RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.2.317-324

Occurrence of *Listeria monocytogenes* in raw milk and chicken meat in Thrissur, Kerala[#]

M. C. B. Prasad[1], V. K. Menon[2]*, A. A. P. Milton[3], B. Sunil[4], C. Latha[5], P. M. Priya[6] and Naicy Thomas[7]
Department of Veterinary Public Health
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University
Kerala, India

Citation: Prasad, M.C.B., Menon, V.K., Milton, A.A.P., Sunil, B., Latha, C., Priya, P.M. and Thomas, N. 2024. Occurrence of *Listeria monocytogenes* in raw milk and chicken meat in Thrissur, Kerala. *J. Vet. Anim. Sci.* **55**(2):317-324
DOI: https://doi.org/10.51966/jvas.2024.55.2.317-324

Received: 26.09.2023 *Accepted: 16.04.2024* *Published: 30.06.2024*

Abstract

The present study was carried out with the objective to detect Listeria monocytogenes from raw milk and chicken meat. Two hundred samples of raw milk and chicken meat were obtained from local dairy farms and retail outlets, respectively in Thrissur, Kerala. These samples were enriched primarily in University of Vermont Medium-I (UVM I) broth, followed by further enrichment in UVM II broth. Subsequently, the enriched samples were streaked onto PALCAM agar for further analysis. Simultaneously, the samples were directly screened for presence of L. monocytogenes by PCR and real time PCR. Bacterial cultures that exhibited standard colony features were subsequently identified at the species level through the phenotypic tests. The isolates obtained were subjected to molecular confirmation, specifically targeting hlyA gene using conventional PCR and real time PCR. Additionally, the isolates were subjected to antibiotic sensitivity test. The overall occurrence of L. monocytogenes was 1.5 per cent, with two and one isolate being recovered from milk and meat, respectively. However, the occurrence was determined to be three per cent by PCR and real time PCR respectively. The identification of L. monocytogenes in raw milk and chicken meat highlights the importance to enforce adequate sanitation and hygiene measures in the food chain in order to protect the health of consumers.

Keywords: *Occurrence, Listeria monocytogenes, hlyA, milk, meat*

[#]*Part of Ph.D thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

1. *PhD Scholar*
2. *Associate Professor, Department of Veterinary Public Health, KVASU*
3. *Scientist, ICAR-Research complex for NEH region*
4. *Professor& Head, Department of Veterinary Public Health, KVASU*
5. *Director of Academics and Research, KVASU*
6. *Professor& Head, Department of Veterinary Microbiology*
7. *Associate Professor, Department of Animal Genetics and Breeding*

**Corresponding author: vrinda@kvasu.ac.in, Ph. 9497655590*

Globally, food borne pathogens are highly diverse in nature causing severe foodborne infections in humans. These infections tend to obstruct socioeconomic progress by causing severe stress on health care services and damaging trade and national economies (FAO and WHO, 2022). *Listeria monocytogenes*, which causes foodborne listeriosis is attributed to wide variety of foods like milk, meat, dairy products, aquatic foods, vegetables and ready-to-eat food products (Shakuntala *et al.,* 2019). As per the World Health Organization (FAO and WHO,2022), incidence of listeriosis range from 0.1 to 10 cases per one million individuals annually. Although, the overall number of listeriosis cases is relatively low, the substantial fatality and hospitalisation rate associated with this infection renders it as a notable public health issue (EFSA, 2022). It is a widely distributed microorganism with the capacity to thrive in diverse pH levels and alkaline environments. Its ability to reproduce even at low temperature enables it to contaminate food at various stages throughout the food production and distribution chain (Du *et al.,* 2017). Milk serves as major source of nutrients for all people across the globe. The natural nutrients and components present in milk create an environment conducive for the multiplication of *L. monocytogenes*, leading to significant food safety concerns (Shamloo *et al.,* 2019). On the other hand, chicken meat and meat products have often been linked to foodborne listeriosis. The arrival of live birds at poultry processing facilities and unhygienic retail establishments could potentially be a contributing factor to these outbreaks (Noll *et al.,* 2018). Additionally, the hot and humid climate prevalent in certain regions leads to higher level of bacterial contamination on the surface of chicken meat that is available in the market (Kalorey *et al.,* 2005). Therefore, the aim of the present study was to generate information on occurrence of *L. monocytogenes* in raw cow milk and chicken meat in Thrissur district of Kerala.

