


Prevalence and Virulence Gene Profiling of *Listeria monocytogenes* from Fish and Meat Samples from Aizawl, Mizoram

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Abstract

This survey aimed to study the occurrence of *Listeria* species in fish and meat samples and characterization of their virulence genes. Over all, *Listeria* spp. was found in 25.22% samples out of which 9.0% and 16.21% were *L. monocytogenes* and *L. innocua*, respectively. *L. monocytogenes* (n=10) belonged to 4b, 4d and 4e serovars. All the isolates revealed presence of virulence genes- *plcA* and *iap*, while *plcB* gene was also present in 90% of the isolates. The occurrence of *L. monocytogenes* in samples shows cogent evidence for their zoonotic potential and has public health significance.

Keywords: *Listeria* spp., Prevalence, Serotypes, Virulence gene, Antimicrobials, Susceptibility

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INTRODUCTION

The genus *Listeria* is facultatively anaerobic, rod-shaped bacteria and dispersed in food, environmental and clinical samples¹. *L. monocytogenes* is a known pathogen, whereas the pathogenicity of *L. seeligeri*, *L. ivanovii* and *L. innocua* have been less documented². *Listeria monocytogenes* is disseminated from animals to humans through fecal-oral route. The fatality rate is higher (30 to 75%) especially in pregnant women, neonates, aged people and people with grievous underlying disease conditions like immune-suppression³. Ninety percent of cases of *Listeria* contamination due to use of contaminated food products are reported⁴.

Listeria monocytogenes was differentiated into 13 serotypes⁵. Only serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for 95% of human illnesses⁶. In 2012 in the US, serotypes 4b (54%) and 1/2a (28%) were most commonly identified⁷. Many virulence determinants act as essential factors for the pathogenesis of *L. monocytogenes*, including *hlyA*, *inlA*, *inlB*, *inlC*, *inlJ*, *actA*, *iap* and *plcA* gene⁸. The *hlyA* gene is the main virulence determinant in *L. monocytogenes*; a hemolysin gene. The pore-forming cytolysin listeriolysin O is encoded by this *hlyA* gene. The product of *hlyA* is the first virulence factor which play a vital role in the *L. monocytogenes* pathogenesis, helps in intracellular parasitism⁹.

Little information is available on *Listeria* contamination in animal originated foods from North-East India. This survey aims to estimate the *Listeria* spp., identify the main serotypes and to screen the *L. monocytogenes* isolates for presence of different virulent genes.

MATERIALS AND METHODS

Collection of Samples

One hundred and eleven (111) samples, including fish gill, fish intestine and beef (15 each), chicken and pork (24 each) and mutton (18) were collected from various markets in Aizawl, Mizoram, a hilly state in the North-Eastern region India. The samples were collected aseptically by maintaining cold chain and were processed for microbiological analysis within 24 h. This study was conducted for a period of one year from July 2018 to June 2019.

Isolation of *Listeria*

For isolation of *Listeria*, ISO 11290 method was used. Each sample was inoculated aseptically in Half Fraser Broth followed by incubation at 30°C. It was subcultured in Fraser broth, further streaked onto PALCAM agar and incubated at 37°C. The grey-green colonies on PALCAM agar with diffuse black-zone was plated on Tryptone Soya Yeast Extract agar. Gram staining and Biochemical tests comprising catalase test, fermentation and acid production from various sugars, followed by MR/VP tests were performed for identification of the isolates. All the *Listeria* isolates and the control strain were preserved in nutrient agar stab (with 1.5% agar agar) and 16% glycerol for subsequent analysis. *L. monocytogenes* ATCC 13832 was utilized as the standard strain.

Confirmation of *Listeria* by PCR

Listeria were cultured in Brain Heart Infusion Broth for overnight for extraction of genomic DNA using Bacterial DNA isolation kit (Genaid, Taiwan). To confirm the suspected isolates, PCR was performed by amplifying *Listeria* genus-specific *prs* gene using specific primer¹⁰ (1st BASE, Malaysia) (Table 1). To confirm *L. monocytogenes* isolates, primers specific for

