Prevalence of *Listeria monocytogenes* in Foods of Animal Origin: Study from Assam, A North-Eastern State of India

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**ABSTRACT**

Listeriosis is an important emerging bacterial food-borne zoonotic disease caused by *Listeria monocytogenes*. During the last two decades, the organism has been implicated in several food-borne disease outbreaks making it a serious public health hazard. The pathogenic strains of Listeria can cause severe illness in man after the ingestion of contaminated food products, especially the refrigerated foods of animal origin. In India, very few studies have been carried out in respect to the occurrence and incidence of *L. monocytogenes*. Assam, one of the eight states of Northeastern India, where approximately 79.0 percent of the total population are non-vegetarians. Chicken meat, pork, chevon and beef are exclusively taken as regular food items throughout the state. The present study describes about isolation, identification and molecular characterization of *L. monocytogenes* from different foods of animal origin from Assam, India. A total of 750 different meat (chicken, chevon, pork and beef) and milk and their products (curd and paneer) samples were collected and analyzed for presence of Listeria. Isolation of *L. monocytogenes* was done from different food samples of animal origin by using selective enrichment method. PCR was employed for confirmation of the suspected cultures of Listeria targeting *psr* and 16SrRNA4gene. Upon confirmation, the isolates were further characterized based on 16S rRNA gene and phylogenetic analysis was performed. Of 750 samples, 48 (6.4%) samples were found positive. The highest rate of isolation of Listeria spp. was recorded in raw milk (8.95%) followed by curd (6.58%), chevon (5.36%), chicken (5.74%), pork (5.98%) and beef (3.75%). Molecular characterization of the isolates based on 16S rRNA gene of *L. monocytogenes* isolated from foods of animal origin from Assam with that of other sequences reported from different parts of the world revealed that all the isolates of the present study are clustered along with other *L. monocytogenes* isolates reported from different parts of the world, however, showing more closely related to isolates reported from China.

**Keywords:** Assam, detection, food borne, foods of animal origin, India *L. monocytogenes*, prevalence, zoonosis.

I. INTRODUCTION

Listeriosis is an important emerging bacterial food-borne zoonotic disease caused by *Listeria monocytogenes* (*L. monocytogenes*). Although *L. monocytogenes* has been recognized as a human pathogen way back in 1929, its recognition as a food-borne pathogen in 1981 [1] is relatively...
new. During the last two decades, the organism has been implicated in several food-borne disease outbreaks. Of late, it has emerged as an important food-borne pathogen and a nagging health concern following large outbreaks in North America and Europe [2]. This organism is ubiquitously found in environments such as intestines of healthy humans or animals, household environment, food products, soil, water and silage or other decaying vegetation [3], [4]. Foods act as a vehicle for 99 percent of human listeriosis cases [5]. Foods most frequently implicated include salad, sea food, meat and dairy products [6]. Occurrence of the pathogenic L. monocytogenes has been reported in various food samples including meat and milk in India [5], [7], [8]. Traditional isolation and identification procedures are the most widely used methods for the detection of the organism from the food. Even though detection of L. monocytogenes from food can be achieved most authentically by cultural methods, these are time consuming and laborious procedures. To overcome these shortcomings, a number of immunological and nucleic acid-based methods have been described for the rapid identification of L. monocytogenes from food [9]. Among them, polymerase chain reaction (PCR) based methods are being increasingly used in food microbiology for the detection of food-borne pathogens. However, the routine use of PCR for direct detection of pathogen in food is modest because of various PCR-inhibitory components in meat and media [10]. Moreover, the presence of low level of L. monocytogenes (<100 cfu/g) in food [11] and false positive reactions because of nucleic-acid amplifications of dead organisms add to the drawbacks of using PCR in the detection of food-borne pathogens.

Assam, a north-eastern state of India having majority of its population as non-vegetarian, there is every possibility of getting such food borne infections to human through meat. The slaughter houses/ butchers also do not maintain proper hygienic measures which again pose a threat to the entire non-vegetarian people of Assam. Reports on prevalence of Listeria in foods of animal origin are very few [12], [13]. This report describes about detection and prevalence of Listeria monocytogenes from foods of animal origin that are retailed in greater Guwahati, a major city of Assam.