Materials and methods

Samples

A total of 100 samples each of raw milk and chicken meat were collected from local dairy farms and retail outlets in Thrissur from May to August 2023. The samples were collected using sterile techniques, transported to the laboratory and then subjected to microbiological analysis. Samples after collection were placed in an icebox to maintain a temperature of 4˚C until they were further analysed at the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

Isolation and identification of L. monocytogenes

The procedure followed in this study was based on the method outlined by USDA (2018) with some necessary modifications. Briefly, one mL or g of milk or meat sample was directly inoculated into University of Vermont Medium-1 (UVM-I) supplemented with Nalidixic acid and Acriflavine hydrochloride at 10.0mg/L and 6.0mg/L concentration and left to incubate overnight at 37˚C. Subsequently, a 0.1 ml portion of the enriched UVM-1 culture was transferred to UVM-2 medium supplemented with 10.0mg/L of Nalidixic acid and Acriflavine hydrochloride of 12.5mg/L and incubated overnight at 37˚C.The enriched inoculum from UVM-II was streaked onto polymyxin-acriflavine-lithium-chloride-ceftazidime-aesculin-mannitol (PALCAM) agar (HiMedia, India). These plates were then incubated at a temperature of 37˚C for a duration of 24 to 48 hours.

Colonies exhibiting a small, round, shiny, greyish-green appearance bordered by a black zone resulting from aesculin hydrolysis on PALCAM agar were suspected for *L. monocytogenes*. A minimum of five suspected colonies were then sub cultured on Brain Heart Infusion (BHI) broth. The suspected colonies were then subjected to gram staining. The typical small, gram positive rods were further identified using biochemical tests *viz*., catalase, oxidase and sugar fermentation (specifically rhamnose, xylose and mannitol) tests.

Antibiotic sensitivity test

Antimicrobial susceptibility was determined using the Kirby-Bauer disc diffusion method according to the standard procedure described by Clinical and Laboratory Standards Institute (CLSI, 2017).

Nine antibiotic discs commonly used were tested, which include tetracycline (30 µg), chloramphenicol (25 µg), streptomycin (10 µg), cefotaxime (30µg), ampicillin (10µg), erythromycin (15µg), nitrofurazone (100 µg), doxycycline (30µg) and ciprofloxacin (10µg). The interpretation of the results was done by comparing the diameter of the inhibition zones with a standard chart of inhibition zones provided by the disc manufacturer (HiMedia, India). The clinical thresholds for determining organism susceptibility to antibiotics were established following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI, 2017). Based on these guidelines, the isolates were categorised as either sensitive, intermediate, or resistant to each antibiotic tested.

Molecular confirmation

A loop full of fully grown *L. monocytogenes* isolates were inoculated in tryptone soy broth individually and incubated at 37˚C for 24 hours. One millilitre of this inoculum was taken and centrifuged at 8000rpm for 10 min. The supernatant was discarded and the pellet formed was subjected to DNA extraction as per manufacture instructions of QIAamp DNA Mini Kit (Qiagen, Germany). While, for molecular screening of milk and meat samples, three-step centrifugation-based DNA extraction method was carried out according to the protocol described by Yamazaki *et al.* (2009). The primers used for PCR and real time PCR were designed using Primer explorer 3 tool of National Centre of Biotechnology Information (NCBI) as a part of the main study. The amplification procedure for PCR was standardised individually to obtain a distinct band of 207amplicon length for primer sequences *hlyA*F2 and *hlyA*R2 (Table 1). The standardisation was done using American Type Culture Collection (ATCC) *L. monocytogenes* 13932 (ATCC, USA). The standardised PCR protocol for a 20µL reaction mixture, consisted of the following components: 10µL of 2X Master mix (Thermo scientific, USA), 10µM of a primer set comprising both forward and reverse primers, 1 µL of DNA template and nuclease free water was added to achieve the desired reaction volume. To ensure accuracy, both positive and negative controls were included in each PCR run. The amplification reaction was conducted using a Gradient Thermocycler (Bio-Rad, USA). The PCR cycling conditions consisted of an initial DNA denaturation step at 95°C for 10 minutes, followed by 32 cycles of 30 seconds denaturation step at 95°C, a 45 seconds annealing step at 60°C, and a 45 seconds extension step at 72°C. This was followed by a final extension step of 10 minutes at 72°C, with a holding temperature of 4°C. The amplified products were subsequently subjected to agarose gel electrophoresis, stained with safe DNA at a concentration of 0.5 µg/ml, and visualised using a gel documentation system (Syngene, UK).

