J.Natn.Sci.Foundation Sri Lanka 2023 51 (3): 533 - 539
DOI: http://dx.doi.org/10.4038/jnsfsr.v51i3.11397

RESEARCH ARTICLE

Microbiology and Molecular Biology

Pulsed-field gel electrophoresis typing and molecular characterization of *Listeria monocytogenes* isolates in raw milk samples from Polonnaruwa District, Sri Lanka

WAS Wijendra*, AGG Kaushalya, KGR Kuruppuarachchi, HBC Harshani, WASI Fonseka and R Ramesh
Department of Molecular Biology, Medical Research Institute, Colombo 8, Sri Lanka

Submitted: 03 August 2022; Revised: 27 March 2023; Accepted: 28 April 2023

Abstract: *Listeria monocytogenes* is an important food borne human pathogen associated with severe diseases in humans and animals. Human infection is associated with high mortality rates. Our previous studies done in 2012 on milk and milk products revealed 4b as the dominant serotype followed by 1/2a, 1/2b and 1/2c. Therefore, this study was conducted to determine the sources of contamination and to identify the strain differences (pulsotypes) by pulsed-field gel electrophoresis (PFGE) typing and serotype profiles of *L. monocytogenes* isolates from farm collected raw milk samples from randomly identified locations in Grama Niladhari divisions of the Polonnaruwa district, Sri Lanka. Eighty isolates obtained from raw milk samples were confirmed as *L. monocytogenes* by Polymerase Chain Reaction, belonging to serotypes 4b, 1/2a, 1/2b and 1/2c. The strains were identified by PFGE. PFGE analysis digested with AscI and ApaI enzymes revealed different banding patterns. All the pulsotypes were found to be serotype 4b. The sub typing indicated the diversity of the *Listeria* species. The presence of serotypes 1/2a, 1⁄2b, 1/2c, and 4b in raw milk is a public health concern, as these serotypes are frequently associated with foodborne outbreaks and sporadic cases of human listeriosis. In our study, PFGE analysis allowed discrimination among isolates of the same serogroup. Further PFGE analysis showed heterogeneity among isolates recovered from both same sampling areas and different areas.

Keywords: Polymerase chain reaction, pulsotypes, serogroup, serotypes.

INTRODUCTION

The genus *Listeria* consists of six species including *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri and Listeria grayi* (Sarfraz *et al*., 2017). *L. monocytogenes* is pathogenic for humans and animals whereas *L. ivanovii* is pathogenic to ruminants, and the other species are nonpathogenic (Osman *et al*., 2016). *L. monocytogenes* is a pathogenic bacterial species that can survive under various conditions of refrigeration, freezing, heating, and drying (Jones & D'Orazio, 2013).

Food borne *L. monocytogenes*causes significant outbreaks of Listeriosis in humans, with mortality rates ranging from 9% to 44% (Clark *et al*., 2010). The virulence or the pathogenicity of *L. monocytogenes* appears to be serotype based, with serotypes 1/2a, 1/2c, 1/2b, and 4b being involved in 98% of documented human listeriosis cases (Wiedmann *et al*., 1997; Jacquet *et al*., 2002). Even though thirteen *L. monocytogenes* serotypes have been recognized, only three serotypes (1/2a, 1/2b, and 4b) cause the vast majority of the clinical cases and 4b is the commonest (Tappero *et al*., 1995; Doumith*et al*., 2004).

Listeriosis is one of the most important zoonotic bacterial illnesses with worldwide distribution. The disease has great economic and public health importance. *L. monocytogenes* has been defined as an opportunistic pathogen mainly affecting children, pregnant women, and aged and immune-challenged individuals (Walter *et al*., 2000).