II. METHODOLOGY

A. Study Area and Collection of Samples

The study was conducted in and around Guwahati (26.1445° N, 91.7362° E), a major city in Assam, a northeastern state of India. A total of 750 samples consisting of different meat (chicken n=122, chevon n=112, pork n=117 and beef n=80), raw milk (n=190) and milk products (curd n=76 and paneer n=53) were collected aseptically from different retail shops. The samples (approximately 150g) were collected in sterilized polyethylene zip sachets and brought to the laboratory under complete cold chain for microbiological analysis.

B. Reference Strain

Reference strains of L. monocytogenes (MTCC 1143) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The strains were maintained by continuous passaging and periodically tested for purity, morphology and biochemical characteristics.

C. Isolation and Identification of L. monocytogenes

Isolation of L. monocytogenes was done from different food samples of animal origin by using selective enrichment method [14]. Briefly, samples (25g/ or 25ml) were added to 45 ml of PolymixinAcriflavin Lithium Chloride Ceftazidime Ascinul Mannitol (PALCAM broth, HiMedia) containing listeria supplement, mixed well and incubated at 30°C for 24 hours. A loopful of broth was then streaked on to PALCAM agar plate and incubated at 30°C for 24 h. The grey greenish colonies with sunken centres surrounded by black zone of ascinul hydrolysis were suspected to be Listeria. Motility, Gram’s staining, oxidase and catalase tests were performed for preliminary conformation of organism. The typical colonies of listeriae, which revealed Gram-positive cocci, were subcultured in Brain Heart Infusion (BHI, Himedia) broth at 25°C overnight. The isolates with characteristic tumbling motility at 25°C, catalase-positivity and oxidase-negativity were further characterized biochemically and tested for their pathogenicity as per standard method [15].

D. Detection of L. monocytogenes by PCR

Confirmation of the suspected cultures of L. monocytogenes isolated from meat/milk or other dairy products were further confirmed using polymerase chain reaction targeting amplification of two genes viz: prs gene and 16s rRNA gene using specific published primers. Details of the primers for each gene are presented in Table I.

E. Preparation of Bacterial Lyase

For molecular detection of genes in the L. monocytogenes by PCR, the bacterial cell lyase was prepared from each isolate as per the method of [16]. Two to three pure culture colonies of L. monocytogenes were inoculated in to about 5 ml of Luria Bertani (LB) broth and incubated overnight at 37°C in a shaking incubator. After overnight incubation, the growth was tested for its purity and was centrifuged at 10,000 xg for 5 min. The pellet collected after centrifugation was resuspended in 75 μl of 1X Tris EDTA (TE) buffer and the whole volume was transferred to a 1.5 ml sterile microcentrifuge tube. Bacterial suspensions were placed at 100°C for 20 min by keeping the tube in a floator in water bath. After boiling, the micro-centrifuge tubes with the suspension were immediately kept in crushed ice for 20 min to impart cold shock. The tubes were then thawed and subjected to high speed centrifugation at 12,000 xg for 10 min and the clear

TABLE I: PRIMERS USED FOR DETECTION OF DIFFERENT GENES IN L. monocytogenes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>prs</td>
<td>F:5’GCTGAAGAGATTTGCAGAAAGAAG3’&lt;br&gt;R:5’CACAAAGAACCTTGGATTGGCGG3’&lt;br&gt;GTTGCAGCCTA3’</td>
<td>370</td>
<td>[17]</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>F:5’GGACGGGGGCTAATTGCTGAA3’&lt;br&gt;R:5’TTCATGTAGCGGCTGGTGGCAGCCTA3’</td>
<td>1200</td>
<td>[18]</td>
</tr>
</tbody>
</table>

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supernatant fluids (lysates) containing the bacterial genomic DNA were collected without disturbing the pellets. The collected lysates were used as template DNA for PCR.

F. Polymerase Chain Reaction (PCR)

Amplification of bacterial DNA for detection of prs and 16S rRNA genes of L. monocytogenes was performed in a thermal cycler (Bio-Rad) in 25µl volume containing 4µl of the prepared sample (lysate) as template, 0.5µM of forward and reverse primer (Table I), 1X PCR mastermix (containing 0.05 unit/ µl Taq DNA polymerase in reaction buffer, 0.4 mM each of dATP, dCTP, dTTP and dGTP, 4 mM MgCl2; PCR buffer) and nuclease free water up to 25 µl. Thermal cycling conditions for amplification of various genes of Listeria were followed as per standard reference [17, 18].