The sequence of primers used for real time PCR were *hlyA*Fin and *hlyARin,* given in Table 1. The standardised 20µL reaction mixture included 10 µL of 2X Applied Biosystems™ Power SYBR Green PCR Master Mix, 5µM each of forward and reverse primer, 2µL of DNA template and nuclease free water was added. The reaction was conducted in Applied Biosystems™ StepOnePlus™ Real-Time PCR system (Foster City, CA, USA). The cycling conditions include initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec., annealing and elongation at 60°C for 1 min each. A Ct value of 32 was taken as cut off threshold for detection of *L. monocytogenes*.

Table 1. List of primers used in the study

Method	Name & Gene	Sequence	Size (bp)
PCR	*hlyA*F2	5'TTTTGACGCTGCCGTAAGTG 3'	207
	*hlyA*R2	5'TAGGCAATGGGAACTCCTGG 3'	
Realtime PCR	*hlyA*Fin	5'GCCGTAATTTACGGTGGCTCC 3'	69
	*hlyA*Rin	5'TCGTAAGTCTCCGAGGTTACCG 3'	

Results and discussion

Out of the two hundred samples analysed, a total of three samples (1.5%) were tested positive for *L. monocytogenes* by culture method. The occurrence of *L. monocytogenes* was more in meat (2%) than in milk (1%). The isolates displayed positive result in the catalase and negative reaction in the oxidase test. Sugar fermentation tests showed positive for L-rhamnose and negative for D-mannitol and D-xylose. When cultures were subjected to PCR and real time PCR, all the isolates revealed the presence of *hlyA* gene (Fig. 1, 2). The *hlyA* gene, which codes for listeriolysin O (LLO) toxin is unique and serves as a major virulence factor, existing as a single copy in the genome of *L. monocytogenes*. The species-specific properties of the *hlyA* gene and LLO make them excellent molecular targets for identifying *L. monocytogenes*, particularly in food samples. It was found that three per cent of milk and three per cent of chicken meat samples from field revealed the presence of *hlyA* gene by PCR and real time PCR. In the current study, the detection of *L. monocytogenes* in bovine raw milk samples is notably lower when compared to the results of previous studies conducted by Bhilegaonkar *et al.* (1997), Barbuddhe *et al.* (2002), Soni *et al.* (2013), and Yousef *et al.* (2020), who reported occurrences of 8.1 per cent, 6.25 per cent, 5.8 per cent and 14 per cent, respectively. This higher occurrence observed in their studies might be attributed to variations in the geographical locations of the research and the number of samples analysed. Conversely, our results indicate a higher occurrence when compared to the study of Rawool *et al.* (2007), who reported an overall occurrence of 0.55 per cent in a sample set of 243 milk samples with a history of subclinical mastitis in India. The source of organism in raw milk have been documented to include faecal contamination and environmental exposure during milking, storage, transportation, poor-quality silage as well as the presence of infected cows in dairy farms (Griffiths, 1989; Bemrah *et al.,* 1998). Lack of proper hygienic measures while milking, storage and transportation attribute to the contamination sources of organism in raw milk. Furthermore, cases of listerial mastitis, encephalitis, or *Listeria*-related abortions in cattle can directly contaminate milk.

The occurrence of *L. monocytogenes* in two per cent of chicken meat samples in the current study was lower than earlier studies conducted by Barbuddhe *et al.* (2003), Kalorey *et al.* (2005), and Shakuntala *et al.* (2019), where they reported a higher occurrence of 8.5% in chicken meat.

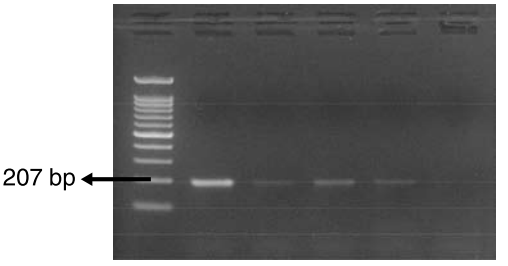

Fig.1. Confirmation of *L. monocytogenes* field isolates by conventional PCR targeting *hlyA* gene. Lane M: 100 bp DNA ladder, Lane PC: Positive control, Lane NTC: Non template control; Lane (1-3): Sample numbers ML 97, MT 90, MT 97;(Milk-ML, Meat-MT).