* Corresponding author (wijendra@mri.gov.lk; https://orcid.org/0000-0002-1449-5492)

Only three studies have been reported from Sri Lanka to ascertain the presence of *L. monocytogenes* in our dairy merchandise. These studies revealed the percentage of *L. monocytogenes* contaminated milk samples in Sri Lanka is comparatively higher than other countries (Gunasena *et al*., 1995; Jayamanne *et al*., 2001; Wijendra *et al*., 2014). For the first time, Wijendra *et al.* (2014) have reported circulating serotypes (1/2a, 1/2b, and 1/2c) isolated from milk and milk products in Sri Lanka. The serotypes may potentially be useful in tracing *L. monocytogenes* strains involved in disease out-breaks, however, the value of serotyping in epidemiological investigations is somewhat limited due to the unavailability of scientific data on strain differences among the *L. monocytogenes*. Therefore, it is very important to identify strain differences by using sub typing methods for source tracking and epidemiologic investigations of infection caused by *L. monocytogenes.* The PFGE is considered as the 'gold standard' sub typing method for epidemiological studies due to its high discrimination power, robustness, and reproducibility (Martin *et al*., 2006), with limitations such as being time consuming and labour intensive (usually taking 2–3 days), and the high cost of the equipment (Olive & Bean, 1999).

The aim of this study is to identify the strain differences (pulsotypes) by PFGE typing of the isolates from farm collected raw milk samples in the Polonnaruwa district, Sri Lanka. Further PFGE identified strains of the circulating serotypes should sound an alarm to the stakeholders of public health sector in Sri Lanka, to take appropriate control measures in preventing outbreaks. Milk farmers will be made aware of the microbial quality of their products, which will ensure the production and supply of hygienic, quality raw milk to the collection centres.

MATERIALS AND METHODS

Organisms used

Standard cultures of *L. monocytogenes* ATCC 51776 as the positive control and *L. innocua* ATCC 33090 as the negative control were used.

Sample collection and preparation

Sampling was carried out according to the proportional sampling method (MINITAB14 software, 2009). In the Polonnaruwa District there are seven divisional secretariats. Two hundred raw milk samples were collected from 21 collection centres located in each divisional secretariat and brought to the laboratory under cold chain. These samples may or may not be taken from a single cow. As enrichment, 25 mL of raw milk samples were incubated in 225 mL of Listeria enrichment broth (LEB, Oxoid, England) at 37 °C for 24 h. After 24 h of incubation, 500 µL of suspension was subjected to DNA extraction and also poured onto the Listeria selective agar plates (Listeria selective agar, Oxoid, England), and incubated at 37 °C for 24 h.

Bacterial DNA extraction from enriched milk samples

Total DNA extraction was carried out for all the 150 of enriched milk samples using NORGEN milk bacterial DNA extraction kit (NORGEN BIOTEK, Canada).

Isolation of *Listeria*

As enrichment, 25 mL of raw milk samples were incubated in 225 mL of LEB at 37 °C for 24 h. After the incubation, 500 µL of suspension was streaked on the Listeria selective agar plates and incubated at 37 °C for another 24 h (Lovett *et al*., 1987).

DNA Extraction from cultures

Total DNA extraction was carried out from bacterial cultures of all samples using a commercial bacterial DNA extraction kit (QIAamp DNA Mini Kit, QIAGEN).

PFGE typing and molecular characterization of *L. monocytogenes*

L. monocytogenes isolates were confirmed using the polymerase chain reaction (PCR). According to Lieve *et al*. (1995) with modifications, nested PCR detection was performed. To achieve the optimal sensitivity, 1 µL of the first PCR mixture was used as a template in the second PCR. PCRswere done using Thermal cycler 9600 (Perkin–Elmer Corp.).

Serotyping by PCR

Molecular serotyping for all positive samples of *L. monocytogenes* was performed using multiplex PCR method, according to the Doumith *et al*. (2004). *L. monocytogenes* ATCC 51776, serotype 1/2a was used as the control.

PFGE analysis

PFGE analysis was performed according to the Centers for Disease Control and Prevention PulseNet standardized procedure for *L. monocytogenes* with AscI and ApaI as the restriction endonucleases (Pulse Net USA, 2017), as described previously (Acciari *et al*., 2017). The PFGE typing was carried out in a CHEF-MAPPER apparatus (Bio-Rad Laboratories, Des Plaines, USA). Isolate similarity dendrograms were generated using open-source software Gel J version 2.0. The similarities between the AscI and ApaI macro-restriction profiles (MRPs) were calculated using the Dice coefficient, applying an optimization coefficient and band tolerance of 1.0% for both enzymes. Isolates with a similarity coefficient equal or above 85% were clustered as the same genotype (Heras *et al*., 2015).