The PCR product obtained was subjected to electrophoresis in 1.5% agarose gel. Containing ethidium bromide (10mg/ml). Electrophoresis is carried out using 1X Tris Acetate EDTA buffer at 100 volts for 60 minutes. After running, the gel was viewed and captured with Gel doc system (DNRS systems).

G. In vitro Pathogenicity Assay

The bacterial isolates of L. monocytogenes identified based on morphological, biochemical characteristics as well as PCR were subjected to in-vitro pathogenicity test for their characterization as pathogenic or non-pathogenic isolates. Three types of pathogenicity assay were performed viz. hemolysis on sheep blood agar, CAMP test and Phosphatidylinositol-specific phospholipase C (PI-PLC) Assay following standard methods [19].

H. Molecular Characterization of L. monocytogenes Isolates

The PCR amplicons of 16S rRNA gene of L. monocytogenes were purified and cloned into pGEMT easy (Promega) vector. The positive clones were selected and sequenced commercially at Xcelris Genomics Limited, Ahmedabad, India. The generated sequences were edited manually and analyzed using MEGA 5.0 software [20]. The 16S rRNA gene sequences of L. monocytogenes reported from different parts of the world were retrieved from the GenBank database and phylogenetic tree was constructed.

III. RESULTS

Listeria monocytogenes is an important emerging bacterial food-borne zoonotic pathogen responsible for causing abortion, septicemia, meningitis, infertility, gastro-enteritis and conjunctivitis in both man and animals. In the present study, out of 750 samples comprising raw milk, curd, paneer, chicken, chevon, pork and beef subjected to bacteriological examination (Table II), overall 48 (6.40%) samples were found positive for Listeria spp. The highest rate of isolation of Listeria spp. was recorded in raw milk (8.95%) followed by curd (6.58%), chevon (5.36%), chicken (5.74%), pork (5.98%) and beef (3.75%). All the 48 isolates showed morphological (Fig. 1), staining and biochemical characteristics typical for Listeria. Biochemically, the organisms fermented rhamnose and mannose but did not ferment xylose and mannitol. All the strains were motile and found positive for catalase, CAMP test and haemolysin production, and negative for oxidase test (data not shown). Further all 48 isolates were confirmed as L. monocytogenes based on PCR amplification of prs gene, while 43 isolates showed positive results in 16s rRNA gene. On agaroase gel electrophoresis, all the genes resulted in specific amplification product of expected 370bp (prs gene) and 1200bp (16s rRNA gene) respectively (Fig. 2A & 2B). The ability to accurately identify Listeria is important in assuring the safety of food products. Detection of Listeria isolates is essential by using the prs and 16s rRNA gene specific primers at genus as well as species level to rapidly identify the organism. In the present study, prs gene specific primer for Listeria spp. was used for confirmation of the organisms.

A. In-vitro Pathogenicity Detection of L. monocytogenes

In the present investigation, the in-vitro detection of virulence factors of L. monocytogenes isolated from foods of animal origin was carried out by using CAMP test, test for hemolysis production and PI-PLC. A total of 43 isolates subjected to the above three tests were comprised of 17 from milk, 5 from curd, 3 from paneer, 7 from chicken, 5 from chevon, 4 from pork and 2 from beef. All the 43 samples showed positive results for CAMP test (Fig.3) and test for hemolysin production (Fig.4). However, no isolates from paneer, chicken, pork and beef were found to be positive for PI-PLC, but 3 (out of 17), 1 (out of 3) and 2 (out of 5) isolates from milk, curd and cheeseon respectively, showed positive result for the test (Table III).

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of sample (s)</th>
<th>Number of samples positive for Listeria spp.</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>190</td>
<td>17</td>
<td>8.95</td>
</tr>
<tr>
<td>Curd</td>
<td>76</td>
<td>5</td>
<td>6.58</td>
</tr>
<tr>
<td>Paneer</td>
<td>53</td>
<td>3</td>
<td>5.66</td>
</tr>
<tr>
<td>Chicken</td>
<td>122</td>
<td>7</td>
<td>5.74</td>
</tr>
<tr>
<td>Chevon</td>
<td>112</td>
<td>6</td>
<td>5.36</td>
</tr>
<tr>
<td>Pork</td>
<td>117</td>
<td>7</td>
<td>5.98</td>
</tr>
<tr>
<td>Beef</td>
<td>80</td>
<td>3</td>
<td>3.75</td>
</tr>
<tr>
<td>Total</td>
<td>750</td>
<td>48</td>
<td>6.40</td>
</tr>
</tbody>
</table>

Fig. 1. Typical colonies of L. monocytogenes on palcam agar.