Table 1. Primers used for identification of *Listeria* species

| Species | Target Gene | Primers | Sequences | Product Size (bp) | References |
|-------------------------|-------------|------------------|---|-------------------|--------------------------------------|
| Genus <i>Listeria</i> | <i>prs</i> | prs-F prs-R | 5'-GCTGAAGAGATTGCGAA-3' 5'-AGAAGCAAAGAAACCTTGG ATTGCGG-3' | 370 | Michel <i>et al.</i> ¹⁰ |
| <i>L. monocytogenes</i> | <i>hlyA</i> | hlyA-F hlyA-R | 5'-GCTTTTGACGCTGCCGTAAG-3' 5'-GCAACGTATCCTCCAGAGTGATCG-3' | 335 | Designed. |
| <i>L. innocua</i> | <i>in9</i> | in9-F in9-R | 5'-GGCTTCAGCGATTCTCCG-3' 5'-GCCCGATTTCCTCACTGTCTAA-3' | 421 | Tingting <i>et al.</i> ²⁴ |

Table 2. Primers used for identification of *L. monocytogenes* serogroups

| Target Gene | Primer | Sequences | Product Size (bp) | Serotype Specificity | References |
|-------------|-----------|-----------------------------|-------------------|----------------------|------------------------------------|
| ORF2819 | ORF2819-F | 5'-AGCAAAATGCCAAAACCTCGT-3' | 470 | 1/2b,3b,4b, 4d,4e | Michel <i>et al.</i> ¹⁰ |
| | ORF2819-R | 5'-CATCACTAAAGCCTCCCATG-3' | | | |
| ORF2110 | ORF2110-F | 5'-AGTGGACAATTGATTGGTGAA-3' | 597 | 4b,4d,4e | Michel <i>et al.</i> ¹⁰ |
| | ORF2110-R | 5'-CATCCATCCCTTACTTTGGAC-3' | | | |

Table 3. Primers used for detection of virulence-associated genes in *L. monocytogenes* isolates

| Target Gene | Primer | Sequences | Product Size (bp) | References |
|-------------|----------------|----------------------------|-------------------|--|
| <i>plcA</i> | <i>plcA</i> -F | 5'-TCCGCTCTACCTGACACAAC-3' | 317 | The primers were designed in this study. |
| | <i>plcA</i> -R | 5'-TCGTTGCTGTTTTGCTCGTC-3' | | |
| <i>plcB</i> | <i>plcB</i> -F | 5'-TTGCCGGGTTTTGCTAATGC-3' | 323 | |
| | <i>plcB</i> -R | 5'-TAGTCCGCTTTGCGCCTTTT-3' | | |
| <i>iap</i> | <i>iap</i> -F | 5'-AGTGGCGCTGGTGTGATAA-3' | 605 | |
| | <i>iap</i> -R | 5'-GCTGTTGTTGTTGCGTTGC-3' | | |

the virulence associated *hlyA* gene¹¹ were self-designed using primer BLAST online tool. For detection of *L. innocua*, PCR amplification of *in9* gene was targeted²⁴. A total volume of 25 µl mixture comprising 12.5 µl of 2X Mastermix, 0.5 µl (10 pmol) each of bi-directional primers, DNA (1 µl) and nuclease-free water (10.5 µl) was prepared for PCR. The amplification of *prs*, *hlyA* and *in9* genes was done by 5 min. denaturation at 95°C, accompanied by thirty cycles of 95°C (30 sec.), 53°C (30 sec.), 72°C (30 sec.) and final elongation at 72°C (5 min.).

Identification of serogroup

A simplex PCR was used to determine the serogroup using group-specific primers (Table 2). Amplification of ORF2819 was used to identify 1/2b and 3b serovars and amplification of both ORF2819 and OFR2110 to identify 4b, 4d and 4e serovars. This PCR does not differentiate within serovars.

A 25 µl volume of reaction mixture was prepared for PCR same as prepared for *Listeria* identification. *Listeria monocytogenes* (ATCC 13832) serotype 4b was used as the positive control. PCR conditions for amplification were as previously described by Michel *et al.*¹⁰

Virulence associated gene detection

After confirming the isolates by *hlyA* gene, a simplex PCR was done to determine the

three virulence genes, *plcA*, *plcB* and *iap* (Table 3). The composition of reaction mixture was the same as for the other genes used in this study. PCR conditions for amplification of all the three virulence genes were standardized at denaturation at 95°C (5 min.), followed by thirty cycles of 95°C (30 sec.), 52°C (30 sec.), 72°C (30 sec.) and a final elongation at 72°C (5 min.).

