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Incidence and Detection *of Listeria monocytogenes* in Milk and Milk Products of Sri Lanka

V.S. Jayamanne and U. Samarajeewa[1]

Postgraduate Institute of Agriculture
University of Peradeniya
Peradeniya, Sri Lanka

ABSTRACT. Listeria monocytogenes is a relatively recent food-borne pathogen reported mostly from developed countries. Listeriosis, the illness caused by the pathogenic virulent strains of the microorganism affects mostly the pregnant, older adults, newborns, and immuno-suppressed adults. Milk and milk products have been reported to harbor L. monocytogenes. This study investigates the presence of L. monocytogenes in milk and milk products of Sri Lanka. Cow milk (55), goat milk (30), pasteurized milk (30), sterilized milk (30), UHT milk (30), cheese (30), curd (30) and yogurt (30) samples were tested for L. monocytogenes using Oxoid Listeria Enrichment Broth and Oxoid Modified Oxford Medium. The presence of Listeria was confirmed by morphological (gram staining and microscopy) and biochemical (catalase, TSI, MR and VP test, H_2S production, hydrolysis of aesculin, and growth on urea agar) tests. Virulent strains of L. monocytogenes were differentiated by testing for hemolysis of sheep blood agar.

Of the samples tested (265), 39 samples (15%) contained virulent L. monocytogenes. Cow milk (29%), goat milk (27%), pasteurized milk (17%), and cheese (33%) samples contained virulent strains of L. monocytogenes. Sterilized milk, UHT milk, curd and yogurt did not contain L. monocytogenes. Commonly used pasteurization protocols appear to be inadequate at times to destroy Listeria in milk products. The absence of Listeria in sterilized and UHT milk suggests that sterilization or UHT treatment ensure total destruction of Listeria. The percentage of Listeria contaminated milk samples in Sri Lanka is much higher compared to developed countries. Regulatory bodies of Sri Lanka should pay immediate attention to control this pathogen.

INTRODUCTION

Listeria monocytogenes is a food-borne pathogen of recent concern to the food industry (Armstrong, 1985; Brackett, 1988). *Listeria* is a gram positive, cold tolerant, non-spore forming and non-acid fast, rod-shaped bacterium, which is often found in soil and water (Seeliger and Welshimer, 1975), and feces of animals (Weis and Seeliger, 1975). The illness caused on ingestion of virulent pathogenic strains of *L. monocytogenes* is called Listeriosis. *Listeria* has emerged as a dangerous intracellular food-borne pathogen during the past two decades, and there have been reports of *Listeria* outbreaks associated with dairy products (Fleming *et al.*, 1985; Marth and Ryser, 1990; Farber and Peterkin, 1991). Pregnant women and newborns, older adults, and people with weakened immune systems due to AIDS, diabetes, cancer, and kidney diseases, are at a high risk on exposure to *L. monocytogenes*. The mortality rate of the disease is approximately 25% (USDA, 1999).

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1 Department of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka.

Infected pregnant women may experience only mild flu-like illness, and the illness can be transmitted from mother to the fetus through the placenta leading to miscarriage, stillbirth, or serious health problems for the newborn child. Cow milk has been implicated in food-borne fatal Listeriosis (Fleming *et al.*, 1985). The ability of *L. monocytogenes* strains to proliferate in raw milk at ambient and under refrigerated conditions is well documented (Donnelly and Briggs, 1986; Lovett *et al.*, 1987; Rosenow and Marth, 1987; Slade *et al.*, 1988). *Listeria* is said to be slightly more resistant to heat processing than other bacteria, especially when buffered by proteinaceous foods.

Only four suspected cases of Listeriosis have been reported in Sri Lanka. The first was two children suffering from meningitis (Jayasundara *et al.*, 1962; Withana and Mirando, 1967), and in the other two cases (children) headache, fever and a rash have been reported (Wijesundera *et al.*, 1992).

