Isolation, Identification and Molecular Characterization of Listeria Species from Milk and Milk Products in Navsari City of South Gujarat

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ABSTRACT

The present study was undertaken with the objective of isolating and identifying Listeria spp. from milk and milk products sold at retail markets in the city of Navsari, Gujarat. A total of 100 samples comprising 50 milk and 50 milk product were collected aseptically from local market. Presumed Listeria spp. were subjected to battery of standard biochemical test for identification of Listeria spp. Cultures identified as Listeria monocytogenes were further subjected to detection of different virulence associated genes viz., inlA, inlB, plcA and plcB. Out of all the 100 samples tested 8 milk and 2 ice cream samples showed presence of Listeria spp. Species-wise 4 isolates of L. seeligeri, 3 isolates of L. monocytogenes and 1 isolate of L. welshimeri were obtained from milk samples and 2 isolates of L. welshimeri were obtained from ice cream samples. All the L. monocytogenes isolates confirmed on the basis of biochemical and in-vitro pathogenicity tests targeting inlA, inlB, plcA and plcB genes.

Keywords: Listeria species, milk and milk products, pathogenicity

Introduction

Foodborne listeriosis is an important cause of human food borne infections worldwide with high fatality rates (20–30%) compared with other food borne microbial pathogens (Liu, 2006). In mammals, L. monocytogenes can cause spontaneous abortions and is the cause of circling disease which is a manifestation of basilar meningitis. Symptoms of listeriosis range from flu-like illness to severe complications including meningitis, septicemia, spontaneous abortion or listeriosis of the newborn. The Listeria species are tolerant to extreme conditions such as low pH, low temperature and high salt conditions (Sleator et al., 2003; Liu et al., 2005). Therefore, they are found in a variety of environments, including soil, sewage, silage, water, effluents and foods. Healthy cattle can serve as reservoirs for L. monocytogenes, whereby the organism can be secreted in their milk. Milk can also become contaminated through accidental contact with faeces and silage. It is also documented that products prepared from raw milk contained L. monocytogenes (Schett et al., 2005). The consumption of raw milk or products made of raw milk has caused several listeriosis outbreaks resulting in several hundred cases (Pal and Awel, 2014).

The bacterium is a facultative intracellular pathogen that induces its own uptake into phagocytic and non-phagocytic cells and spreads from cell to cell using an actin-motility process. It enters the host primarily through the intestine with contaminated food then adheres to and then internalized by host cells with the assistance of a family of surface proteins called ‘internalins’ (Gaillard et al., 1991). inlA virulent gene codes for internalin A protein, which interacts with E-cathedrin and inlB, which codes for a 65-kDa protein recognizes C1q-R (or Met) mediate entry of L. monocytogenes into the host cell (Gaillard et al., 1991). The genes plcA encodes a phosphatidylinositol specific phospholipase C which along with plcB gene, helps in spreading the organism between cells (Sun et al., 1990).

Various foods have been implicated in the spread of L. monocytogenes, namely meat and meat products, raw milk, soft cheese, pasteurized dairy products including ice cream, fish and fish products and ready-to eat foods (Mahmood et al., 2003; Aurora et al., 2006; Shrinithivihahshini et al., 2011 and Rahimi et al., 2012).

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Keeping in view the above points in the present investigation an attempt was made to study occurrence of *Listeria* spp. and its virulence gene characterization from milk and milk products collected from markets/retail shops as well as roadside vendors in Navsari city of south Gujarat.

**Materials and Methods**

**Sample Collection**

A total of 100 samples, comprising milk (50), milk products *viz.*, ice cream (20), milk shake (15) and fruit salad (15) were collected from randomly selected retail shops and vendors from Navsari city, Gujarat.

**Isolation of *Listeria* species**

The FDA-BAM (Hitckins, 2001) method was used for isolation, wherein enrichment of 25 g. milk/milk product sample was carried out in 225 ml *Listeria* enrichment broth containing selective supplements (Hi Media Pvt. Ltd., Mumbai) at 35° ±1°C for 48 h followed, by plating on Oxford agar (Hi Media Pvt. Ltd., Mumbai) and incubation for 24-48 h at 37°C. The black luxuriant colonies surrounded by diffuse black zone were subjected to Gram’s staining, catalase test and oxidase test and characteristic Gram positive, coccobacillary or rod shaped organisms, which were catalase +ve and oxidase -ve, were sub-cultured in brain heart infusion broth at 25°C for 12-18 h. Cultures showing typical tumbling motility were considered as “presumptive” *Listeria* isolates, which were in turn subjected to a battery of biochemical test like MR, VP, nitrate reduction and sugar fermentation tests to identify isolates up to species level.

**In-vitro pathogenicity tests**

All the isolates were streaked on 5% sheep blood agar (SBA) and examined for zone of haemolysis surrounding the colonies. The Christie Atkins Munch-Peterson (CAMP) test was also performed using standard strains of *Staphylococcus aureus* (MTCC 3160) and *Rhodococcus equi* (MTCC 1135) to differentiate *L. monocytogenes* and *L. ivanovii*, wherein synergistic zone of hemolysis is observed with *S. aureus* and *L. monocytogenes* and *R. equi* and *L. ivanovii*, respectively.