RESULTS AND DISCUSSION

Out of 200 raw milk samples, 150 were enriched. After culturing, only 80 samples tested positive for PCR detection. Among the PCR-positive samples, 42.5% were of serotype 4b, while 30% were serotype 1/2b, 10% were serotype 1/2c, and 11.25% were serotype 1/2a. (Figure 1).

Serotyping was done for all the positive samples among the enriched samples by the multiplex PCR method. Amplified fragment combinations were analyzed to identify the serotypes. Identification by this method could not be completed in some of these samples due to the non-amplifications and the presence of non-specific fragments (Figure 1 and Figure 2).

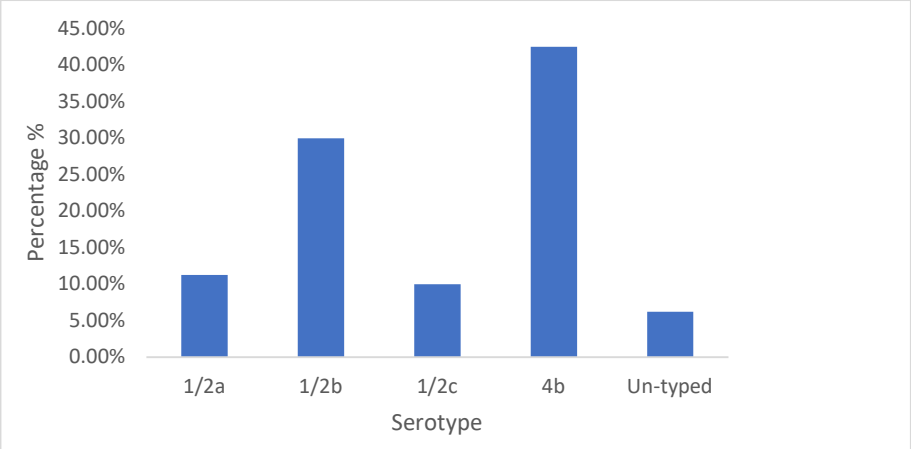


Figure 1: Serotype distributions (%) of *Listeria monocytogenes* in Polonnaruwa District

This study reveals that 53% of the collected raw milk samples were contaminated with *L. monocytogenes*. One major finding is thatthe majority of the isolates found in raw milk samples belong to 1/2b or 4b (Figure 1).

With regard to the distribution of serotype patterns in the areas of the Grama Niladhari divisions (GN divisions), Bakamuna, Madirigiriya, and Sangabodigama had all five serotypes. The areas of Unagalawehera, Nawanagaraya, Nagapokuna, Mahakirimatiyaya, Kaudulla, Kaduruwela, Chandanapokuna, Bisobandaragama and Thuna-Ela showed only a single serotype. Patunugama became a unique area in which no isolates were detected. Apart from that, the isolates from areas Sungawila and Deka-Ela were not typed (Figure 2).

