Isolation and Molecular Characterization of *Listeria monocytogenes* from Mutton and Paneer

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**ABSTRACT**

The present study was undertaken to study the occurrence and molecular characterization of *Listeria monocytogenes* from mutton and paneer sold at retail outlets of Kashmir. A total of 110 samples (mutton-60, paneer-50) were tested for the presence of *L. monocytogenes* using a two step enrichment procedure followed by plating on two selective agars (PALCAM and LSA). The organism was isolated from three mutton samples with an overall prevalence of 2.72%. The prevalence in mutton was found to be 5%. *L. monocytogenes* was not isolated from any of the paneer sample. The confirmation of the organism was done on the basis of biochemical tests and by Christie, Atkins and Munch Petersen (CAMP) test. The isolates were subjected to PCR assay for virulence associated with *hly A* gene. The pathogenicity of the isolates was confirmed by demonstration of monocytosis and kerato-conjunctivitis in rabbits. The isolates recovered were sensitive to gentamicin, doxycycline, enrofloxacin, amoxycillin/clavulanic acid, ciprofloxacin, oxytetracycline ampicillin/cloxicillin, oxytetracycline and resistant to streptomycin, cefodoxime, cefotaxime.

**Keywords:** Antibiogram, *Listeria monocytogenes*, molecular characterization, pathogenicity.

**Introduction**

Listeriosis, an important bacterial zoonosis is caused by an intracellular pathogen *Listeria monocytogenes*. *L. monocytogenes* is one of most virulent food-borne pathogens with case fatality rate of 20-30% (Ramaswamy *et al.*, 2007), which can even go up to 50% in neonatal population (MacGowan *et al.*, 1991; Baron *et al.*, 1994). Immune-compromised patients (HIV/AIDS infection), pregnant women, newborn babies, cancer patients and the elderly individuals are particularly susceptible to listeriosis (DiMaio, 2000; McLauchin *et al.*, 2004). *L. monocytogenes* is responsible for causing abortion, septicemia, meningitis, infertility, gastroenteritis and conjunctivitis in both humans and animals (Aureli *et al.*, 2000; Va’zquez-Boland *et al.*, 2001). *L. monocytogenes* is ubiquitous in environment and is found in decaying vegetation, soil, water and sewage. Food acts as a vehicle for 99% of human listeriosis cases (Mead *et al.*, 1999). Foods most frequently implicated include salads, sea-foods, meat and dairy products (WHO, 1988). The occurrence of the pathogenic *L. monocytogenes* has been reported in various food samples including meat and milk in India (Barbuddhe *et al.*, 2002; Chaudhari *et al.*, 2004). The public health concern of *L. monocytogenes* is further added by its resistance to antibiotics. The first multiresistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart-Salmeron *et al.*, 1990), thereafter, *L. monocytogenes* strains resistant to one or more antibiotics have been isolated (Charpentier *et al.*, 1995).
Material and Methods

Bacterial strains

The reference strains of *L. monocytogenes* (ATCC 19112) and *Staphylococcus aureus* (ATCC 11632) used in this study were procured from Hi-media (Mumbai).

Sampling

A total of 110 food samples comprising of mutton (60) and paneer (50) were collected aseptically from different retail outlets in and around Srinagar city (Jammu and Kashmir), during the period of October 2009 to March 2010. The samples were collected in UV sterilized polyethylene sachets and then transported to the laboratory immediately in ice packs and kept at 4°C until processing.

Isolation and biochemical identification

Isolation of *L. monocytogenes* from food samples was carried as per USDA protocol (McClain and Lee, 1988) with slight modifications. The primary enrichment was carried out in University of Vermont Medium (UVM), containing nalidixic acid and acriflavine and the secondary enrichment was carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine. Briefly, 25 g of food sample was thoroughly homogenized with 225 ml of UVM broth and the mixture was incubated at 30°C for 24 h. About 0.5 ml of the UVM enrichment was then transferred into 10 ml of Fraser broth and incubated at 37°C for 24 h. A loop full of culture from the Fraser broth was streaked on *Listeria* selective agar and incubated at 37°C for 24 h. Greenish-yellow colonies typical of *Listeria* spp. were considered positive. At least five presumptive colonies from *Listeria* selective agar were streaked on PALCAM agar and incubated at 37°C for 24-36 h for confirmation of *L. monocytogenes*.