B. Molecular Characterization of L. monocytogenes

During the present investigation, the 16s rRNA gene from representative isolates of L. monocytogenes (one representative isolate each from all types of foods of animal origin considered for study) were sequenced and phylogenetic study was carried out. The generated sequences were edited and submitted to GenBank (Accession nos. KM010016, KM010017, KM010018, KM010019,
KM010020, KM010021, and KM010022). Phylogenetic analysis was performed using MEGA 6.0 software by using neighbour joining method. Alignment of 16S rRNA gene sequences of the 7 (seven) *L. monocytogenes* isolates of the present study were compared with related sequences retrieved from GenBank database. The published sequences were reported from China (FJ774227, FJ774230, JF967620, JF967623), Canada (EU090894), USA (JQ901483), France (AJ535701) and Tezpur, Assam (KF894986).

On phylogenetic analysis (Fig.5) of 16S rRNA gene of *L. monocytogenes* isolated from foods of animal origin from Assam with that of other sequences reported from different parts of the world revealed that all the isolates of the present study are clustered along with other *L. monocytogenes* isolates reported from different parts of the world. Within the isolates of the present study, they shared a homology percentage ranging from 91.7-96% at nucleotide level. It was evident from the analysis that, the *L. monocytogenes* isolates of the present study from milk (Accession. No KM010016) and chicken (Accession. No KM010020) are closely related to KF894986, a previously reported isolate from Assam. On the other hand, other isolates viz. isolates from beef (Accession. No KM010017), chevon (Accession. No KM010018), paneer (Accession. No KM010019) and pork (Accession. No KM010021) are showing closer relationship with isolates reported from China. It was interesting to note that, the *L. monocytogenes* isolate from curd (Accession. No KM010022) in the present study is placed distantly from the other isolates forming a separate clade in the phylogenetic tree. This may be due to mutation in the bacterial isolate

<table>
<thead>
<tr>
<th>Source</th>
<th>Total number of isolates tested</th>
<th>Number of isolates showing positive result to (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Curd</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Paneer</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Chicken</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Chevon</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pork</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Beef</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

**TABLE III: IN VITRO DETECTION OF VIRULENCE FACTORS IN L. MONOCYTGENES ISOLATED FROM FOODS OF ANIMAL ORIGIN**

Fig.2. Amplification various genes of *L.* *monocytogens* by PCR. (a) Amplification of *prs* gene of *Listeria* isolates (370 bp); M: 100 bp Marker, Lane 1- 10: *Listeria* isolate, Lane11: Positive control, Lane 12: Negative control; (b) Amplification of 16S rRNA gene of *Listeria* isolates (1200bp); M: 100 bp plus Marker, Lane 1- 8: *Listeria* isolate, Lane 9: Positive control, Lane 10: Negative control.

Fig.3. *L. monocytogenes* isolate showing positive for CAMP test.

Fig.4. *L. monocytogenes* isolate showing haemolysin production on sheep blood agar.
IV. DISCUSSION

Listeria monocytogenes has become an important food borne pathogen and it can be found in a variety of foods which include raw foods and processed foods. Prevalence of Listeria in meat, milk and related dairy products has been well documented by various workers. In milk and milk products, a prevalence rate ranging from 3-10% have been reported [21]-[25]. Prevalence of a higher percentage of Listeria in milk and milk products in the present study is also in accordance with the earlier reports. The source of contamination of L. monocytogenes in raw milk is mostly from the gastrointestinal tract of animals, environment and skin of teat [26] and shedding of Listeria in to milk is due to chronic mastitis [27]. Similarly, various workers have reported presence of L monocytogenes in meat, both fresh as well as processed meat of different species of animals with varying levels. For example from beef and pork [28] a prevalence rate of 22. 94% have been reported, while a lower prevalence of L. monocytogenes (16.6%) in various foods of animal origin has been recorded [29] with highest prevalence in milk, as recorded in the present study.