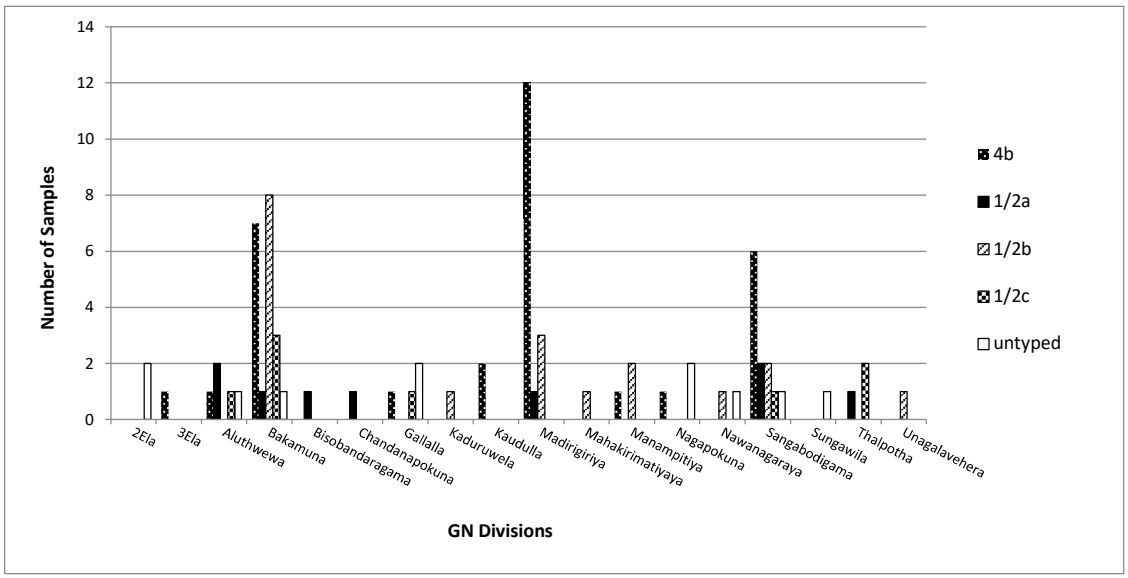
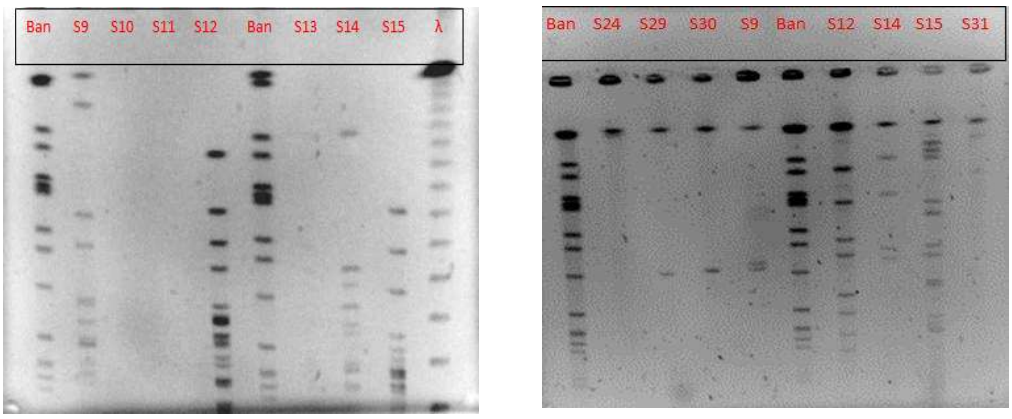


Figure 2: Distribution of serotypes in GN Divisions of Polonnaruwa District

In the PFGE analysis, digestion with AscI and ApaI enzymes revealed different banding patterns (Figure 3a and 3b).



a b

Figure 3: PFGE analysis of Sangabodigama samples a) Digestion with AscI; b) Digestion with ApaI (S- Sangabodigama samples, Ban-Standard culture λ- Ladder)

Additionally, the results of another twenty-three isolates which were digested only with ApaI, revealed that there are 12 different pulsotypes. The overall PFGE results indicated that 11 out of 33 were serotype 4b isolates, and are part of a single pulsogroup (similarity ≥ 85%).

This study revealed that 53% of the raw milk samples had been contaminated with *L. monocytogenes* which agrees with our previous study (Wijendra *et al*., 2014). High to low percentages of prevalence were reported by Waak *et al*. (2002), Vardar-Unlu *et al*. (1998), Hayes *et al*. (1986), Holko *et al*. (2002), Rahimi *et al*. (2010), and Kells and Gilmour (2004). In comparison to the above studies, we observed a higher prevalence of *Listeria* in raw milk. A higher prevalence of pathogens represents a potential risk to consumers of raw milk and raw milk products.

It is important to note here the seriousness of the health risk in supplying contaminated milk to the dairy industry. Such contaminated milk increases the chances of post pasteurization contamination through biofilms (Husu, 1990).

Despite the high importance of diary-foods listeriosis, only a few studies have been reported to determine the presence of *L. monocytogenes* in our dairy products, and also only four suspected cases of listeriosis have been reported in Sri Lanka (Gunasena*et al*., 1995; Jayamanne*et al*., 2001; Wijendra *et al*., 2014). According to their results, the percentage of *L. monocytogenes* contaminated milk samples in Sri Lanka is much higher compared to developed countries. But the determinations of pathogenicity and the presence of virulence factors of the *L. monocytogenes* isolates present in Sri Lanka needs in-depth research.