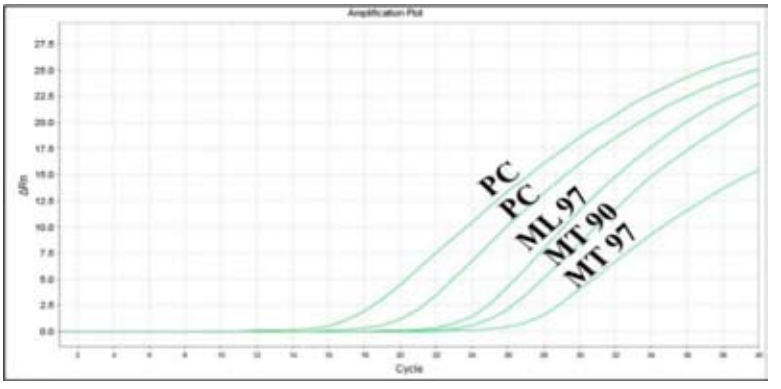

Fig 2: Amplification plots of *L. monocytogenes* field isolates by real time PCR. PC: Positive control; Milk-ML, Meat-MT

However, the occurrence of the organism observed in the study was greater than the reports of Latha *et al.* (2017) in Thrissur. They reported a much lower occurrence of 0.08 per cent using culture methods. An occurrence of 2.67 per cent reported by Anu (2012) from chicken meat is higher than the present study. The identification of *L. monocytogenes* in meat samples highlight the significance of maintaining sanitation and hygiene in the food preparation environment and to avoid faecal contamination of the meat during slaughter. The presence of *L. monocytogenes* in chicken meat carries potential risks, primarily through cross-contamination when handling both cooked and raw foods. The bacterial contamination has its origin from areas where the meat is cleaned and the surfaces of cutting boards and knives. Consequently, it is strongly advised to avoid consuming undercooked meat to mitigate potential health risks.

The antimicrobial susceptibility of the three isolates was assessed using the standard disc diffusion method. All three isolates and ATCC 13932 displayed sensitivity to chloramphenicol, doxycycline, streptomycin, nitrofurazone, ciprofloxacin, and tetracycline. The resistance to ampicillin observed in our study aligns well with the findings of previous research conducted by Srinivasan *et al.* (2005) and Vasu *et al.* (2014). While, the isolates obtained from meat exhibited resistance to cefotaxime and ampicillin. This resistance to cephalosporins in isolates from chicken meat corresponds with studies conducted by Noll *et al.* (2018) and Menon *et al.* (2021). The resistance to cefotaxime and ampicillin can be linked to the natural resistance of the organism, which results from the limited or absent affinity of listerial penicillin-binding protein 3 and 5 for cephalosporins (Hakenbeck and Hof, 1991).

Conventional microbiological techniques for detecting *L. monocytogenes* are both labour-intensive and time-consuming, involving several steps from isolation to confirmation, which typically takes around 3-4 days. Consequently, present study employed direct screening of milk and meat samples for *L. monocytogenes* using both conventional and real-time PCR methods. Out of the 200 samples tested, three samples each from meat and milk were found to be positive for the *hlyA* gene of *L. monocytogenes* (Fig. 3A, B). The overall occurrence of *L. monocytogenes*

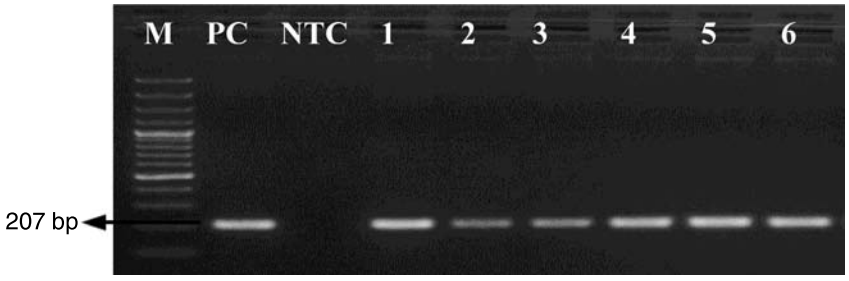

Fig. 3A

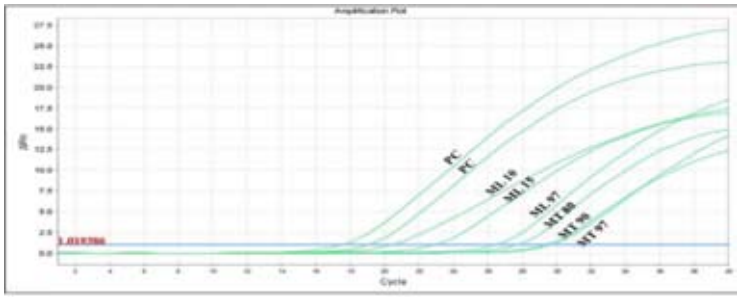

Fig. 3B

Fig.3: Direct detection of *L. monocytogenes* in milk (ML) and chicken meat (MT). A. Conventional PCR;B. Real time PCR; Lane (1-3): Sample numbers ML 10, ML 15, ML 97;Lane (4-6): MT 80, MT 90, MT 97; Lane M: 100 bp DNA ladder, Lane PC: Positive control, Lane NTC: Non template control

