



## Research Article

### First Report of *Listeria monocytogenes* Serotypes Detected from Milk and Milk Products in Sri Lanka

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#### ABSTRACT

*Listeria monocytogenes* is the causative organism of listeriosis, which primarily affects immune-compromised individuals, including pregnant women. Contamination of milk and milk products with *L. monocytogenes* is a serious problem to the world and to the developing countries like Sri Lanka. Even the presence of low numbers of *L. monocytogenes* is a potential risk since this organism is capable of multiplying at ambient and under refrigerated conditions. The main aim of the study is to detect the circulating serotypes in dairy industries using molecular methods and primarily to trace the lineage of these serotypes with a focus on food safety and disease prevention. Altogether total of 266 samples from raw milk, pasteurized milk, ice cream, curd, yogurt and cheese were collected from many parts of the country, tested for the presence of *L. monocytogenes* and studied the serotype. *L. monocytogenes* was detected in all types of the above-mentioned milk and milk products. Out of total *L. monocytogenes* strains detected by PCR, 61.51%, 11.53% and 4% belong to serotypes 1/2a (or 3a) [lineage II], 1/2b (or 3b) [lineage I] and, 1/2c (or 3c) [lineage II] respectively. It has been observed previously that serotypes (1/2a), 1/2b) isolated from raw milk samples been linked to multiple human listeriosis outbreaks. This study revealed the serotypes of *L. monocytogenes* circulating in Sri Lanka, thus makes an alarm to the health authorities to be more vigilant about the probable outbreaks of listeriosis in Sri Lanka. This is the first report on circulating *L. monocytogenes* serotypes in Sri Lanka.

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#### INTRODUCTION

*Listeria monocytogenes* is a pathogenic bacterium that can cause Listeriosis in humans and various animal species. In humans, foodborne *L. monocytogenes* causes large outbreaks of Listeriosis, with a mortality rate of 9% to 44% (Clark et al., 2010). The primary mode of infection is through the ingestion of contaminated food. Therefore, contamination of milk and milk products with *L. monocytogenes* is a serious problem to the world (Kozak et al., 1996; Donnelly, 2004; Rudolf and Siegfried 2001; Conly and Johnston, 2008; Pan et al. 2006; Molla et al., 2004).

Different strains of *L. monocytogenes* express different antigenic determinations, thus each strain can be serologically identified. *L. monocytogenes* is divided into at least 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) (Seeliger and Jones, 1986; Kathariou, 2002; Kerouanton et al. 2010; Kasalica et al., 2011). The virulence of *L. monocytogenes* seems to be serotype dependent 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).

The largest listeriosis outbreak in U.S. history occurred in 2011, (CDC MMWR, 2011). Two large outbreaks in human population were reported from California, (Linna et al., 1988), and Switzerland, (Bille, 1990). Another listeriosis outbreak occurred in Switzerland during 2005 (Bille et al., 2006). Two outbreaks of listeriosis were reported in Finland, in 1998 and 1999 (Lyytikäinen et al., 2000; Mäijälä et al., 2001).

*L. monocytogenes* can now be further classified into three evolutionary lineages. Lineage I encompasses serotypes 1/2b, 3b, 4b, 4d and 4e, lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c; and lineage III comprises serotypes 4a, 4c as well as 4b (Brosch et al., 1994; Graves et al., 1994; Rasmussen et al., 1995; Chen and Knabel, 2007).

Only two studies have been reported in Sri Lanka to ascertain the presence of *L. monocytogenes* in our dairy products. According to their results the percentage of *L. monocytogenes* contaminated milk samples in Sri Lanka is much higher compared to developed countries (Gunasena et al., 1995 and Jayamanne and Samarajeewa, 2001). Only four suspected cases of listeriosis have been reported in Sri Lanka so far (Wijesundera et al., 1992, Withana and

Mirando, 1967). There is no information about the circulating serotypes in Sri Lanka so far.

The main aim of our study is to detect the circulating serotypes in dairy industries using molecular methods and primarily to trace the lineage of these serotypes, as these serotypes are responsible for outbreaks and epidemics.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*L. monocytogenes* ATCC 51776 and *L. innocua* ATCC 33090 was used in the sensitivity & specificity studies respectively. All the bacterial strains were grown on blood agar (Oxoid-UK) at 37°C for 24 hrs. and sub cultured in Brain Heart Infusion (BHI) broth. Listeria Enrichment broth (LEB) from Oxoid-UK was used for enrichment and the Listeria selective agar medium, (Oxoid-UK) was used for selective plating. Stock cultures were made using skimmed milk (William et al., 2008).