Though milk and milk products were reported to transmit food-borne Listeriosis, only one study has been reported in Sri Lanka to ascertain the presence of *Listeria* in our dairy products. A study carried out by Gunasena *et al.* (1995), on the occurrence of *L. monocytogenes* in market samples of different food items, indicated that 38% of the samples contained *L. monocytogenes*. Of them 49% of vegetables, 34% of chicken and 26% of dairy products were contaminated.

The objective of this study is to investigate the possible presence of *L. monocytogenes* in cow milk, goat milk, pasteurized milk, sterilized milk, ultra high temperature (UHT) milk, yogurt, buffalo curd, and cheese produced and processed in Sri Lanka and to observe the hemolytic activity of the isolates.

MATERIALS AND METHODS

Collection of samples

Samples of cow milk (55), goat milk (30), pasteurized milk (30), sterilized milk (30), UHT milk (30), curd (30), yogurt (30), and locally produced soft cheese (30) were collected aseptically. Cow milk was collected from farms and milk collecting centres in Halloluwa, Kulugammana, Menikhinna, Pilimatalawa and Polgahamula in the Kandy district and Kamburupitiya and Thihagoda in the Matara district. Goat milk was collected from small-scale farms in Matara and the research farm of the Faculty of Agriculture, University of Ruhuna, Matara. Cow and goat milk were collected into McCartney bottles and pipettes heat-sterilized at 170°C for 2 h. Pasteurized milk of two brands, sterilized milk of one brand, and UHT milk of three brands were purchased from markets in Colombo, Kandy, Matara and Peradeniya. Buffalo curd pots were purchased from Peradeniya, Kandy, Matara and Galle. Two brands of locally made soft cheese were also tested.

Raw cow milk and goat milk were collected over a 6 month period and tested. Pasteurized milk, sterilized milk, UHT milk, curd, yogurt and cheese samples were collected and tested over a 3 month period so that the samples represent different batches. All samples were collected and transported aseptically to the laboratory and stored at 3±2°C pending analysis. Cow milk, goat milk, curd samples collected from Matara, Kamburupitiya and Galle were transferred aseptically to McCartney bottles containing Listeria Enrichment Broth (Oxoid) immediately and transported to the laboratory.

Pasteurized milk, sterilized milk, UHT milk, yogurt and cheese samples were transported to the laboratory in their original packages intact.

Microbiological tests

Listeria in milk was enriched in FDA Listeria Enrichment Broth (LEB) (Oxoid). Samples (25 ml or g) were blended with Listeria Enrichment Broth (225 ml) at 12000 rpm for 2 min in a Waring blender. The resulting solution was incubated at 35°C for 48 h for selective enrichment. The selective isolation of *Listeria* was done in Modified Oxford (MOX) agar (Oxoid). Enriched samples were next streaked onto MOX agar plates and incubated at 35°C and examined for colonies after 24, 48 and 72 h. Single colonies growing on the MOX agar were isolated and transferred to MOX agar slants in McCartney bottles and incubated (35°C) until there was a sufficient growth. The MOX agar slants were stored in the refrigerator (3±2°C) pending confirmatory morphological and biochemical tests. MOX agar slants were sub-cultured at 3 month intervals to ensure the viability of organisms. The tests [microscopy on wet mount, gram staining, catalase test, methyl red (MR) test, Vogus-Proskauer (VP) test, H_2S production, colour changes in triple sugar iron agar, and colour changes in urea agar, hydrolysis of aesculin] described by Food and Agriculture Organization (1992), were carried out to confirm the presence of *Listeria* in the food samples.

Hemolytic activity

Of the species in the genus *Listeria, L. monocytogenes* is the most virulent and pathogenic because of its production of hemolysin (listeriolysin) (Cossart *et al.*, 1989). Sheep blood agar plates were stabbed with *L. monocytogenes* cultures, incubated at 35°C, and examined for hemolytic clearing zones after 24, 48, 72, 96 and 120 h. The diameters of hemolytic clearing zones were measured.