was determined to be three per cent which is higher than conventional methods observed in the study. Studies by Niederhauser *et al.* (1992) and Aznar and Alarcón (2003) reported a higher occurrence of *L. monocytogenes* by PCR than culture methods. Niederhauser *et al.* (1992) reported a sample initially deemed culture-negative, was found to be positive using PCR. This finding is in line with the results in our study and could be accredited to the particular strain entering a viable but non-culturable state. Additionally, the percentage of occurrence determined through molecular methods in our study exceeded the results reported by Sharma *et al.* (2017), who found a lower occurrence rate of 1.1 percent in raw milk using PCR.

Conclusion

The presence of *L. monocytogenes* in raw milk and chicken meat represents a significant public health concern. Consumption of foods that are contaminated raises the likelihood of contracting foodborne listeriosis. To mitigate this risk, it is crucial to rigorously enforce sanitary conditions in dairy farms and meat processing units, ensure proper storage and promote personal hygiene among individuals involved in handling of foods in the food chain. These measures can help to reduce the potential contamination of foods with organism at the retail and farm level, and subsequently decrease the bacterial load in processed milk and meat products. Furthermore, the antibiotic susceptibility profile of this organism underscores the need for cautious use of antimicrobials in the fields of veterinary and human medicine. Regular screening of foods and understanding of antibiotic resistance patterns will be instrumental in effectively controlling the emergence of *L. monocytogenes*.

Acknowledgement

The authors are thankful to the Kerala Veterinary and Animal Sciences University for providing the facilities needed for carrying out the research.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Anu S, 2012.Occurrence of *Listeria* species in beef, chicken and sea foods. *M.V.Sc. thesis*, Kerala Veterinary and Animal Sciences University, Pookode, 100p.

Aznar, R. and Alarcón, B., 2003. PCR detection of *Listeria monocytogenes*: a study of multiple factors affecting sensitivity. *J. Appl. Microbiol*.**95**: 958-966.

Barbuddhe, S.B., Chaudhari, S.P. and Malik, S.V.S. 2002.The occurrence of pathogenic *Listeria monocytogenes* and antibodies against listeriolysin-O in buffaloes.*J. Vet. Med*.**49**: 181-184.

Barbuddhe, S.B., Swain, B.K., Chakurkar, E.B. and Sundaram, R.N.S. 2003.Microbial quality of poultry meat with special reference to *Listeria monocytogenes*. *Indian J. Poult. Sci*.**38**: 305-307.

Bemrah, N., Sanaa, M., Cassin, M.H., Griffiths, M.W. and Cerf, O. 1998. Quantitative risk assessment of human listeriosis from consumption of soft cheese made from raw milk. *Prev. Vet. Med*.**37**: 129-145.

Bhilegaonkar, K.N., Kulshrestha, S.B., Kapoor, K.N., Kumar, A., Agarwal, R.K. and Singh, B.R. 1997. Isolation of *Listeria monocytogenes* from milk. *J. Food Sci. Technol*.**34**: 248-250.

Clinical and Laboratory Standards Institute (CLSI). 2017. Performance Standards for Antimicrobial Susceptibility Testing. Twenty Seventh Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA. 250p.

Du, X.J., Zhang, X., Wang, X.Y., Su, Y.L., Li, P. and Wang, S. 2017. Isolation and characterization of *Listeria monocytogenes* in Chinese food obtained from the central area of China. *Food Control*.**74**: 9-16.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2022.

The European Union One Health 2021 Zoonoses Report, Italy. 273 p.

FAO and WHO. 2022. *Listeria monocytogenes* in ready-to-eat (RTE) foods: attribution, characterization and monitoring. Microbiological Risk Assessment Series No. 38, Rome. 202p

Griffiths, M.W. 1989. *Listeria monocytogenes*: its importance in the dairy industry. *J. Sci. Food Agric*.**47**: 133-158.