Figure I: Locations of the sample collection

### Sample Collection

Samples were collected randomly from different parts of the country (Figure I). A total of 154 samples of raw and pasteurized milk samples were collected from farms and milk collecting centers and 112 samples of milk products such as cheese, curd, yogurt and ice cream were obtained from retail outlets including supermarkets, farm shops, groceries, milk booths and public markets (Table I).

### Enrichment and DNA Extraction

A total of 25 g or 25 ml of dairy samples were incubated in 225 ml of LEB at 30 ± 1 °C for 24 and 48 hrs. After 24 hrs. 500µL of suspension was extracted according to Gerrit et al (2005) with modifications include three washing steps using PBS. Extracted DNA was kept at -20°C for further analysis.

### PCR Amplifications:

Nested PCR detection was performed according to Lieve et al., 1995. As a modification, to achieve the optimal sensitivity 5 µL, 2 µL and 1 µL of the first PCR mixture were used as template in the second PCR. Thermal cyclor 9600 (Perkin-Elmer Corp.) was used to run the PCR reactions.

### Serotyping by PCR

Serotyping was done according to Borucki and Call, (2003).

### Statistical Analysis

Chi-square test analysis was performed and differences were considered significant at values of P<0.05.

## RESULTS

### Sensitivity of the Nested PCR

The specificity of the PCR primers has previously been validated (Chiba *et al* (1998), Border *et al*) but the sensitivity was reassessed in our laboratory by amplification of 10-fold serial dilutions in milk by spiking axenically grown *L. monocytogenes* cells. The minimal amount of *L. monocytogenes* that could be reliably detected was 1cfu. To remove the presence of potential PCR inhibitor(s) in the milk samples, washing steps were added in the DNA extraction as a modification. One micro liter of first PCR gave the optimal sensitivity in the nested PCR.

Table I: Details of sample collections

District	Sites	Number of samples collected in each type of Dairy products						Total
		Raw milk	Pasteurized Milk	Ice cream	Curd	Yoghurt	Cheese	
Colombo	Homagama	10	2	2	2	2	2	20
	Piliyandala	05	5	2	2	2	2	18
Gampaha	Mirigama	10	2	2	2	2	2	20
	Badalgama	05	5	2	2	2	2	18
Galle	Arachchikanda	10	2	2	2	2	2	20
	Batapola	05	5	2	2	2	2	18
Hambantota	Tissamaharamaya	10	2	2	2	2	2	20
	Hambantota	05	5	2	2	2	2	18
Anuradhapura	Godage Mawatha	10	2	2	2	2	2	20
	Wijayapura	05	5	2	2	2	2	18
Polonnaruwa	Sewagama	10	2	2	2	2	2	20
	Bediwewa	05	5	2	2	2	2	18
Kandy	Pilimathalawa	10	2	2	2	2	2	20
	Gatambe	05	5	2	2	2	2	18
<b>Total</b>		<b>105</b>	<b>49</b>	<b>28</b>	<b>28</b>	<b>28</b>	<b>28</b>	<b>266</b>

Figure II: Positive findings of *L. monocytogenes* from Dairy products by PCR

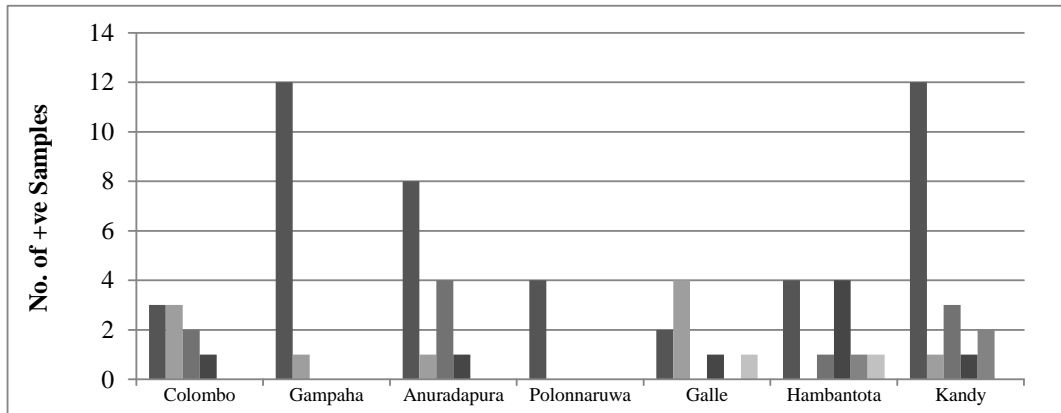
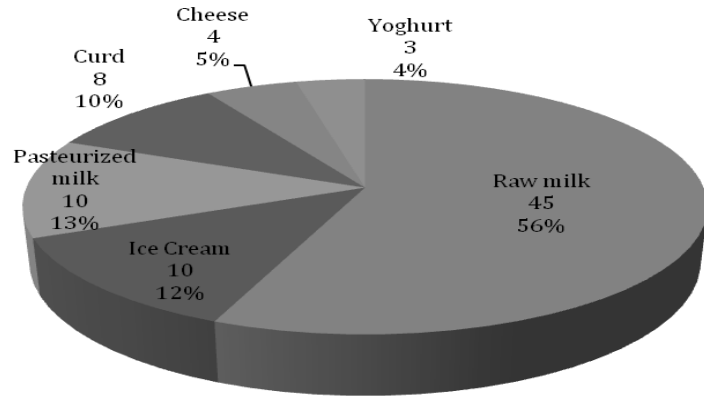