Presence of L. monocytogenes in chicken meat, both fresh as well as processed meat have been well documented. However, the detection level of L. monocytogenes are found to vary greatly from place to place. From India a very high level (25%) of L. monocytogenes in fresh chicken meat has been reported [4] but a comparatively lower percent (8.5%) was reported from Meghalaya [13]. Another researcher reported the distribution of L. monocytogenes as 8.33% in chicken meat from Bangladesh [30]. The findings of the present study is lower with regard to prevalence of L. monocytogenes on fresh chicken meat in Guwahati city, Assam.

Presence of L. monocytogenes in food samples can be achieved by using both conventional as well as molecular methods. However, the conventional method is simple, sensitive, and inexpensive if compared with molecular methods [31], [32]. But conventional methods are laborious and time consuming as they require more than a week for the detection and confirmation of pathogen [32]-[34]. Recent advances in molecular technology and molecular methods have led to identify these techniques as an alternative to culture and serological methods for food testing [35].

Reference[36] reported that out of 188 number of Listeria spp. isolates, 92 were L monocytogenes including 7 non haemolytic and PI-PLC negative strains. They also reported that PI-PLC production is correlated with hemolysis except for four haemolytic, PI-PLC strains. Haemolytic L. monocytogenes isolates lacking the plcA gene and PI-PLC activity were deemed non-pathogenic when assessed by mouse and chick embryo inoculation tests, in spite of having the hlyA gene[37]. The mechanism of pathogenicity of L. monocytogenes is usually associated with haemolysin production [38].The haemolytic activity of L. monocytogenes and the level of haemolysin production may be dependent on enrichment procedures, selective media, temperature and virulence of the bacteria [11], [19]. A correlation between the activity of the enzyme PI-PLC and the virulence of L. monocytogenes has been demonstrated by in vivo pathogenesis test [39]-[41]. L. monocytogenes isolates in the present study found positive for CAMP test can not be confirmed as pathogenic as the CAMP test is not sufficient to indicate whether the isolate is virulent or not [11]. However, on the basis of haemolysin production, all the 43 isolates could be considered as pathogenic. Since phospholipases are essential determinants of pathogenicity for L. monocytogenes[42]. Therefore, the isolates (n=6, Table III) which were found positive for PI-PLC test along with other pathogenicity tests in the present study could be considered as virulent and may be regarded as potential threat to public health.

DNA sequencing and phylogenetic analysis have been used by many workers to identify Listeria spp. in various food samples. A number of genes of Listeria spp have been targeted and studied for the purpose. Polymorphism of rRNA genes is commonly used to characterize bacterial species [43]. The 16S RNA gene of Listeria spp. is one of the important gene for molecular characterization of the isolates [44], [45]. Similarly, [46] studied the phylogenetic analysis of 16S rRNA gene sequence of L. monocytogenes isolated from chicken, beef and seafoods and reported the phylogenetic distinctiveness and phenotypic disparities within the species. In the current study, all the L. monocytogenes isolates from foods of animal origin showed the genetic similarity with that of isolates reported from China, Canada, France, USA and Tezpur, Assam. Assam, one of the 8 states of north eastern part of India strategically shares porous international boundaries with neighbouring countries. The present finding suggests the circulation of pathogenic strains of L. monocytogenes strains in the foods of animal origin in Assam or introduction of these pathogens due to transboundary transport of food and animal products to Assam making the public vulnerable to infection.

V. CONCLUSION

Listeria monocytogenes considered as a highly pathogenic bacterium that contaminate a wide range of food products with a high mortality rate in the world. Presence of L. monocytogenes in foods of animal origin are undesirable but unavoidable. There is very little information available on prevalence of Listeria in foods of animal origin from Assam. Presence of Listeria monocytogenes in foods of animal origin as recorded in the present study is a public health concern and needs due attention. The findings of the present study will serve as baseline information on this aspect. Detailed molecular epidemiological studies can provide further insights into the actual status and it will be helpful to adopt adequate managemental strategies to prevent and control the same. Also, further study on the processing method to reduce and eliminate this kind of bacteria from foods of animal origin before consumption is necessary.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest exists.


O’Donnell ET. The incidence of Salmonella and Listeria in raw milk from...