RESULTS AND DISCUSSION

Prevalence of *Listeria* in milk-foods

Of 265 milk and milk-food samples tested, 79 (30%) were contaminated with strains of *L. monocytogenes*. However, only 39 (15%) isolates showed hemolysis of sheep blood agar confirming them to be virulent strains of *L. monocytogenes*. Contaminated samples included raw cow milk, raw goat milk, pasteurized milk, and cheese. Sterilized milk, UHT milk, curd and yogurt did not contain *L. monocytogenes* (Table 1).

Based on the results given in Table 1, the distribution pattern of virulent strains of *L. monocytogenes*, in relation to number of samples and strains belonging to genus *Listeria*, is shown in Fig. 1. Cow milk (29%) and goat milk (27%) samples contained virulent strains of *L. monocytogenes*. There was no notable geographic difference in the percentage of contaminated samples between the two locations, Matara and Kandy. The percentage of milk samples containing *L. monocytogenes* in the present study is much higher than what is reported elsewhere (Table 2). This situation may be the result of inadequate attention paid at collection and transport of milk in Sri Lanka. The food

Table 1. Results of presumptive and confirmatory tests for *Listeria* performed on milk and milk-food samples.

Product	Number of samples				
	Tested	Growing on MOX agar	Giving morphological and biochemical reactions*	Producing hydrogen sulfide	Hemolytic
Raw cow milk	55	35	35	0	16
Raw goat milk	30	19	19	0	8
Pasteurized milk	30	5	5	0	5
Cheese	30	20	20	0	10
UHT milk	30	0	ND	ND	ND
Curd	30	0	ND	ND	ND
Yogurt	30	0	ND	ND	ND
Sterilized milk	30	0	ND	ND	ND
Total	265	79	79	0	39

*Tests used - Microscopy on wet mount, gram staining, catalase test, MR and VP test, colour changes in TSI agar, hydrolysis of aesculin and colour changes in urea agar.
ND - not determined.

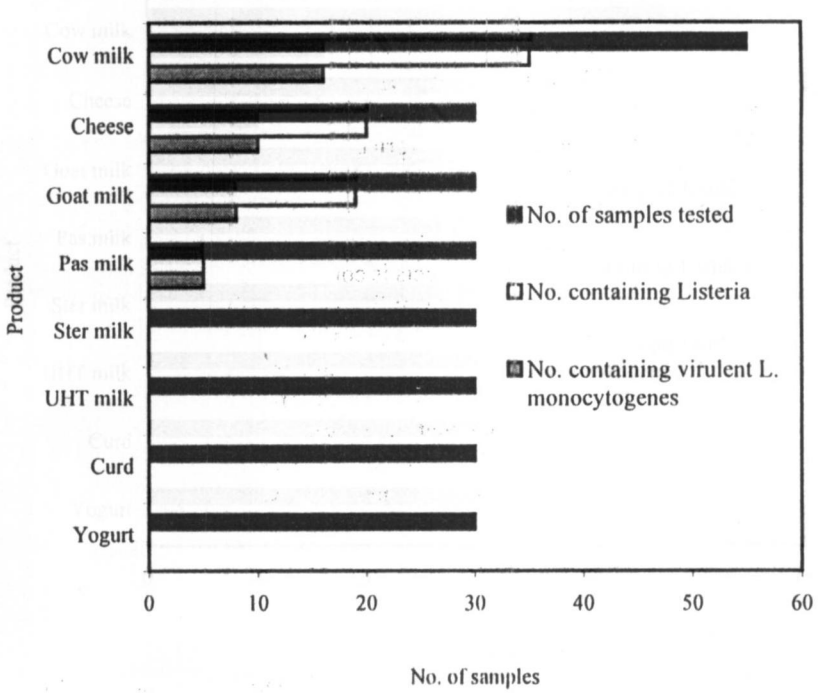

Fig. 1. Incidence of *L. monocytogenes* in milk and milk products of Sri Lanka.