The isolates were also subjected to standard biochemical tests such as catalase, oxidase, motility at 22°C and acid production from mannitol, rhamnose and xylose, nitrate reduction, urea hydrolysis, gelatin liquefaction, methyl red test and Voges-Proskauer test as per Cowan (1993) Steel (1993). *L. monocytogenes* isolates identified biochemically were tested for hemolytic activity by blood agar plate method and CAMP test with *S. aureus*.

Demonstration of monocytosis in rabbits and Anton test

*L. monocytogenes* grown in brain heart infusion broth (BHIB) at 22°C for 24 h was inoculated in three healthy New Zealand white rabbits via intra-peritoneal route. The blood was collected from the animals prior and following the inoculations. Blood smears were made at regular intervals of 24 h up to 120 h post inoculation and stained with Giemsa’s stain and observed for development of monocytosis. The isolates were also tested for development of kerato-conjunctivitis by instillation of approximately one million actively motile *L. monocytogenes* in the eye of two adult healthy New Zealand white rabbits. The rabbits were observed for development of kerato-conjunctivitis up to eight days following instillation.

Detection of virulence associated *hly A* gene

All the isolates confirmed by cultural, biochemical and in-vivo pathogenicity test were screened for the presence or absence of *Listeriolysin O (hly A)* gene as per Noterman et al. (1991a) with slight modifications.

DNA extraction: Isolates of *L. monocytogenes* were grown overnight in brain heart infusion broth at 37°C. Out of this broth culture, 0.5 ml culture was transformed to micro-centrifuge tube and centrifuged at 12000 rpm for 10 min. The supernatant was decanted off and pellet was resuspended in 500 µl of phosphate buffer saline solution (PBSS) and re-centrifuged at 12000 rpm for 10 min. The recovered pellet was resuspended in 100 µl of sterilized DNase and RNase free milliQ water. The pellet was suspended in the added water by vortexing and the lysis of the bacteria was performed by boiling the tube for 10 min, followed by snap chilling in crushed ice. The obtained lysate was utilized as template DNA in amplification reaction.
PCR amplification: A pair of forward and reverse primers (Table 1) were used for amplification of 456 bp region of the \textit{hly} A gene in 25 µl of reaction mixture containing 2.5 µl of lysate DNA, 2.5 µl of 10x PCR buffer, 0.5 µl of 10 mM dNTP, 2.0 µl of 25 mM MgCl$_2$, 3 µl of 10 ng/µl of the primers (forward + reverse), 0.1 µl of 5 U/µl Taq DNA polymerase and 14.4 µl MilliQ water. The amplification parameters included an initial denaturation at 94°C for 2 min, followed by 35 cycles each of denaturation at 94°C for 15 sec, annealing at 57°C for 30 sec, extension at 72°C for 1.30 min and a final extension at 72°C for 5 min. The PCR products were analyzed by submersive agarose gel electrophoresis to resolve the amplified DNA fragments of the target gene.

\textbf{Antibiotic sensitivity testing}

All the \textit{Listeria} isolates were subjected to antibiotic sensitivity by disc diffusion method as described by Bauer \textit{et al}. (1966). The following panel of antimicrobial discs and concentrations were used; enrofloxacin (10 mcg), streptomycin (10 mcg), amoxycillin (30 mcg), cephodoxime (10 mcg), amoxycillin/clavulanic acid (30 mcg), norfloxacin (10 mcg), oxytetracycline (30 mcg), cephotaxime (10 mcg), gentamicin (10 mcg), ampicillin (10 mcg), doxycycline (30 mcg), cephalexin (30 mcg), amikacin (10 mcg), ampicillin/cloxacillin (10 mcg), ciprofloxacin (30 mcg).