One major finding of this study was that the majority of the isolates found in raw milk samples belong to 1/2a, 1/2b, and 4b serotypes, and these serotypes are linked with most of the human listeriosis cases (Jersek *et al*., 1996; Ryser *et al*., 1999; Wojciech *et al*., 2004; Liu, 2006; De Santis *et al*., 2007; O'Connor *et al*., 2010). These results have demonstrated that the molecular method used in this study has a good discriminatory capacity for identifying the serovars.

PFGE analysis revealed that there are strain differences among the serotypes. The data obtained from the study indicated that the molecular comparison of these isolates can be used in epidemiological investigations and even to trace outbreak sources in a case where the products may be implicated.

These results also emphasize the need for implementing programmes which will employ molecular identification of pathogenic strains along with measures that may guide infection control in the food industry (Graves & Swaminathan, 2001; Zdolec*et al*, 2019; Warke *et al*., 2023). There may be geographical and other differences in areas of the GN divisions which contribute variation in the prevalence of *L. monocytogenes.* Since the area under study only covered some areas of the GN divisions in the entire district, further study is warranted.

CONCLUSION

The presence of serotype 1/2a, 1⁄2b, 1/2c, and 4b in raw milk is of public health concern, as these serotypes are frequently associated with food borne outbreaks and sporadic cases of human listeriosis. PFGE analysis has identified the different strains of isolates belonging to various serotypes.

In this study, PFGE analysis enables us to distinguish between isolates of the same serogroup, as well as isolates retrieved from the same or different sampling areas. Finally, the obtained data indicated that comparison of these isolates can be used in epidemiological investigations and even to trace outbreak sources in a case where the products may be implicated. These results also emphasize the need to implement programmes thatwill employ molecular identification of pathogenic strains in the food industry along with measures that may guide infection control. However, these pathogens represent a potential risk to consumers of raw milk and raw milk products. We suggest that it may be reasonable to give priority to PFGE analysis of serotype 4b isolates from non-human sources, as the information yielded may be of greater value to public health.

Acknowledgements

The authors wish to convey sincere gratitude and thanks to Medical Research Institute (MRI), Ministry of Health, Sri Lanka, for providing financial assistance in the form of research fund for this research work.

REFERENCES

Acciari *et al.* (15 authors) (2017). *Listeria monocytogenes* in smoked salmon and other smoked fish at retail in Italy: Frequency of contamination and strain characterization in products from different manufacturers. *Journal of Food Protection* **80**: 271–278.
DOI: https://doi.org/10.4315/0362-028X.JFP-15-599

Clark C.G., Farber J., Pagotto F., Ciampa N., Dore K., Nadon C., Bernard K. & Ng L.K. (2010). Surveillance for *Listeria monocytogenes* and listeriosis, 1995–2004. *Epidemiology and Infection* **138**: 559–572.
DOI: https://doi.org/10.1017/S0950268809990914

De Santis E.P.L., Pilo A.L., Cosseddu A.M., Canu N.A., Scarano C. & Marongiu P. (2007). Multiplex PCR for the identification and serotyping of *L. monocytogenes* isolated from sheep cheese–processing plants. *Veterinary Research Communications*. **31**: 359–363.
DOI: https://doi.org/10.1007/s11259-007-0037-0

Doumith M., Cazalet C., Simoes N., Frangeul L., Jacquet C., Kunst F., Martin P., Cossart P., Glaser P. &Buchrieser C. (2004). New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infection and Immunity* **72**:1072–1083.
DOI: https://doi.org/10.1128/IAI.72.2.1072-1083.2004

Graves M.L. & Swaminathan B. (2001). Pulse Net standardized protocol for subtyping *Listeria monocytogenes* by macro restriction and pulsed-field gel electrophoresis. *International Journal of Food Microbiology* **65**: 55–62.
DOI: https://doi.org/10.1016/s0168-1605(00)00501-8

