RESEARCH ARTICLE

# PFGE Analysis of *Listeria monocytogenes* Isolates from Milk, Bovine Clinical and Bovine Environment Origin from Nagpur

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## Abstract

*Listeria monocytogenes*, a foodborne pathogen, causes listeriosis, that mainly affects immunocompromised persons. Epidemiological investigation and molecular subtyping can be helpful in linking the specific sources of infection. Thirty-eight isolates of *Listeria monocytogenes* recovered from the bovine environment, milk, and bovine clinical cases were characterized by serotyping and pulse field gel electrophoresis (PFGE) to correlate the genetic links between the isolates. Serotyping revealed 3 *L. monocytogenes* isolates to be of serovar group 4b, 4d and 4e, 34 isolates of serovar group 1/2b, 4b, 3b, 4d, 4e, and one isolate to be serovar group 1/2a, 1/2c,3a, and 3c. The PFGE analysis revealed 38 *L. monocytogenes* isolate from seven different clusters combined *Apa*I and *Asc*I with 80% relative genetic similarity. In comparison majority of the strains showed location and serovar wise homology in PFGE pattern than source wise. It was also observed that at the place of collection, the organisms circulate in the environment. The present findings suggested that the dairy/bovine farm environment is a potential source of contamination of *L. monocytogenes* in milk and animals.

**Key words:** Bovine environment, *L.monocytogenes* Pulsed field gel electrophoresis, Serotyping.

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## Introduction

*Listeria monocytogenes*, a causative agent of listeriosis, is a Gram positive, facultative intracellular pathogen causing severe invasive disease in animals and humans. Infection may result in severe illness, such as meningitis, meningoencephalitis, septicemia, abortion and stillbirth in pregnant women (Barbuddhe and Chakraborty, 2009). Infants, immunocompromised, and the elderly are typically suffered from the most severe illness.

Farm animals being reservoirs of *L. monocytogenes* can be involved indirectly in human listeriosis. It has been known for a long time that silage is the main source of *L. monocytogenes* infection in farm animals (Wiedmann *et al.,* 1996). The more serious source of infection seems to be dairy products as well as milk from affected cows (Jensen *et al.,* 1993).

*L. monocytogene* strains are serogrouped according to variation in the somatic (o) and flagellar (H) antigens. *L. monocytogenes* can be classified into four lineages: lineage I encompass serotypes 1/2b, 3b, 4b and 3c; lineage II includes serotypes 1/2a, 1/2c, 3a, lineage III comprising of serotypes 4a, 4b and 4c and lineage IV is independent lineage (Orsi *et al.,* 2011). It is also noteworthy that *L. monocytogenes* strains of a common serotype vary in virulence. Molecular typing of *L .monocytogenes* serotypes assist in the quick detection of isolates involved in disease outbreaks and thus adds to the efficiency of diagnosis (Hofer *et al.,* 2000).

At present pulse field gel electrophoresis (PFGE) scores high rank in the detection and phylogenetic analysis of *L. monocytogenes*. PFGE is considered gold standard for typing of *L. monocytogenes* because of its reproducibility, sensitivity, and epidemiological concordance (Chen and Knabel, 2008). PFGE offers to study the genetic variability (Wang *et al*., 2012), which could be able to discriminate the situations like outbreak or epidemiology or non-outbreak clones and also useful to retract the source of contamination.

The method is featured by high discriminatory power, reproducible and can be standardized easily and is specific at genetic level (Connor *et al.,* 2010). Very few studies have demonstrated the direct comparison of milk, bovine clinical cases and bovine environment isolates. For this reason, the present study was aimed to investigate the level of genetic diversity and possible relationships among *L. monocytogenes* strains isolated from milk, bovine clinical cases, and bovine environments using serotyping and PFGE profiling.

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## Materials and Methods

**Species Level Identification:** This study included 38 isolates available in the Department from different sources, including 12 from the bovine environment, 12 from milk, and 14 clinical cases from bovines.

Presumptive positive *L. monocytogenes* isolates were confirmed using biochemical testing as described in the international standard isolation protocols (McClain and Lee,1988)

**Multiplex PCR For Serotyping:** The multiplex –PCR was performed for serotyping as per the methodology described by Doumith *et al.* (2004). The primers used for multiplex PCR (Table 1) were procured from Sigma Aldrich, USA.

