

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

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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 *Apal* 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).

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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 (Gunaseena *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. PCRs were 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 *Apal* 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 *Apal* 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).

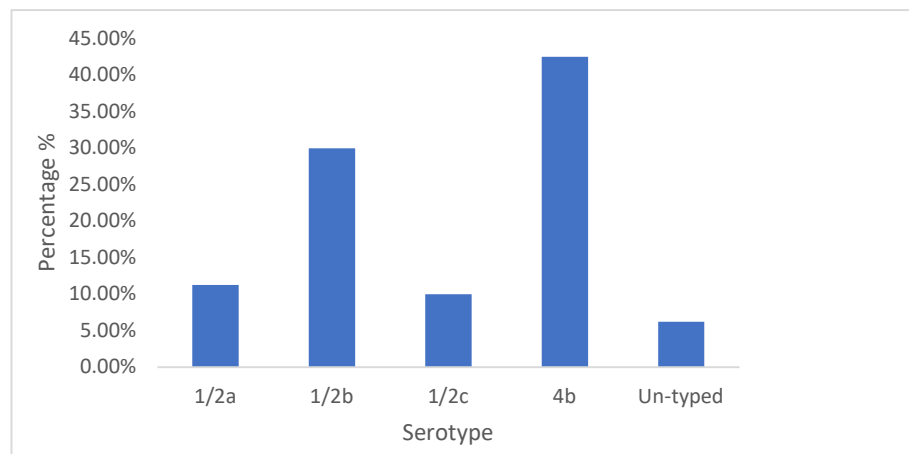


Figure 1: Serotype distributions (%) of *Listeria monocytogenes* in Polonnaruwa District

This study reveals that 53% of the collected raw milk samples were contaminated with *L. monocytogenes*. One major finding is that the 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).

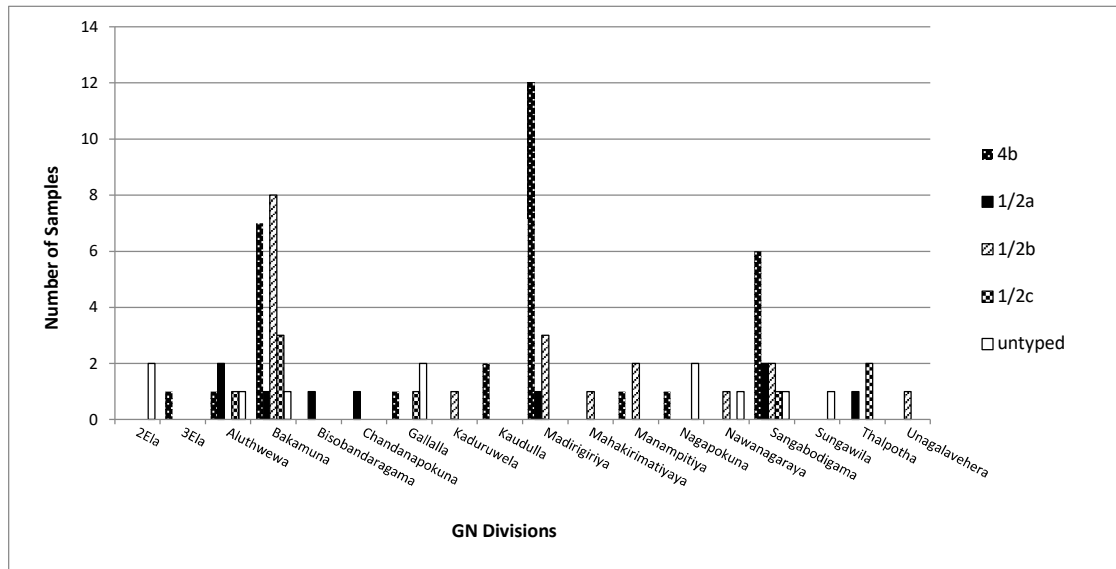


Figure 2: Distribution of serotypes in GN Divisions of Polonnaruwa District

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

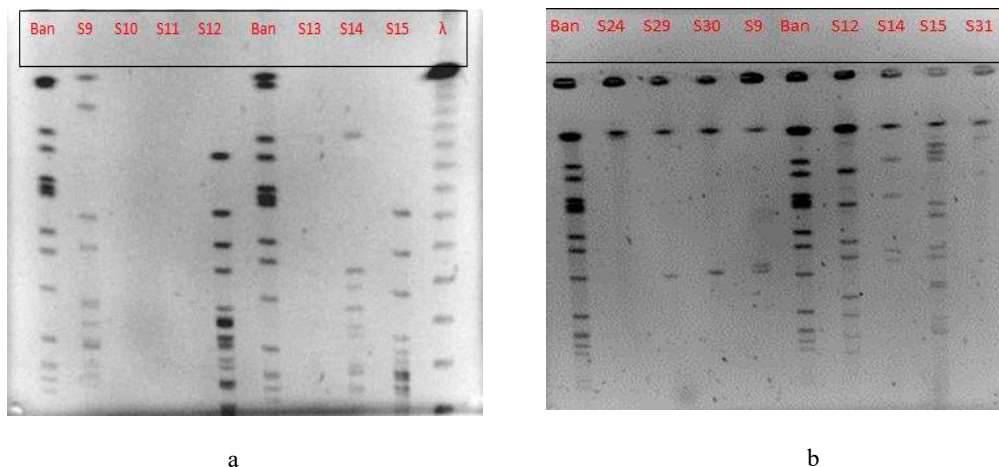


Figure 3: PFGE analysis of Sangabodigama samples a) Digestion with *AscI*; b) Digestion with *ApaI* (S- Sangabodigama samples, Ban-Standard culture λ- Ladder)

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 $\geq 85\%$).

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 dairy-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 (Gunasekera *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; Zdolet *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 that will 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.

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