RESULTS AND DISCUSSION

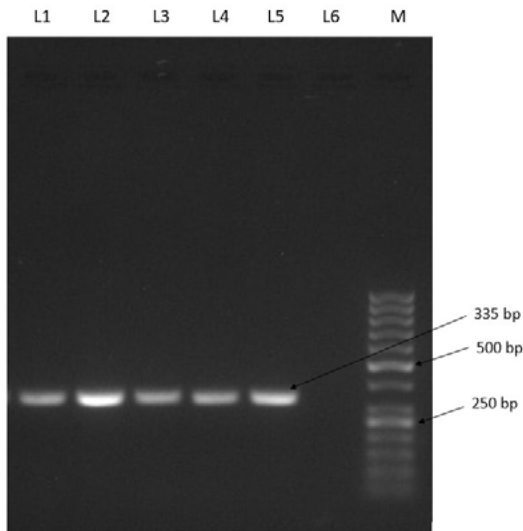
Prevalence of *Listeria*

Occurrence of *Listeria* spp. collected from various markets in Aizawl is shown in Table 4. Out of 111 samples, 28 (25.22%) were positive for *Listeria*., 10 (9.0%) for *L. monocytogenes* and 18 (16.21%) for *L. innocua*. The *hlyA* gene was found in *L. monocytogenes* using PCR (Fig. 1). Incidence of *L. monocytogenes* rated higher in mutton and pork (16.6% each) followed by chicken (12.5%). The incidence of *L. innocua* was found in mutton (50%) followed by fish gill and pork (33.33% each), chicken (16.66%), fish intestine and beef (6.66%).

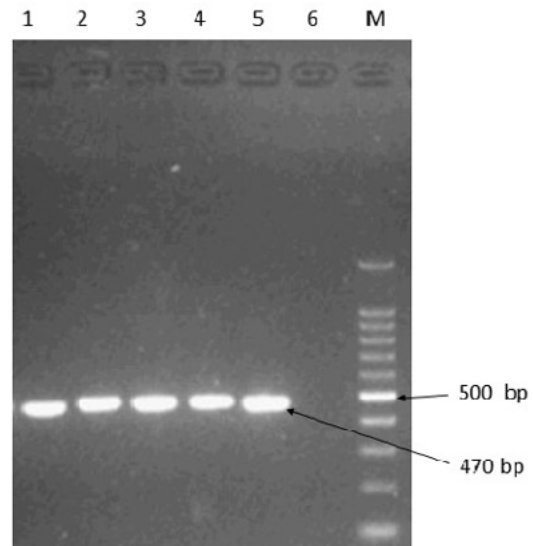
Nayak *et al.*¹² recorded the overall occurrence of *Listeria* in 9% of foods and the maximum incidence was found in milk samples (16%) followed by meat and fish (8% each), and milk (4%). Barbuddhe¹³ and Nayak¹⁴ with their co-workers reported occurrence of *Listeria* from 10.17% and 6.7% meat samples, respectively.

Murtiningsih and Sunarya¹⁵ reported *L. innocua* as the superior species isolated from fish and seafood samples (11.3%). Variation in occurrence of *Listeria* spp. reported by different workers might

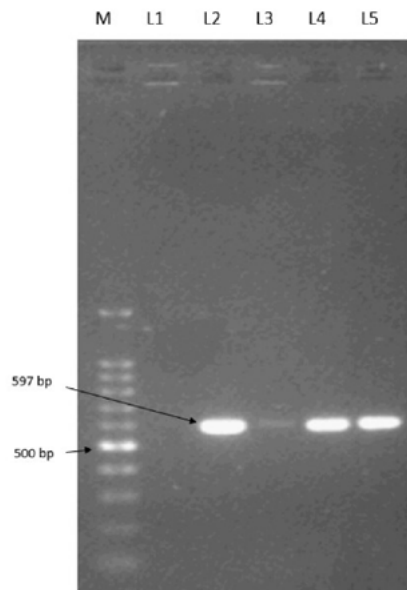
be owing to the difference in specimen, source and variation in hygienic condition maintained in different locations.



