- D mannopyranoside) and hemolysis in order to differentiate the *L. monocytogenes* isolates.

2) Polymerase chain reaction (PCR)

The primers for detection of the hemolysin gene (hlyA, 234bp), invasive associated protein gene (iap, 131bp) and 16SrRNA (1200 bp) gene of *L. monocytogenes* used in this study (Table-1) were synthesized by Hysel India Pvt. Ltd.

Gene	Orientation	Sequence (5'→3')	Length	G+C content	References
hlyA	Forward	CGG AGG TTC CGC AAA AGA TG	20	55%	[7]
	Reverse	CCT CCA GAG TGA TCG ATG	18	55.5%	
iap	Forward	ACA AGC ACC TGT TGC AG	17	52.9%	[8]
	Reverse	TGA CAG CGT GTG TAG TAG CA	20	50%	
16SrRNA	Forward	GGA CCG GGG CTA ATA CCG AAT GAT AA	26	50%	[9]
	Reverse	TTC ATG TAG GCG AGT TGC AGC CTA	24	50%	

Table1: Nucleotide sequence of oligonucleotide primers used in this study

The PCR was standardized for the detection of the hlyA and iap gene of *L. monocytogenes* as per the method described [10] and for 16SrRNA gene [9]. *L. monocytogenes* isolates were grown overnight in brain heart infusion broth at 37°C. The cultures (approximately 1 ml) were then centrifuged in appendrof tube at 10,000 rpm for 10 min. The recovered pellet was resuspended in 100 ml of sterilized

DNase and RNase-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 min. and then chilled in crushed ice. The reaction mixture was prepared in a total volume of 25µl consisting 10 µl of 2.5 mastermix (Fermentas), 0.125 µl of each primer (Hysel India pvt.ltd) and 2.5 µl of the boiled samples was used as DNA template. PCR amplification was carried out in the thermal cycler (Biorad).

PCR cycling parameters for hlyA and iap genes were as follows: Initial denaturation at 94°C for 4min (1 cycle), followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1min, and extension at 72°C for 1min. Final extension at 72°C for 10 min. And for 16SrRNA gene Initial denaturation at 95°C for 2.5min (1 cycle), followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 57°C for 1min, and extension at 72°C for 1min. Final extension at 72°C for 5 min.

For both reactions finally the PCR products were kept at 4°C until collection. The resultant products were further analyzed by agarose gel (1.2%) electrophoresis, stained with ethidium bromide (0.51g/ml) and visualized by a UV transilluminator.

Place	Cow		Buffaloes		Total Samples	Total Isolates
	No. of sample	No. of isolates	No. of sample	No. of isolates		
Assam	30	2	6	1	36	3 (8.33%)
Meghalaya	55	2	9	0	64	2 (3.12%)
Total	85	4 (4.70%)	15	1 (6.66%)	100	5 (5%)

Table 2: Recovery of *L. Monocytogenes* from different place.

The PCR result shows about the virulence efficiency of the isolates. PCR assay allowed amplification of hlyA, iap and 16SrRNA gene of *L. monocytogenes* to its respective base pair 234 bp, 131bp and 1200bp products represented by three different bands in the corresponding region of the DNA marker ladder.



Fig. 1: Detection of hlyA (234 bp) and iap (131 bp) gene of *L. monocytogenes* by multiplex polymerase chain reaction.

Lane M 100 bp marker, lane 1-9 isolates.

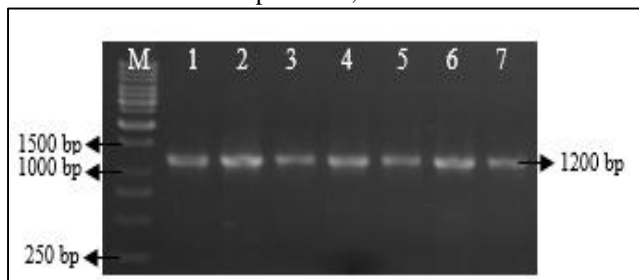


Fig. 2: Detection of 16srRNA (1200 bp) gene of *L. monocytogenes* by polymerase chain reaction. M= Marker (1 kb), 1-7 isolates.

IV. CONCLUSION

The occurrence of virulence genes in the isolates revealed that the *L. monocytogenes* were equally virulent and pathogenic as it found all over the world. Thus, the presence of *L. monocytogenes* in dairy milk is maybe due to indirect contamination from farm or from infected animals. The contamination sources of raw milk by *L. monocytogenes* are fecal [6] and environmental contamination during milking, storage and transport, infected cows in dairy farms and poor

III. RESULTS

A total of 100 milk samples, of which 85 from dairy cow and 15 from buffaloes were collected and examined for *L. monocytogenes*. *L. monocytogenes* were isolated from 5 samples (5%). Incidence of *L. monocytogenes* was higher in Assam (3 isolates out of 36 milk samples; 8.33%) than Meghalaya (2 out of 64 milk samples; 3.12%) (Table-2). Incidence was also found to be higher in buffalo milk (1 out of 15 milk samples; 6.66%) than cow milk (4 out of 85 milk samples; 4.70%). From PCR based detection it has been found that all the isolates are positive to 16SrRNA, hlyA and iap genes.

silage quality [11]. It is also conceivable that milk can be contaminated from unclean udders, teats, human hands, and equipment.

As milk is a complete food in nutrition list, it has led to increase its consumption around the world. But milk and milk products is an important vehicle of listeriosis infection when it is unpasteurized. Consumption of unpasteurized milk leading man towards more unhealthy, as raw milk has previously been suggested as a vehicle of transmission of listeriosis, pasteurized milk has not.

India is the largest milk producer of milk in the world. In India, the report of isolation of *L. monocytogenes* from milk has been revealed by [12]. However, systematic studies on raw milk samples for *L. monocytogenes* in the North East region of India are lacking. The aim of the present study was to evaluate the association between raw milk contaminations by *L. monocytogenes* and to identify the main risk related to management and to specify the critical control points that must be monitored in order to avoid milk contamination.

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