**In vitro detection of virulence genes of *Listeria* isolates by PCR**

All the *Listeria* isolates were screened for the presence or absence of virulence associated genes by using the PCR protocols separately standardized for the detection of *inlA*, *inlB*, *pleA*, *pleB*, as per the methodology described by Jaradat *et al.* (2002) and Jallewar *et al.* (2007). The PCR protocol was standardized using *L. monocytogenes* (MTCC 1143).

The DNA of the *Listeria* spp. was extracted using the protocol of QIAIEN DNeasy blood and tissue kit with minor modification in centrifugation parameters. A loop-full of culture grown overnight in 4 ml BHI broth at 37°C was used to isolate the DNA as per the protocol provided in the kit for Gram positive bacteria employing lysis, wash and elution buffers using the DNeasy Mini spin column, wherein 5 µl of the eluted DNA was used as template in PCR reaction mixture.

To visualize the targeted PCR amplification, 5 µl of the PCR product was mixed with 1 µl of 6x gel loading buffer and electrophoresed along with DNA molecular weight marker (3B BlackBio Biotech) on 2.0 per cent agarose gel containing Ethidium bromide (@ 0.5 µg/ml) at 5 V.cm for 60 min in 0.5x TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, Gene Genius BiImaging System, UK).

**Results and Discussions**

Microbiological and biochemical analysis of a total 100 samples resulted in isolation of 10 (10%) *Listeria* spp. of which 8 were isolated from milk and 2 from milk products. Out of 8 positive milk samples 4 samples turned out to be positive for *L. seeligeri*, 3 for *L. monocytogenes* and 1 for *L. welshimeri*. The overall prevalence of *L. monocytogenes* detected from milk samples in this study was 6%, which is in congruence to results of Beigh *et al.* (2014) who also reported same 6% prevalence of *Listeria* spp. from milk with the isolation of one isolate of *L. monocytogenes*; Bhilegaonkar *et al.* (1997) who reported 5.78% (7/121) prevalence of *L. monocytogenes* in farm and bulk milk samples; Barbuddhe *et al.* (2002) who found 6.25% out of 64 milk samples analyzed positive for *L. monocytogenes*, Van-Kessel *et al.* (2005) who observed 6.5% cent from 861 milk samples as well as Waghmare (2006) who recorded 5.88% prevalence of *L. monocytogenes* from raw milk samples. Sharma *et al.* (2012) also recovered 25 (21.7%) isolates of *L. monocytogenes* from 115 raw cattle milk samples and Shrinitivahashini *et al.* (2011) found 60.6% of milk samples positive for *L. monocytogenes* out of 134 samples of various foods analyzed. These values are higher than those found in the present study, indicative of lower degree of contamination of milk in the area under study.

Examination of 50 milk product samples comprising of 20 ice cream, 15 milk shake and 15 fruit salad samples showed the presence of 2 *Listeria* spp. in ice cream and both of them were identified as *L. welshimeri* (Table 3). *L. seeligeri* and *L. welshimeri* were recovered from 4 (8%) and 1 (2%) milk samples, respectively, as depicted in Table 4.
Table 1. Primer pairs used in virulence characterization of *L. monocytogenes*

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Primer sequence (5'-3')</th>
<th>Length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>inlB</em></td>
<td>F: AAA GCA CGA TTT CAT GGG AG R: ACA TAG CCT TGT TTG GTC GG</td>
<td>146</td>
<td>Ericsson et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>plcB</em></td>
<td>F: GGG AAA TTT GAC ACA GCG TT R: ATT TTC GGG TAG TCC GCT TT</td>
<td>261</td>
<td>Vazquez-Boland et al. (1992)</td>
</tr>
</tbody>
</table>

Table 2. Steps and conditions of thermal cycling for different primer pairs

<table>
<thead>
<tr>
<th>Primers (Forward and Reverse)</th>
<th>Cycling conditions</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>inlA</em>(F) <em>inlA</em>(R)</td>
<td>94°C, 3 min</td>
<td>94°C, 1 min</td>
<td>54°C, 2 min Repeated for 35 cycles</td>
<td>72°C, 1 min</td>
<td>72°C, 10 min</td>
</tr>
<tr>
<td><em>inlB</em>(F) <em>inlB</em>(R)</td>
<td>94°C, 3 min</td>
<td>94°C, 1 min</td>
<td>50°C, 2 min Repeated for 35 cycles</td>
<td>72°C, 1 min</td>
<td>72°C, 10 min</td>
</tr>
<tr>
<td><em>plcB</em>(F) <em>plcB</em>(R)</td>
<td>94°C, 3 min</td>
<td>94°C, 1 min</td>
<td>52°C, 2 min Repeated for 35 cycles</td>
<td>72°C, 1 min</td>
<td>72°C, 10 min</td>
</tr>
<tr>
<td><em>plcA</em>(F) <em>plcA</em>(R)</td>
<td>95°C, 2 min</td>
<td>95°C, 15 sec</td>
<td>60°C, 30 sec Repeated for 35 cycles</td>
<td>72°C, 1.5 min</td>
<td>72°C, 10 min</td>
</tr>
</tbody>
</table>