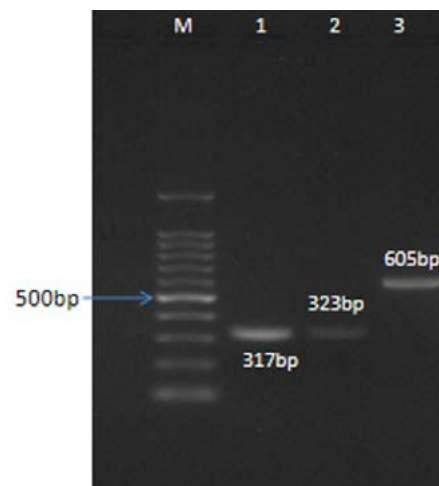
L1: Positive control (ATCC 13832), L2-L5: *Listeria* isolates, L6: Negative control, M: 50bp Ladder
Fig.1. Identification of *L. monocytogenes* by PCR



L1: Positive control (ATCC 13832), L2-L5: *Listeria* isolates, L6: Negative control, M: 100 bp Ladder
Fig.2. Identification of *L.monocytogenes* serotypes (1/2b,3b,4b,4d,4e) isolates by PCR



M: 100 bp Ladder, L1: Negative control, L2: Positive control (ATCC 13832), L3-L5: *Listeria* isolates
Fig.3. Identification of *L.monocytogenes* serotypes (4b, 4d, 4e) isolates by PCR



M: 100 bp Ladder, L1: PCR Product (*plcA* gene), L2: PCR Product (*plcB* gene), L3: PCR Product (*iap* gene)
Fig.4. Virulent gene detection in *L.monocytogenes* by PCR

Table 4. Prevalence of *Listeria* spp. in various food samples

| Type of Sample | No. of samples tested | No. of samples positive for <i>Listeria</i> spp. (%) | | Total No. of Positive Samples (%) |
|----------------|-----------------------|--|-------------------|-----------------------------------|
| | | <i>L. monocytogenes</i> | <i>L. innocua</i> | |
| Fish gill | 15 | 0 | 5 (33.3%) | 5 (33.33%) |
| Fish intestine | 15 | 0 | 1 (6.6%) | 1 (6.66%) |
| Pork | 24 | 4 (16.6%) | 4 (16.6%) | 8 (33.33%) |
| Mutton | 18 | 3 (16.6%) | 6 (33.3%) | 9 (50%) |
| Chicken | 24 | 3 (12.5%) | 1 (4.16%) | 4 (16.66%) |
| Beef | 15 | 0 | 1 (6.6%) | 1 (6.66%) |
| Total | 111 | 10 (9%) | 18 (16.21%) | 28 (25.22%) |

Serotyping

All *L. monocytogenes* showed PCR amplification of both ORF2819 and ORF2110, which identified them as serovars 4b, 4d or 4e (Fig.'s 2 and 3). Serovars 1/2a, 1/2b and 4b are linked with listeriosis¹⁶ and 4b was linked with major outbreaks of listeriosis¹⁷. Thus, the presence of serovar 4b in foods inflicts greater warnings on human health¹⁸. Serotype 4b was proclaimed from 37.2% of foods in Turkey¹⁹ as well in India and the United States of America with 60.4%²⁰ and 16.4%²¹, respectively. The difference in rate of the prevalence might be due to contradiction in the types of foods selected for each investigation. According to Michel *et al.*¹⁰, the serotypes 3a, 4d, and 4e are infrequent in foods, therefore the serovars 4b, 4d and 4e were marked as 4b serogroup. In the present study, all the isolated *L. monocytogenes* were confirmed as 4b serogroup which is a major public health concern, since isolates of *L. monocytogenes* belonging to this serogroup are potential human pathogens.

Virulence-associated genes

Virulence-associated gene profiling in *L. monocytogenes* was done by PCR amplification of three genes (Fig. 4). The *plcA* and *iap* genes showed presence in all the isolates, while *plcB* gene was present in 90% of the isolates.

In a study in North-East India, Pegu *et al.*²² found virulence-associated genes, *hlyA* (40.7%), *iap* (29.6%), *plcA* (40.7%) and *plcB* (22.2%) of 27 isolates from fish. The mechanism of pathogenicity of *L. monocytogenes* is stated to be usually associated with production of haemolysin encoded by *hlyA* gene²³. In our study, all the suspected isolates exhibited existence of *hlyA*, *plcA* and *iap* genes. This indicated that the

isolates are potentially pathogenic. Occurrence of such pathogenic bacteria in meat has significant public health implications.

CONCLUSION

This seems to be the first study on isolation and serogrouping of *L. monocytogenes* in foods from Mizoram. The prevalence of serogroup 4b reported to be associated frequently with human listeriosis seems to be of serious public health concern. These virulent strains can contaminate other foods during processing, packaging or storage. Hence, large-scale screening of various food and food products sold in the markets of northeast India at regular intervals for the incidence of virulent strains is of utmost importance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors .

DATA AVAILABILITY

All datasets generated or analyzed in this research are included in the manuscript.

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