Hakenbeck, R. and Hof, H. 1991. Relatedness of penicillin-binding proteins from various *Listeria* species.*FEMS Microbiol.Lett*.**84**: 191-195.

Kalorey, D.R., Barbuddhe, S.B., Kurkure, N.V. and Gunjal, P.S. 2005.Prevalence of *Listeria monocytogenes* in poultry meat in Vidharba region of India.In XVIIth European Symposium on the Quality of Poultry Meat. 23rd May, 2005, Doorwerth. The Netherlands.

Latha, C., Anu, C.J., Ajaykumar, V.J. and Sunil, B. 2017.Prevalence of *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Salmonella enterica* Typhimurium in meat and meat products using multiplex polymerase chain reaction.*Vet. World*.**10**: 927.

Menon, K.V., Sunil, B. and Latha, C., 2021. Prevalence and antibiotic resistance profile of *Listeria* spp. associated with seafoods from fish catchment areas in Kerala, India. *Vet. World*.**14**: 777.

Niederhauser, C., Candrian, U., Höfelein, C., Jermini, M., Bühler, H.P. and Lüthy, J., 1992.Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Appl. Environ. Microbiol*.**58**: 1564-1568.

Noll, M., Kleta, S. and Al Dahouk, S.2018. Antibiotic susceptibility of 259 *Listeria monocytogenes* strains isolated from food, food-processing plants and human samples in Germany. *J. Infect. Public Health*.**11**: 572-577.

Primer explorer 3.0, National Centre of Biotechnology Information (NCBI). https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi, Accessed on May 15th 2022.

Rawool, D.B., Malik, S.V.S., Shakuntala, I., Sahare, A.M. and Barbuddhe, S.B., 2007. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases.*Int. J. Food Microbiol*.**113**: 201-207.

Shakuntala, I., Das, S., Ghatak, S., Milton, A.A.P., Sanjukta, R., Puro, K.U., Pegu, R.K., Duarah, A., Barbuddhe, S.B. and Sen, A. 2019. Prevalence, characterization, and genetic diversity of *Listeria monocytogenes* isolated from foods of animal origin in North East India. *Food Biotech*.**33:** 237-250**.**

Shamloo, E., Hosseini, H., Moghadam, Z.A., Larsen, M.H., Haslberger, A. and Alebouyeh, M. 2019. Importance of *Listeria monocytogenes* in food safety: A review of its prevalence, detection, and antibiotic resistance. *Iran. J. Vet. Res*.**20**: 241.

Sharma, S., Sharma, V., Dahiya, D.K., Khan, A., Mathur, M. and Sharma, A., 2017. Prevalence, virulence potential, and antibiotic susceptibility profile of *Listeria monocytogenes* isolated from bovine raw milk samples obtained from Rajasthan, India. *Foodborne Pathog. Dis*.**14**: 132-140**.**

Soni, D.K., Singh, R.K., Singh, D.V. and Dubey, S.K. 2013. Characterization of *Listeria monocytogenes* isolated from Ganges water, human clinical and milk samples at Varanasi, India. *Infect. Genet.Evol*.**14**: 83-91.

Srinivasan, V., Nam, H.M., Nguyen, L.T., Tamilselvam, B., Murinda, S.E. and Oliver, S.P. 2005. Prevalence of antimicrobial resistance genes in *Listeria monocytogenes* isolated from dairy farms. *Foodborne Pathog. Dis*. **2**: 201-211.

USDA, 2018. United States Food and Drug Administration-Bacteriological Analytical Manual (U.S.FDA-BAM). **10**: 35pp.

Vasu, R.K., Sunil, B., Latha, C., Menon, V. and Kumar, A. 2014. Prevalence of *Listeria* species in meat processing environments. *Int. J. Curr. Microbiol. App. Sci*. **3**: 542-546.

Yamazaki, W., Taguchi, M., Kawai, T., Kawatsu, K., Sakata, J., Inoue, K. and Misawa, N. 2009. Comparison of loop-mediated isothermal amplification assay and conventional culture methods for detection of *Campylobacter jejuni* and *Campylobacter coli* in naturally contaminated chicken meat samples. *Appl. Environ. Microbiol*.**75**: 1597-1603.

Youssef, M., Ramadan, H. and Al-Ashmawy, M. 2020. Prevalence of *Listeria* species in raw milk, ice cream and yogurt and effect of selected natural herbal extract on its survival. *Mansoura Vet. Med. J*.**21**: 99-106. ■