Gunasena D.K., Kodikara C.P., Ganepola K. & Widanapathirana S. (1995). Occurrence of *Listeria monocytogenes* in food in Sri Lanka. *Journal of National Science CouncilSri Lanka* **23**(3): 107– 114.
DOI: http://dx.doi.org/10.4038/jnsfsr.v23i3.5848

Hayes P.S., Feeley J.C., Graves L.M., Ajello G.W. & Fleming D.W. (1986). Isolation of *Listeria monocytogenes* from raw milk. *Applied Environmental Microbiololy* **51:** 438–440.
DOI: https://doi.org/10.1128/aem.51.2.438-440.1986

Heras J., Dominguez C., Mata E., Pascual V., Lozano C., Torres C. &Zarazaga M. (2015). GelJ-a tool for analyzing DNA fingerprint gel images. *BMC Bioinformatics* **16**: 270.
DOI: https://doi.org/10.1186/s12859-015-0703-0

Holko I., Urbanova J., Kantikova M., Pastorova K. & Kmet' V. (2002). PCR Detection of *Listeria monocytogenes* in milk and milk products and differentiation of suspect isolates. *Acta Veterinaria Brunensis* **71**: 125–131.
DOI: https://doi.org/10.2754/avb200271010125

Husu J. (1990). Epidemiological and experimental studies of *Listeria* infections with special reference to faecal excretion in ruminants, contaminations of fresh milk, presence of the silage and growth at low temperatures. *Dissertation*, College of Veterinary Medicine, Helsinki, Finland.

Jacquet C., Gouin E., Jeannel D., Cossart P. & Rocourt J. (2002). Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. *Applied Environmental Microbiology* **68**(1): 616–622.
DOI: https://doi.org/10.1128/AEM.68.2.616-622.2002

Jayamanne V.S. & Samarajeewa U. (2001). Incidence and detection of *Listeria monocytogenes* in milk and milk products of Sri Lanka. *Tropical Agricultural Research* **13**: 42–50

Jersek B., Tcherneva E., Rijpens N. & Herman L. (1996). Repetitive element sequence-based PCR for species and strain discrimination in the genus *Listeria*. *Letters in Applied Microbiology* **23**: 55–60.
DOI: https://doi.org/10.1111/j.1472-765x.1996.tb00028.x

Jones G.S. & D'Orazio S. (2013). *Listeria monocytogenes*: Cultivation and laboratory maintenance. *Current Protocols in Microbiology* **31(**9B): 2.1–2.7.
DOI: https://doi.org/10.1002/9780471729259.mc09b02s31

Kells S. & Gilmour A. (2004). Incidence of *Listeria monocytogenes* in two milk processing environments and assessment of *Listeria monocytogenes* blood agar for isolation. *International Journal of Food Microbiology* **91**:167–174.
DOI: https://doi.org/10.1016/S0168-1605(03)00378-7

Lieve M.H., De Block J.H. &Moermans R.J. (1995). Direct Detection of *Listeria monocytogenes* in 25 Milliliters of Raw Milk by a Two–Step PCR with Nested Primers. *Applied Environmental Microbiology* **61**(2): 817–819.

Liu D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology* **55**: 645–659.
DOI: https://doi.org/10.1099/jmm.0.46495-0

Lovett J., Francis D.W. & Hunt J.M. (1987) Listeria in raw milk: Detection, incidence and pathogenicity. *Journal of Food Protection* **50**: 188–192.
DOI: https://doi.org/10.4315/0362-028X-50.3.188

Martin P., Jacquet C., Goulet V., Vaillant V. & De Valk H. Participants in the PulseNet Europe Feasibility Study (2006). Pulsed-field gel electrophoresis of *Listeria monocytogenes*strains: the PulseNet Europe Feasibility Study. *Foodborne Pathogen Disease* **3**: 303–308.
DOI: https://doi.org/10.1089/fpd.2006.3.303

Michael Evans University of Toronto (2009). *MINITAB Manual.* W.H. Freeman and Company, New York, USA.