**Pulsed–Field Gel Electrophoresis:** Pulsed-field gel electrophoresis (PFGE) was carried out as per the protocol described by the CDC pulseNet standardized procedure suggested by Graves and Swaminathan (2001) adopting a standard apparatus (Bio-Rad Laboratories, Hercules, USA) for *L. monocytogenes* using the restriction endonucleases *Asc*I and *Apa*I. Bio Numerics software package (version 3.0 : Applied Maths) was used for generation of dendrograms.

### Data Processing

All the 38 samples, EGDe strain and marker lane were imported into BioNumerics for processing and analysis. Further band based dendrograms were prepared by using Dice coefficients (Dice, 1945). Furthermore, for an unweightedpair group method, arithmetic averages (UPGMA) were used (Tenover *et al.*, 1995). Default clustering setting of 0.50% optimization (*i.e.*, the relative distance an entire lane is allowed to shift in matching attempts) and 1.00% band position tolerance (*i.e.*, the relative distance a single band within a lane is allowed to shift during matching sample) was used.

## Results and Discussion

Out of the 38 *L. monocytogenes* isolates recovered from the bovine environment, milk, and bovine clinical cases majority of isolates (n=34) represented the group corresponding to serovars 1/2b, 3b, 4b, 4d, 4e, and three isolates represented serogroup corresponding to serovars 4b, 4d and 4e while one isolate serogrouped as 1/2a, 1/2c, 3a, and 3c (plate 1,Table 2).

According to Cheng *et al.* (2008), 13 serotypes of *L. monocytogenes* are known, out of which serotypes 1/2a, 1/2b, and 4b contributed to more than 98% of outbreaks. Our study showed most of the positive milk, bovine clinical samples, and bovine environment possessed 1/2b ,3b,4b,4d,4e serotype. A similar report has been demonstrated by Doumith *et al* (2004), showing that foodborne outbreaks generally involve serovar 1/2b. Fox *et al.* (2009) reported the presence of 1/2a, 1/2b and 4b serotypes in dairy farm environment and milking facility. Recovery of *L. monocytogenes* serotype 1/2a strains along with 1/2b and 4b is responsible for outbreaks in 98% of cases as reported in milk processing environments from Brazil, where 1/2a was a serotype (Brito *et al.*, 2008). Equal distribution of 1/2b or 3b and 1/2a or 3a serotypes in Portugal dairy has also been reported by Chambel *et al.*(2007).

**Table 1:** Nucleotide sequences of primer sets used in this study

| Gene target | Primer sequence (5'-3') | Product Size (bp) | Serovar Specificity | Protein encoded by the target gene |
|---|---|---|---|---|
| *lmo0737* | For: AGGGCTTCAAGGACTTACCC | 691 | *L. monocytogenes* serovars 1/2a, 1/2c,3a, and 3c | Unknown, no similarity |
| | Rev: ACGATTTCTGCTTGCCATTC | | | |
| ORF 2819 | For: AGCAAAATGCCAAAACTCGT | 471 | *L. monocytogenes* serovars 1/2b, 3b, 4b,4d, and 4e | Putative, transcriptional regulator |
| | Rev: CATCACTAAAGCCTCCCATTG | | | |
| ORF2110 | For: AGTGGACAATTGATTGGTGAA | 597 | *L. monocytogenes* serovars 4b, 4d, and 4e | Putative secreted protein |
| | Rev: CATCCATCCCTTACTTTGGAC | | | |
| *prs* | For: GCTGAAGAGATTGCGAAAGAAG | 370 | All *Listeria* species | Putative phosphoribosyl pyrophosphate synthetase |
| | Rev: CAAAGAAACCTTGGATTTGCGG | | | |

[a]For, forward; Rev, reverse.

**Table 2:** Serovar grouping of the isolates

| Sr. No | Serogroup | Milk | Bovine Clinical Cases | Bovine Environment | Total |
|---|---|---|---|---|---|
| 1 | 4b,4d,4e | 2(M8,M14) | ----- | 1(E3) | **3** |
| 2 | 1/2a,1/2c,3a,3c | **----** | **------** | 1(E16) | **1** |
| 3 | 1/2b,3b,4b,4d,4e | 10(M1,M2,M5,M6,M7,M9, M11,M13,M16,M17 | 14 (ACC-2,4,5,6,8,9,10,11,12,13,14, 15,16,17) | 10(E5,E9,E11,E12,E13,E14,E15, E18,E24,E25) | **34** |
| | **Total** | **12** | **14** | **12** | **38** |