Figure III: No. of samples detected +ve for *Listeria* by PCR at district level

Figure IV: Distribution of raw milk contamination

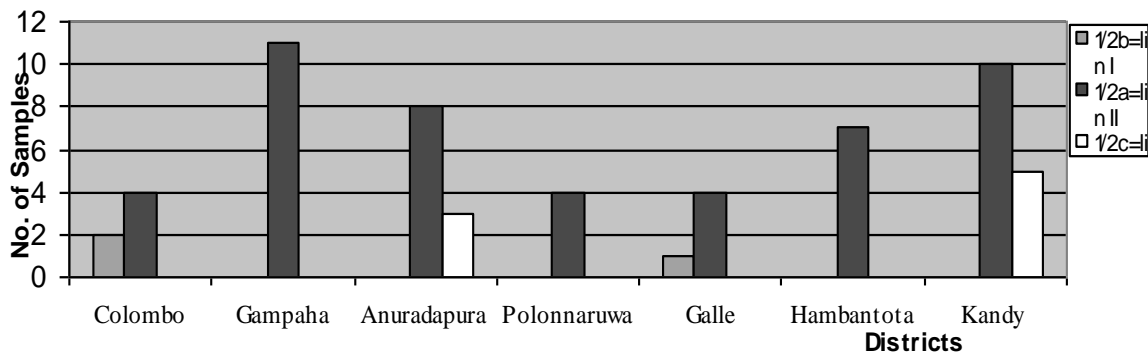
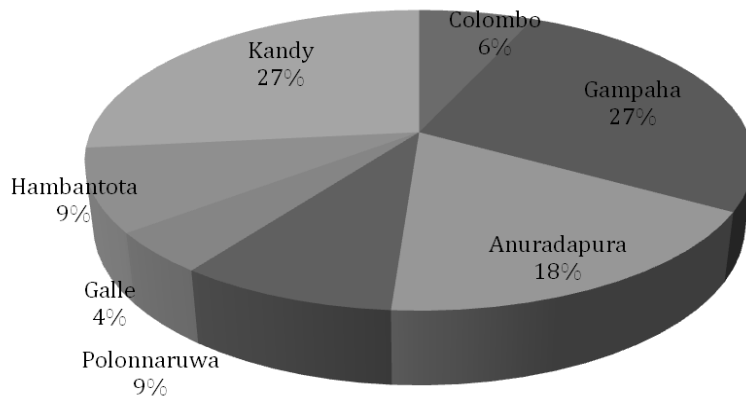


Figure V: Serotypes detected at district level (lin. means lineage)

In the present study, a total of 266 raw milk and dairy product samples from all over the country were examined. The study revealed 45 samples of (41.19%) raw milk, 10 samples of (35.71%) ice cream, 10 samples of (18.36%) pasteurized milk, 8 samples of (28.57%) curd, 4 samples of (14.2%) cheese and 3 samples of (10.71%) yoghurt were contaminated with *L. monocytogenes* (Figure II). The number of samples contaminated by *L. monocytogenes* found to be significant with the type of dairy products. Although *Listeria* contamination was generally found in all types of

dairy products in all districts, numbers of raw milk samples contaminated at district levels were found to be significant. However, numbers of other dairy products contaminated at district level were not significant (Figure III). With regard to the distribution of raw milk contamination it was observed that the highest contamination was found in districts of Kandy and Gampaha (26%). Moderate amount of raw milk contaminants were observed in Anuradhapura (17%), Polonnaruwa (9%), Hambantota (9%) & Colombo (7%) districts. In contrast lowest contamination occurred in districts of Galle (4%) (Figure IV).