O'Connor L., O'Leary M., Leonard N., Godinho M., O'Reilly C., Egan J. & O'Mahony R. (2010). The characterization of *Listeria* spp. isolated from food products and the food-processing environment. *Letters in Applied Microbiology* **51**(5): 490–498.
DOI: https://doi.org/10.1111/j.1472-765X.2010. 02928.x

Olive D.M. & Bean P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology* **37**(6): 1661–1669.
DOI: https://doi.org/10.1128/JCM.37.6.1661-1669.1999

Osman K.M., Samir A., Abo-Shama U.H., Mohamed E.H., Orabi A. & Zolnicov T. (2016). Determination of virulence and antibiotic resistance pattern of biofilm producing Listeria species isolated from retail raw milk. *BMC Microbiology* **16**(1): 263.
DOI: https://doi.org/10.1186/s12866-016-0880-7

PulseNet-International (2013). *Standard Operating Procedure for PulseNet PFGE ofListeria monocytogenes.* Available at *http://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.pdf,* Accessed 21 November 2019.

Rahimi E., Ameri M. & Momtaz H. (2010). Prevalence and antimicrobial resistance of *Listeria* species isolated from milk and dairy products in Iran. *Food Contro l***21**: 1448–1452.
DOI: https://doi.org/10.1016/j.foodcont.2010.03.014

Ryser T. & Marth E. (1999). *Listeria, Listeriosis and Food Safety*, 2nd edition.. Marcel Dekker Inc., New York, USA.

Sarfraz M., Ashraf Y. & Ashraf S. (2017). A Review: Prevalence and antimicrobial susceptibility profile of listeria species in milkproducts.*Matrix Science Medica* **1**(1): 03–09.
DOI: https://doi.org/10.26480/msm.01.2017.03.09

Tappero *et al.* (29 authors) (1995). Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts? *Journal of the American Medical Association* **273**:1118–1122.
DOI: https://doi.org/10.1001/jama.1995.03520380054035

Vardar-Unlu G., Candan F., Sokmen A., Daferera D., Polissiou M., Sokmen M., Donmez E. & Tepe B. (2003). Antimicrobial and antioxidant activity of the essential oil and methanol extracts of Thymus pectinatus Fisch. etMey. Var. pectinatus (Lamiaceae). *Journal of Agricultural and Food Chemistry* **51**: 63–67.
DOI: https://doi.org/10.1021/jf025753e

Waak E., Tham W. & Danielsson-Tham M.L. (2002). Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in *farm* bulk tanks and in dairy plant receiving tanks. *Applied Environmental Microbiology* **68**: 3366–3370.
DOI: https://doi.org/10.1128/AEM.68.7.3366-3370.2002

Walter F.S. & Acheson D. (2000). Foodborne listeriosis. *Clinical Infectious Diseases*. **31**(3): 770–775.
DOI: https://doi.org/10.1086/314008

Warke S.R., Ingle V.C. & Kurkure N.V. (2023). PFGE analysis of *Listeria monocytogenes* isolates from milk, bovine clinical and bovine environment origin from Nagpur. *The Indian Journal of Veterinary Sciences and Biotechnology* **19**(2): 78–82.
DOI: https://doi.org/10.48165/ijvsbt.19.2.15

Wiedmann M., Bruce J.L., Keating C., Johnson A.E., McDonough P.L. & Batt C.A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity* **65**: 2707–2716.
DOI: https://doi.org/10.1128 / iai.65.7.2707-2716.1997

Wijendra W.A.S., Kulathunga K.A.C.C. & Ramesh R. (2014). First report of *Listeriamonocytogenes* serotypes detected from milk and milk products in Sri Lanka. *Advances in Animal and Veterinary Sciences* **2**:11–16.
DOI: https://doi.org/10.14737/journal.aavs/2014/2.5s.11.16

Wojciech L., Kowalczyk K., Staroniewicz Z., Kosek K., Molenda J. & Ugorski M. (2004). Genotypic characterisation of *Listeria monocytogenes* isolated from foodstuffs and farm animals in Poland. *Bulletin of the Veterinary Institute in Pulawy* **48**: 427–435.

Zdolec N., Jankuloski D., Kiš M., Hengl B. & Mikulec N. (2019). Detection and pulsed-field gel electrophoresis typing of *Listeria monocytogenes* isolates from milk vending machines in Croatia. *Beverages* **5**(3):46.
DOI: https://doi.org/10.3390/beverages5030046