M: Milk isolates, ACC: Animal Clinical cases isolates & E: Environment isolates

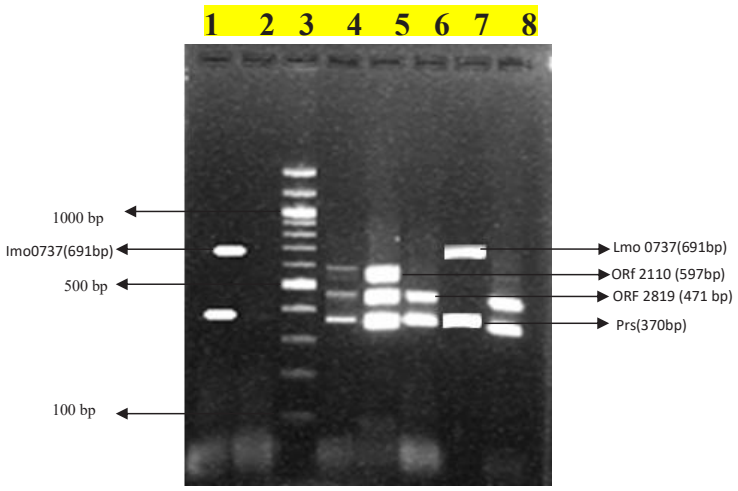
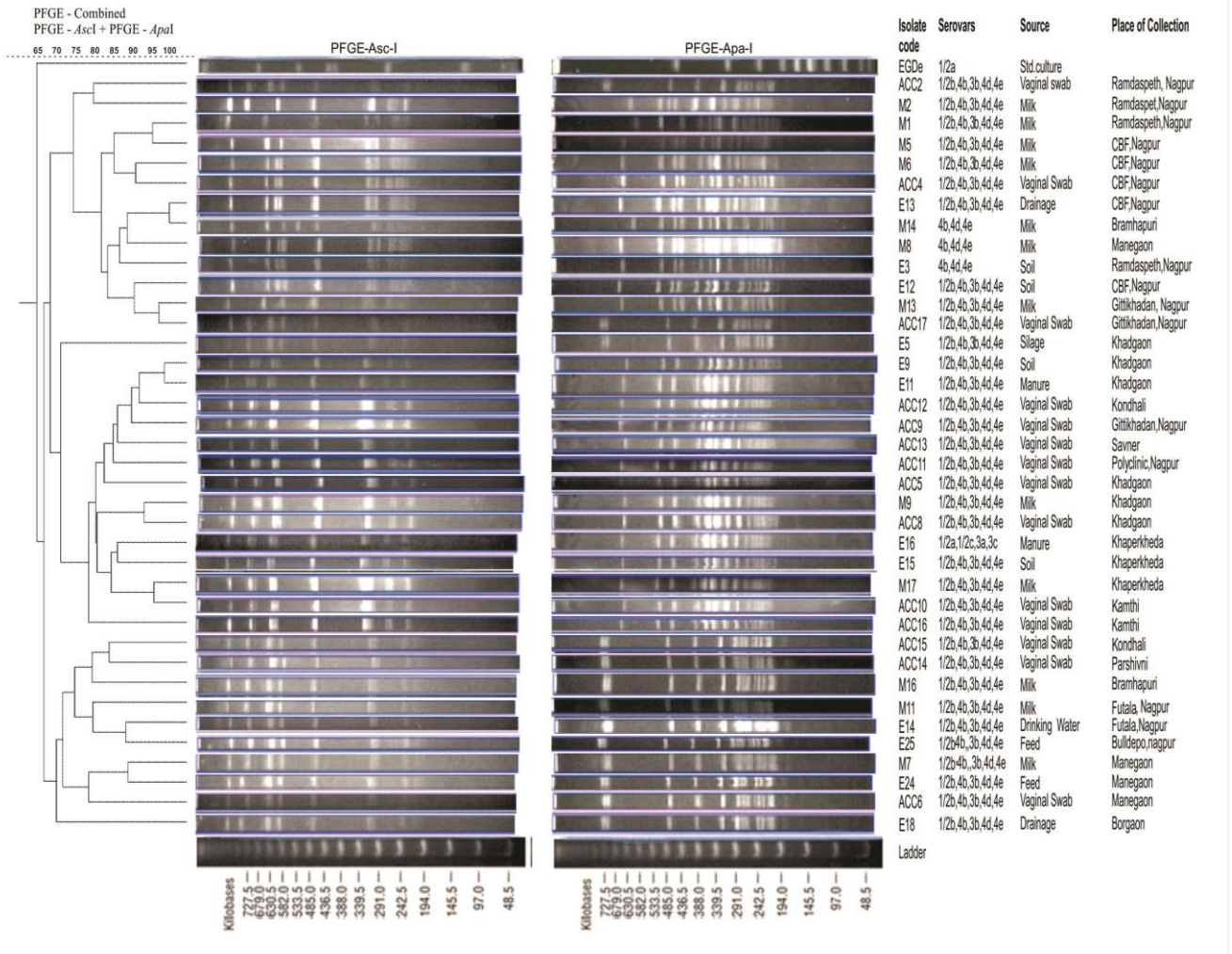