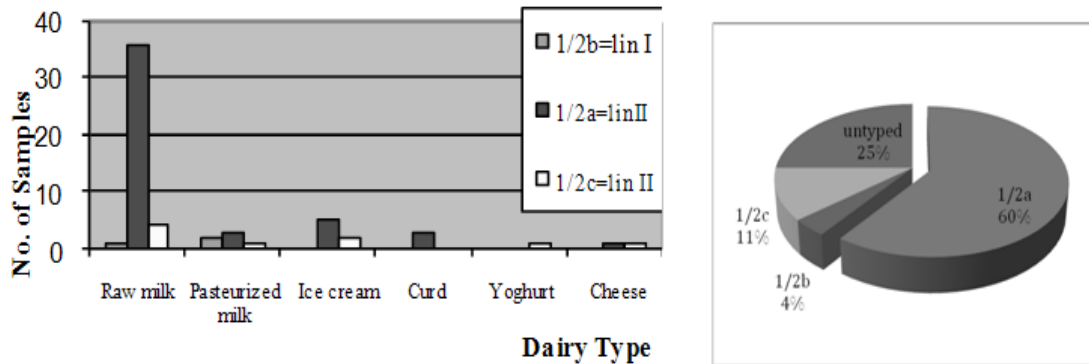


Figure VI: Distribution of serotypes in each dairy type

\*Table II: Distribution of serotypes in each dairy type

Dairy Type	No (%) of Serotype (by molecular method)			No. of un-typed
	1/2a	1/2b	1/2c	
Raw milk	36 (80%)	01 (2.2%)	04 (8.9%)	04 (8.9%)
Ice Cream	05 (50%)	00	02 (20%)	03 (30%)
Pasteurized milk	03 (30%)	02 (20%)	01 (10%)	04 (40%)
Curd	03 (37.5%)	00	00	05 (62.5%)
Cheese	01 (25%)	00	01 (25%)	02 (50%)
Yogurt	00	00	01 (33.3%)	02 (66.7%)
Total	48 (60%)	03 (4%)	09 (11%)	20 (25%)

\*Values are significant at p<0.05

### Molecular Serotyping

The distribution pattern of 1/2a (or 3a) serotype was 36 (81.81%) in raw milk, 3 (33.33%) in pasteurized milk, 5 (50%) in ice cream, 3 (37.5%) in curd and 1 (25%) in cheese while the pattern for the 1/2b (or 3b) serotype was 1 (2.27%) in raw milk and 2 (22.22%) in pasteurized milk, further the pattern for 1/2c (or 3c) serotype was 4 (8.8%) in raw milk, 1 (10%) in pasteurized milk, 2 (20%) in ice cream, 1 (25%) in cheese and 1 (33.33%) in yogurt. The statistical analysis shows that the numbers of different serotypes found in various dairy products were significant. Details are given in (Table II and Figure V).

The most prevalent serotype 1/2a (or 3a) was found in all districts. Apart from 1/2a (or 3a), 1/2b (or 3b) serotype was detected in Colombo, Galle districts and 1/2c (or 3c) serotype found in Anuradhapura, Kandy districts. In districts of Gampaha, Polonnaruwa and Hambantota only 1/2a (or 3a) serotype was found. The numbers of serotype distribution among districts were significant. Lineage I & II were found in both Colombo & Galle districts but in all other districts lineage II was found exclusively (Figure VI).

### DISCUSSIONS

Dairy products are known to be one of the vehicles through which *L. monocytogenes* is transferred to human. Therefore many countries have initiated a zero tolerance policy prohibiting the sale of processed ready-to-eat (RTE) food products contaminated with *L. monocytogenes*. This policy designates *L. monocytogenes* as a contaminant. Previous studies have shown that *L. monocytogenes* has a very low occurrence in dairy products (Jalali and Abedi, 2007, Moshtaghi and Mohamadpour, 2007). Anyhow in our study as we used molecular detection method with high sensitivity (1 cfu detected) very low level of *L. monocytogenes* contaminants were able to be detected in the variety of dairy products.

Our results revealed higher number of *L. monocytogenes* (42%) detected from raw milk samples in comparison to other countries like 0% in center of Iran (Jalali and Abedi, 2008) 0.018% in United States (Frye and Donnelly, 2005) 1.1% in England and Wales (Greenwood et al., 1991) and 1.6% in west of Iran (Moshtaghi and Mohamadpour, 2007). In contrast Pintado et al., (2005) reported higher rate of incidence (up to 46%) in Portugal. These differences may be due to varying environmental condition between different

locations as well as method of detection. These figures cautious those hygienic conditions of milking and the subsequent manipulations in our country are substandard.