\textbf{Results and Discussion}

\textit{L. monocytogenes} has frequently been isolated from various foods of animal origin throughout the globe (Awaisheh, 2010; Vasiliev \textit{et al}.., 2010). In India, the presence of \textit{L. monocytogenes} in meat, milk and milk products has already been reported (Barbuddhe \textit{et al}.., 2002; Chaudhari \textit{et al}.., 2004). In our study, 110 food samples (mutton-60, paneer-50) were tested for the isolation and identification of \textit{L. monocytogenes}. The organism was isolated only from three mutton samples showing an overall prevalence 2.72%. The prevalence of \textit{L. monocytogenes} in mutton was 5%. Similar findings have also been observed by Zhou and Jiao (2006) in China; Jalali and Abedi (2008) in Iran; Little \textit{et al}. (2009) in United Kingdom and Staonsaovapak and Boonyaratanakornit (2010) in Bangkok. Latorre \textit{et al}. (2007) reported a lower prevalence of 0.3% in Italy, while Filiousis \textit{et al}. (2009) reported higher prevalence of 18% in

\begin{table}[h]
\centering
\caption{Details of the primers used for amplification of \textit{hly} A gene}
\begin{tabular}{|c|c|c|}
\hline
Target gene & Primer sequence & Product size (bp) & G+C content \\
\hline
\textit{hly} A Forward & 5'-GCAGTTGCAAGCCTTGGAGTGAA-3' & 456 & 54\% \\
Reverse & 5'-GCAACGTATCCTCCAGAGTGATCG-3' & & 54\% \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Time course of monocyte (%) increase and decrease in rabbits following intra-peritoneal inoculation of \textit{L. monocytogenes} (10$^8$ cells/ml/animal).}
\begin{tabular}{|c|c|c|c|c|}
\hline
Time (hrs) & Rabbit-1 & Rabbit-2 & Rabbit-3 & Average (mean) \\
\hline
0 & 2 & 2 & 2 & 2.00 \\
24 & 4 & 5 & 4 & 4.33 \\
48 & 5 & 6 & 6 & 5.66 \\
72 & 8 & 7 & 8 & 7.66 \\
96 & 5 & 5 & 6 & 5.33 \\
120 & 3 & 4 & 4 & 3.66 \\
\hline
\end{tabular}
\end{table}
Greece. *L. monocytogenes* could not be isolated from any of the samples of paneer under study. Failure to isolate the organism from paneer may be attributed to the method of making of paneer. Paneer is a type of cheese local to the Indian sub-continent prepared by coagulation of boiling milk with lime or tartaric acid. As *L. monocytogenes* is very sensitive to boiling and pasteurization temperatures, this organism may not survive in paneer. Failure to isolate *L. monocytogenes* from samples of cheese has also been reported by some workers (Kongo et al., 2006).