**Plate 1:**Agarose gel showing Multiplex PCR serotyping for determination of the serovargroups of isolates obtained from bovine environment, bovine clinical cases and milk . Lane1: isolates E-16 *L. monocytogenes* serogroup1/2a ;Lane 2*: E coli*- Negative Control Lane3: 100 bp DNA ladder; Lane 4 : isolates M-5 *L. monocytogenes* serogroup1/2b and 4b; Lane5: isolatesACC-*3 L. monocytogenes* serogroup 1/2band 4b; Lane6: isolate-E-*3 L. monocytogenes* serogroup 4b; Lane7: *L.monocytogenes* EGDe as a standard for serotypes 1/2a; Lane8 : isolate M-14 *L. monocytogenes* serogroup 4b.




| Isolate code | Serovars | Source | Place of Collection |
|---|---|---|---|
| EGDe | 1/2a | Std.culture | |
| ACC2 | 1/2b,4b,3b,4d,4e | Vaginal swab | Ramdaspeth, Nagpur |
| M2 | 1/2b,4b,3b,4d,4e | Milk | Ramdaspet,Nagpur |
| M1 | 1/2b,4b,3b,4d,4e | Milk | Ramdaspeth,Nagpur |
| M5 | 1/2b,4b,3b,4d,4e | Milk | CBF,Nagpur |
| M6 | 1/2b,4b,3b,4d,4e | Milk | CBF,Nagpur |
| ACC4 | 1/2b,4b,3b,4d,4e | Vaginal Swab | CBF,Nagpur |
| E13 | 1/2b,4b,3b,4d,4e | Drainage | CBF,Nagpur |
| M14 | 4b,4d,4e | Milk | Bramhapuri |
| M8 | 4b,4d,4e | Milk | Manegaon |
| E3 | 4b,4d,4e | Soil | Ramdaspeth,Nagpur |
| E12 | 1/2b,4b,3b,4d,4e | Soil | CBF,Nagpur |
| M13 | 1/2b,4b,3b,4d,4e | Milk | Gittikhadan, Nagpur |
| ACC17 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Gittikhadan,Nagpur |
| E5 | 1/2b,4b,3b,4d,4e | Silage | Khadgaon |
| E9 | 1/2b,4b,3b,4d,4e | Soil | Khadgaon |
| E11 | 1/2b,4b,3b,4d,4e | Manure | Khadgaon |
| ACC12 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Kondhali |
| ACC9 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Gittikhadan,Nagpur |
| ACC13 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Savner |
| ACC11 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Polyclinic,Nagpur |
| ACC5 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Khadgaon |
| M9 | 1/2b,4b,3b,4d,4e | Milk | Khadgaon |
| ACC8 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Khadgaon |
| E16 | 1/2a,1/2c,3a,3c | Manure | Khaperkheda |
| E15 | 1/2b,4b,3b,4d,4e | Soil | Khaperkheda |
| M17 | 1/2b,4b,3b,4d,4e | Milk | Khaperkheda |
| ACC10 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Kamthi |
| ACC16 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Kamthi |
| ACC15 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Kondhali |
| ACC14 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Parshivni |
| M16 | 1/2b,4b,3b,4d,4e | Milk | Bramhapuri |
| M11 | 1/2b,4b,3b,4d,4e | Milk | Futala, Nagpur |
| E14 | 1/2b,4b,3b,4d,4e | Drinking Water | Futala,Nagpur |
| E25 | 1/2b4b,,3b,4d,4e | Feed | Builddepo,nagpur |
| M7 | 1/2b4b,,3b,4d,4e | Milk | Manegaon |
| E24 | 1/2b,4b,3b,4d,4e | Feed | Manegaon |
| ACC6 | 1/2b,4b,3b,4d,4e | Vaginal Swab | Manegaon |
| E18 | 1/2b,4b,3b,4d,4e | Drainage | Borgaon |
| Ladder | | | |