Isolation of *L. monocytogenes* from ice cream (35.7%) in this study is another proof that this microorganism can survive freezing temperatures (Cordano and Rocourt, 2001). Increased contamination of ice cream by this bacterium is explained by suitable conditions for its growth, such as pH value, water activity, availability of nutrients and storage temperature (Molla et al., 2004).

Significant amount (20.4%) of contamination of *L. monocytogenes* of pasteurized milk due to the presence of abnormally high pathogen load in the raw milk may have overwhelmed the pasteurization process (Jayamanne and Samarajeewa, 2010.) or that localization of some *L. monocytogenes* within bovine cells may have protected them from heat inactivation (Swaminathan, 2001). In Massachusetts, the listeriosis outbreak in 1983 was associated with consumption of pasteurized milk (Fleming et al., 1985).

In fermented products such as curd, yogurt, *L. monocytogenes* has the ability to survive during production process and storing. Further, low amount of *Listeria* contaminants were found in yogurt (10.71%) and cheese (14.28%) in this study. The survival of *L. monocytogenes* in yogurt depends on the sample acidity and this bacterium disappears when the pH falls to 3.5 (Vermeulen et al., 2007).

When consider about the distribution pattern of *Listeria* contamination of raw milk the highest contamination occur in selected areas of Kandy and Gampaha district (wet zone). It may be due to the high moisture content of the soil. Previous studies also indicated that the favourable places for *Listeria* growth is high moisture content in the environments where the organism has had opportunity to incubate (Bunning et al., 1992). Apart from this, in coastal areas such as Galle, Hambantota and dry zone areas such as Anuradhapura, Polonnaruwa encountered moderately low amount of raw milk contaminants due to the high salinity and low moisture content of the environment respectively. Previous studies indicated that the growth of *L. monocytogenes*; the pH values ranged from 5.96 - 5.10, with a mean of 5.48; the salt concentrations ranged from 6.30% - 3.20%, and the moisture levels averaged 37.79% (Kongo et al., 2006).

In this study serogrouping by PCR suggests serotypes belonging to phylogenetic lineage of division I comprises serogroups 1/2b (or 3b) and division II comprises 1/2a (or 3a), 1/2c (or 3c). Isolates within this group appeared in all dairy types. It was found that serotype 1/2b (or 3b) was rare with only one positive in raw milk and two positives being picked up in pasteurized milk. This is in line with most studies that have found serotypes 1/2a and 1/2b was to be the most common serotypes in food (Aarnisalo et al., 2003; Wallace et al., 2003; Cetinkaya et al. 2014).

Although 13 serovars of *L. monocytogenes* have been described, 3 serotypes (1/2a, 1/2b and 4b) account for the majority of clinical cases (Borucki and Call 2003; De Santis et al. 2007). The majority of strains, which have caused large outbreaks, are serovars 1/2a and 1/2b (Kathariou, 2000; Jacquet et al., 2002; Zhang et al., 2007 and Chen and Knabel, 2007). The results of this study demonstrated that *L. monocytogenes* 1/2a (or 3a) was the most frequently isolated

serotype from the raw milk samples (81.81%). In our study, three strains were found to belong to serogroup 1/2b (or 3b), which is the serotype most commonly causing human listeriosis. As our findings include serotypes 1/2a and 1/2b, occurring large outbreaks in future could be predicted in Sri Lanka.

One major finding in this study is that the majority of the isolates found in dairy samples belong to lineage II [1/2a (or 3a), 1/2c (or 3c)] this is in line with the previous studies (Gray et al., 2004; Ward et al., 2004).

This study alarms us the possible out breaks of *Listeriosis*. The presence of serotype of 1/2a & 1/2b further supports this view. From a public health standpoint, it is extremely important to identify the contaminated food vehicle and remove it from food distribution channels as rapidly as possible.

This is the first report in Sri Lanka on serotypes circulating in the country, which was identified from the dairy products representing different parts of the country. These findings open the avenue for more research on circulating serotypes and their lineages. The identification and characterization of *L. monocytogenes* lineages with unique epidemiological, ecological, genetic, and phenotypic characteristics also has important practical implications. For example, it is increasingly apparent that representatives of all lineages need to be included in strain sets that are used to evaluate new detection and sub typing methods. The identified serotypes could be used in genetic analysis and lineage studies, which in turn shed new light on Sri Lankan, isolate characteristics.

Further this study will help in formulating National *Listeria* Risk Assessment guidelines for dairy industry in Sri Lanka. In future similar study will be done in human samples thus different serotypes of *L. monocytogenes* prevailing in the Sri Lankan population can be identified which in turn will help us to investigate the molecular epidemiology of the disease listeriosis in the future.

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