Table 2. Reported incidence of *L. monocytogenes* in milk in different countries.

Product	Country	No. of samples	Incidence (%)		Reference
			Listeria monocytogenes	*Listeria*	
Cow milk	Canada	426	1.9%	ND	Fedio and Jackson (1990)
Cow milk	England	2009	5.08%	15.43%	O'Donnel (1995)
Cow milk	Germany	1095	0.31%	ND	Gasparovic *et al.* (1989)
Cow milk	Ireland	805	15.3%	25%	Harvey and Gilmour (1992)
Cow milk	Spain	774	3.62%	5.9%	Gaya *et al.* (1998)
Goat milk	Spain	1445	2.56%	4.15%	Gaya *et al.* (1996)
Goat milk	USA	450	3.8%	7.8%	Abdel *et al.* (2000)
*	Sri Lanka	46	26.0%	ND	Gunasena *et al.* (1995)
**	Sri Lanka	265	30.0%	ND	Present study

* - Raw milk, pasteurized milk, ice cream, cheese, and fresh cream
** - Cow milk, goat milk, pasteurized milk, sterilized milk, UHT milk, curd, yogurt and cheese
ND - Not determined

regulatory system in Sri Lanka has not addressed this issue yet. In view of the high percentage of *Listeria* in milk including pasteurized milk, greater attention needs to be paid to initiate preventive actions. Regulatory bodies such as Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) have established a zero tolerance limit for *Listeria*in food. In a previous survey carried out in Sri Lanka on milk products, chicken and vegetables (Gunasena *et al.*, 1995), *L. monocytogenes* has been observed in vegetables (49%), chicken (34%) and dairy products (26%). Their detection of *L. monocytogenes* in 26% of dairy products is comparable with the present observation of virulent *L. monocytogenes* in 29% of cow milk and 27% of goat milk but is much higher than the percentage of *L. monocytogenes* reported in milk from other countries. The observation of *Listeria* in 31% of pasteurized milk and absence in cheese by Gunasena *et al.* (1995) is not comparable with the present observation of *Listeria*. Moreover, the number of samples they tested was inadequate to draw statistically valid conclusions. There were differences in the methods used for isolation of *L. monocytogenes* in the present study and the study by Gunasena *et al.* (1995). In this study Modified Oxford Agar, which is highly specific for *L. monocytogenes* was used for isolation of *Listeria*.

Pasteurized milk

In examining the pattern of contamination of *L. monocytogenes* in different types of milk, the heavy contamination of 17% observed with pasteurized milk was notable and unacceptable. This could either be due to inadequate pasteurization temperatures or post-pasteurization contamination. Langeveld *et al.* (1993) concluded that the risk of secondary contamination of pasteurized milk with *Listeria* was low. In addition, milk factories adhere to strict hygienic manufacturing practices. The unacceptable contamination observed in this study may probably be due to inadequate pasteurization temperatures. *Listeria* is known

to be thermo-tolerant (Bearns and Girard, 1958; Rowan and Anderson, 1998). Atypical long cell chains of *L. monocytogenes* are reported to be more heat stable than typical dispersed cell forms (Rowan and Anderson, 1998). In a different study where *L. monocytogenes* was observed in pasteurized milk, the authors attributed the problem to inherent thermal resistance of *L. monocytogenes* (David *et al.*, 1985). These observations suggest that the probable cause of the presence of *L. monocytogenes* to be inadequacy of presently used pasteurization treatment of milk. If the milk contains a large proportion of *Listeria* prior to pasteurization, some of the organisms may survive the process. Post-pasteurization storage of contaminated milk at refrigerator temperatures also may permit selective growth of the remaining organisms (Twedt, 1984). The ability of *L. monocytogenes* to exist as an intracellular parasite too increases the possibility of survival of organisms during pasteurization. Modern dairy processing factories clarify the milk by a centrifugal filtering process prior to pasteurization. Clarification removes leukocytes (Somer, 1935) thus reducing the protective ability of microorganisms to survive as intracellular parasites in milk. The absence of the clarification step prior to pasteurization may have added to the extent of the problem. Pasteurization alone is only partially effective due to protective effect of intracellular lipid. The absence of *L. monocytogenes* in sterilized (120°C for 15 min) and UHT (140°C for 5 sec) milk further supports the view that inadequacy of heat treatment at pasteurization may be the key reason for the contamination in pasteurized milk.