**Plate 2:** Dendrogram derived from PFGE Profile of *AscI* and *ApaI* macroristriction showing restriction pattern similarity among 38 *L. moncytogenes* bovine environment, animal clinical and milk isolates with strain *EGDe*.

The serovar 4b dominates in Europe and the serovar 1/2a, 1/2b and 4b in Canada and United States, where the epidemiological association has been established in perinatal listeriosis with 1/2b and 4b (Gellin *et al*., 1991). The present study also recorded the common presence of 1/2b, 4b, and 1/2a. Singh (2012) obtained *L. monocytogenes* from vegetables, humans, and milk and observed the 4b serotype, especially in milk, while the serotype 1/2b in meat products.

By PFGE analysis, all 38 *L. monocytogenes* 1/2b, 3b, 4b, 4d, 4e (n= 34); 4b, 4d, 4e (n=3); 1/2a, 1/2c, 3a, 3c (n=1) were tested by *Apa*I and *Asc*I enzymes (Plate 2). After digestion with enzyme *Apa*I, 10-14 fragments ranging from approximately 194.0 to 727.5 kb were recorded. Similarly, about 8-12 fragments ranging from 194.0 to 824.5 kb were recorded by *Asc* I digestion.

The fingerprints generated by PFGE with *Asc* I and *Apa* I digestion was indistinguishable based on exact numbers and matching position of all bands. A single band difference was used to differentiate between strains and to assign a specific profile. This criterion was based on the assumption that identical strains will produce identical restriction fragment patterns regardless of the restriction endonuclease used for the DNA digestion (same genotype), but non-identical strains will produce non-matching banding patterns when different restriction endonucleases are used (different genotype). For many species, comparative studies indicated that isolates are indistinguishable by PFGE and are unlikely to show substantial differences by other typing methods (Olsen *et al*.,2005). PFGE analysis grouped the isolates into seven clusters; with 80% relative genetic identity. The strains are grouped as per location and serovar group (Plate 2).

In the present study, isolates from different sources showed a similar PFGE pattern and thus indicated a prevalence of a single clone. These results are in agreement with Doumith *et al*. (2004), who reported a DNA macroarray-based subtyping method by which three lineages of *L. monocytogenes* could be classified into five phylogenetic groups, each correlated with serovars. A number of workers used a similar clustering analysis of PFGE pattern to detect the association between molecular types to ascertain its specific sources. In contrast, PFGE profile indistinguishable among isolates from human, food, and environmental sources has also been reported by Fugett *et al*. (2007). Furthermore, the use of PFGE with restriction endonucleases could resolve the result in two forms with certain clarity.

Analysis of PFGE dendrogram has three genetic lineages: lineage I (serovar 1/2b, 3b, 4b and 4e); lineage II (serovar 1/2a, 1/2c, 3a, and 3c), and lineage III (serovar 4b, 4d and 4e) and represented distinct subgroups. Our study findings are more or less similar to previous studies (Neves *et al*., 2008). Shakuntala *et al*. (2022) reported clonal relationships amongst *L. monocytogenes* strains isolated from foods of animal origin milk, beef, chevon, pork , chicken and fish. Zdolec *et al*.( 2019) reported that identical PFGE patterns of *L. monocytogenes* occurred in milk samples of the same producer over a three-year period, indicating the persistence of pathogens in raw milk vending machines. The results obtained support the need for more effective control of milk in the entire food chain.

## Conclusions

Most of the *L. monocytogenes* strains prevalent in the bovine environment, milk, and bovine clinical cases were confirmed to be of serovar group 1/2b, 3b, 4b, 4d, 4e, posing a serious public health hazard. The majority of the strains isolated were location specific as well as to the serovar group indicated by their PFGE pattern. The strains exhibited clonality. There was the correlation between pulsotype and serovar with identical PFGE patterns belonging to the same serovar. Thus, the understanding of genetic diversity will contribute to the development of rational and workable strategies to control this important zoonotic infection

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