Table 3. Isolation of *Listeria* spp. from milk and milk products

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Number of samples analyzed</th>
<th>No. of samples positive for <em>Listeria</em> spp.</th>
<th>Total positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>L. monocytogenes</em></td>
<td><em>L. seeligeri</em></td>
</tr>
<tr>
<td>Cow milk</td>
<td>25</td>
<td>1 (4%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Buffalo milk</td>
<td>25</td>
<td>2 (8%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Total</td>
<td>M =50</td>
<td>3.50 (6%)</td>
<td>4.50 (8%)</td>
</tr>
<tr>
<td>Ice cream</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Milk shake</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fruit salad</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>MP = 50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

M-Milk; MP-Milk product

Table 4. Hemolysis and CAMP test pattern of *Listeria* spp. on SBA

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th><em>Listeria</em> spp.</th>
<th>Haemolysis</th>
<th>CAMP test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
<td>+</td>
<td>R. equi</td>
</tr>
<tr>
<td></td>
<td><em>L. seeligeri</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>L. welshimeri</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Though they have value concerned with shelf life of milk, their entry in the milk might be from environmental sources. Findings of present work are high compared to 0.1 per cent prevalence each of *L. seeligeri* and *L. welshimeri* obtained by Kalorey et al. (2008) from 2060 milk samples. Moura et al. (1993) also reported 0.9 per cent prevalence of *L. welshimeri* in milk samples. Variation in the results of present study might be due to comparatively small number of the milk samples. The prevalence of *Listeria* spp. in ice cream i.e. 4% in the present study was much lower as compared to those observed by Molla et al. (2004) as 43.5% out of 137 ice cream samples. Shrinithivihashini et al. (2011) who observed 41.7% *Listeria* spp. in ice cream from 134 food samples analyzed and Rahimi et al. (2012)
recorded 16.7% prevalence in ice-cream from 290 samples of traditional milk products thereby demanding care in preparation of the milk product to protect health of consumers. Comparatively lower degrees of incidence had been recorded by Moharram (2007), Muhammed Wosila et al. (2013) and Beak et al. (2000) in ice cream samples viz., 5%, 6% and 6.1%, respectively; which is indicative of marginally safe food hygiene practices might have followed at places under the studies. In India, isolation of L. monocytogenes from milk and milk products is comparatively low suggesting low rate of contamination (Bhilegaonkar et al., 1997; Aurora et al., 2006 and Parihar, 2008).

Results of in vitro pathogenicity tests showed that L. monocytogenes and L. seeligeri produced hemolysis on SBA, whereas L. welshimeri was negative for hemolysis similar to the earlier records of Seeliger (1981), Farber and Peterkin (1991), McAulchlin (1997) and Blanco et al. (2008). The CAMP test performed using the Listeria isolates and the S. aureus or R. equi. The haemolytic listerial isolates in CAMP test showed enhancement of haemolytic zone and developed purulent conjunctivitis in Anton’s test (Beigh et al., 2014).

In the present study, all the 3 isolates identified as L. monocytogenes on the basis of biochemical analysis revealed amplification of targeted virulence associated genes viz., inlA, inlB, plcA and plcB genes (Fig. 1 and 2), which yielded a desired amplified product of 255 bp for inlA, 146 bp similar to the primer pair for inlB (Fig. 1), 1484 bp for plcA and 261 bp for plcB gene (Fig. 2) as reported by Jaradat et al. (2002), Jallewar et al. (2007), Rawool et al. (2007), Nayak et al. (2010), Momtaz (2013) and Soni et al. (2013) from various samples.

Fig.1. Agarose gel showing PCR amplified product of 255 bp for inlA and 146 bp for inlB genes in L. monocytogenes isolates. Lane 1: Positive control (MTCC 1143 L. monocytogenes strain); Lane 2-4: Samples positive for inlA; Lane 5: Negative control; Lane M: 50 bp DNA ladder; Lane 6: Negative control; Lane 7-9: Samples positive for inlB; Lane 10: Positive control (MTCC 1143 L. monocytogenes strain).

Fig.2. Agarose gel showing PCR amplified product of 148 bp for plcA and 261 bp for plcB genes in L. monocytogenes isolates. Lane 1: Negative control; Lane M1: 100 bp DNA ladder; Lane 2-4: Samples positive for plcA; Lane 5: Positive control (MTCC 1143 L. monocytogenes strain); Lane 6: Positive control (MTCC 1143 L. monocytogenes strain); Lane 7: Negative control; Lane 8-10: Samples positive for plcB; Lane 11: 50 bp DNA ladder.

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