Cheese

Cheese recorded the highest percentage of virulent *L. monocytogenes* positive samples (33%). Of 4172 samples of milk, cheese and other dairy products tested by Greenwood *et al.* (1991), *L. monocytogenes* were found most frequently (8.2%) in soft ripened cow cheese. The high contamination observed in cheese could be the result of use of heavily contaminated milk, inadequate heat treatment, post-treatment contamination, or salt tolerance of *Listeria*. *L. monocytogenes* contamination is more common in cheese than in any other dairy products. One factor influencing high *Listeria* level is the protective effects of milk fats on the bacterium at pasteurization (McDonald and Sutherland, 1993). Cheese contains 0.5-2% of salt. Under laboratory conditions *L. monocytogenes* is tolerant to 16-20% salt concentration (Buchanan *et al.*, 1989). Salt suppresses growth of other competitive organisms in cheese giving a relative advantage to *L. monocytogenes* to proliferate in a less competitive environment.

Effect of lactic acid bacteria

Curd and yogurt did not contain *Listeria*. In Sri Lanka, milk is boiled heavily prior to manufacture of curd and yogurt. The boiling temperature, in contrast to pasteurization temperatures, is more effective in destroying *Listeria*. In addition, the majority of the flora in curd and yogurt compose of lactic acid bacteria, which suppresses the growth of pathogens. Stanczak *et al.* (1997) demonstrated that micro-flora of fermented milk products increased the death rate of *L. monocytogenes* during storage at 20°C. Zuniga *et al.* (1995) reported that inhibition of *L. monocytogenes* in fermented milk was associated with a decrease in pH to less than 4.0, and an increase in acidity due to the action of lactic acid bacteria. Conversion of milk to curd or yogurt appears to be a more dependable method to keep *L. monocytogenes* under control than pasteurization.

Virulence

L. monocytogenes produces variable hemolysis of sheep blood agar. The degree of hemolysin (listeriolysin) production is a measure of virulence associated with *L. monocytogenes* (Cossart *et al.*, 1989). Table 3 shows the diameters of hemolytic clearing zones of sheep blood agar by *Listeria* isolates after 48 h of incubation at 35°C.

Table 3. The degree of hemolysis of sheep blood agar by *Listeria* isolates from milk.

Product	No. of samples	Diameter			Non-hemolytic
		> 10 mm	5-10 mm	< 5 mm	
Cow milk	35	5	4	7	19
Goat milk	19	0	3	5	11
Pasteurized milk	5	5	0	0	0
Cheese	20	3	4	3	10
Total	79	13	11	15	40

If the diameter of the clearing zone is considered as a measure of degree of virulence, the large clearing zones observed in all samples of pasteurized milk suggest high virulence of pasteurized milk. High virulence could either be due to increased activity of *L. monocytogenes* in the absence of other competitive organisms in pasteurized milk, or even resistance to heat inherently associated with highly virulent strains, which makes them survive pasteurization temperatures.

CONCLUSIONS

Cow milk, goat milk, cheese and pasteurized milk can carry virulent strains of *L. monocytogenes*, which may cause the fatal food-borne disease Listeriosis. Sterilization, UHT treatments and fermentation appear to destroy *L. monocytogenes* in milk. More virulent strains of *L. monocytogenes* appear to survive pasteurization. The percentage of milk samples contaminated with *Listeria* in Sri Lanka is much higher compared to those in developed countries.

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