The pathogenic potential of *Listeria* isolates have been assessed by *in-vitro* pathogenicity tests like beta-hemolysis on sheep or horse blood agar (Schonberg et al., 1989) and Christie, Atkins, Munch- Peterson (CAMP) test (Mckellar, 1994).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Antimicrobial agents</th>
<th>Sensitive</th>
<th>Moderately sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gentamicin (10 mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>Doxycycline (30 mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3.</td>
<td>Oxytetracycline (30 mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>Ampicillin (10 mcg)</td>
<td>2/3 (66.7)</td>
<td>1/3 (33.3)</td>
<td>–</td>
</tr>
<tr>
<td>5.</td>
<td>Enrofloxacin (10mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6.</td>
<td>Amoxycillin (30 mcg)</td>
<td>1/3 (33.3)</td>
<td>2/3 (66.67)</td>
<td>–</td>
</tr>
<tr>
<td>7.</td>
<td>Streptomycin (10mcg)</td>
<td>–</td>
<td>–</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>8.</td>
<td>Amikacin (10 mcg)</td>
<td>–</td>
<td>1/3 (33.3)</td>
<td>2/3 (66.7)</td>
</tr>
<tr>
<td>9.</td>
<td>Cefpodoxime (10mcg)</td>
<td>–</td>
<td>–</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>10.</td>
<td>Amoxycillin/Clavulanic acid (30mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11.</td>
<td>Ciprofloxacin (30 mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12.</td>
<td>Norfloxacin (10mcg)</td>
<td>–</td>
<td>3/3 (100)</td>
<td>–</td>
</tr>
<tr>
<td>13.</td>
<td>Cephotoxime (10mcg)</td>
<td>–</td>
<td>–</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>14.</td>
<td>Cephalexin (30mcg)</td>
<td>–</td>
<td>3/3 (100)</td>
<td>–</td>
</tr>
<tr>
<td>15.</td>
<td>Ampicillin/Cloxacillin (10mcg)</td>
<td>3/3 (100)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Figures in parenthesis in columns 2, 3 and 4 indicate percentage.
Demonstration of beta haemolysis in sheep/horse blood agar has been used as a tool for differentiating pathogenic *Listeria* from non-pathogenic ones (Courteio, 1991). All the isolates in the present study produced a prominent zone of beta hemolysis in blood agar plates in 24-36 h, as well as showed positive CAMP test with *S. aureus*. Pathogenic nature of *L. monocytogenes* has been linked to the development of keratoconjunctivitis in laboratory animals like rabbits following instillation of live culture into the conjunctival sac. In the present study, 18 h old broth culture produced severe keratoconjunctivitis in rabbits within 2-5 days by intraocular route. Severe keratoconjunctivitis leading to fatal purulent meningitis in rabbits has been reported by some workers (Seeliger and Finger, 1976). On intra-peritoneal inoculation of *L. monocytogenes* in rabbits, there was increase in the monocyte count from 2% (Pre-inoculation level) to 8% (72 h post-inoculation). The results and the time course of monocytopsis increase and decrease in the rabbits following intra-peritoneal inoculation of approximately 10^8 cells/ml/animal are depicted in Table 2. Similar observations have been made previously (Radostitis *et al.*, 1994).

The isolates of *L. monocytogenes* were also screened for their virulent and non-virulent status by verifying the presence or absence of *Listeriolysin O* (*hly* A) gene. The isolates produced a strong amplification of the target gene thereby confirming the isolates as *L. monocytogenes* (Fig. 1). Several virulence associated genes have been sequenced and used as PCR-targets for the detection of *L. monocytogenes* e.g. *hly*A gene (Border *et al.*, 1990), *Dth 18* gene (Wernars *et al.*, 1991), *iap* gene (Jaton *et al.*, 1992) and the *plcA* gene (Notermans *et al.*, 1991b). *Listeriolysin O* encoded by *hly*A gene has been regarded as the most important virulence factor.

The isolates were 100% sensitive to gentamicin, doxycycline, enrofloxacin, amoxycillin/clavulanic acid, ciprofloxacin, oxytetracycline, ampicillin/cloxacillin and 66.7% for ampicillin. The isolates registered an intermediate response for norfloxacin (100%), cephalexin (100%) and amoxyccillin (66.67%). The isolates were, however, found resistant to streptomycin (100%), cefotaxime (100%), cephodoxime (100%) and amikacin (66.67%) (Table 3). While studying the antimicrobial sensitivity of *L. monocytogenes*, Vela *et al.* (1998) found erythromycin, doxycycline and ampicillin as the most effective antibiotics. Kiss *et al.* (2006) reported that *L. monocytogenes* isolated from foods and clinical samples in Hungary were sensitive to penicillin and aminoglycoside antibiotics or a combination thereof was found to be effective. Stonsaovapak and Boonyaratankonrit (2010) reported that the *Listeria* spp. isolated from foods in Bangkok, Thailand were sensitive to amoxyccillin, vancomycin, ampicillin, rifampicin and sulfamethoxazole and resistant to pencillin, chloramphenicol, and tetracycline. Variation in antibiotic susceptibility pattern of *L. monocytogenes* to different antibiotics could be due to strain variation and/or as a result of drug resistance due to indiscriminate use of antibiotics in veterinary and human practice in different geographical areas.

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