

Gülhan VARDAR-ÜNLÜ  
Mehmet ÜNLÜ  
Mustafa Zahir BAKICI

## Incidence of *Listeria* spp. from Raw Milk in Sivas

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Department of Microbiology and Clinical  
Microbiology, Faculty of Medicine,  
Cumhuriyet University, Sivas-Turkey

**Abstract:** Raw milk samples obtained from the Foundation of Milk Industry, three private dairy companies and individual dairy farms in Sivas, were tested for the presence of *Listeria* species. A total of 100 raw milk samples were analysed by Food and Drug Administration (FDA) and two-stage enrichment techniques. Two-stage enrichment procedure was found to be more effective than FDA method in isolating

*Listeria* species from raw milk. The overall incidence of *Listeria* species in raw milk was 6%. *Listeria monocytogenes* was found in 4% of the raw milk samples, while *L. innocua* was found in 2% of the samples. One sample was revealed to contain two serotypes of *L. monocytogenes*.

**Key Words:** *Listeria*, raw milk, food microbiology.

### Introduction

*Listeria* microorganisms are widely distributed in nature and can be isolated from soil, water, vegetable. Of all *Listeria* species, only *Listeria monocytogenes* has been regularly implicated as being pathogenic to humans and animals (1). *L. monocytogenes* is a motile, gram-positive coccobacillus, non-sporeforming, aerobic to facultative anaerobic and an intracellular bacterium within leukocytes (2). Both the natural reservation and mode of transmission to man have been difficult to determine. Consumption of foods containing *L. monocytogenes* can lead to listeriosis in susceptible humans, adults with a compromised immune system, pregnant women and infants (3).

*L. monocytogenes* has been causative agent of major food-borne epidemics (1) in which dairy products including cheese (4, 5), raw milk (6, 7) and pasteurized milk (8) have been incriminated as the contaminated foods. Some investigators suggest that an intracellular stage may confer heat resistance of the organism and allow some *Listeria* within leukocytes to survive pasteurization. The bacteria can stay in injured state resulting from various food-processing operations that produce physical and/or chemical stresses and can also remain virulent upon recovery (9). Various conditions in foods, including refrigeration, extended shelf life and lack of oxygen, may permit the resuscitation and growth of

injured pathogens. Food associated outbreaks of listeriosis involving many deaths and because of presence of *L. monocytogenes* have prompted increased concern for detecting the bacteria in milk and dairy products (1). Except in modern cheese plants, raw milk is widely used in cheese production by domestic manufacturers in Turkey.

Isolation of *Listeria* species is not unduly difficult, except from heavily polluted material because of overgrowth by other microbial species. Past experiences in clinical laboratories indicate that attempt to isolate the organism from positive biologic specimens by directly plating suspect material onto conventional media occasionally fail, but isolation ratio is improved when specimens undergo a period of cold enrichment. The bacteria, contrary to other food-borne pathogens, is able to multiply at refrigeration temperatures (4-6°C). Because food typically contain large numbers of background bacteria, cold enrichment procedure is more successful than direct plating for detecting the organism in foods. For confirmation procedures, the old optical method of Henry (1933) was used (10). In this method, the organism is first cultured for 18-24 h on a transparent medium, then scanned by stereomicroscopy using oblique transmitted light. *Listeria* colonies were distinguished by characteristic blue-green edges when observed with low power magnification using obliquely

transmitted light.

The objective of this study was to determine the prevalence of *L. monocytogenes* in raw milk samples by Food and Drug Administration (FDA) and two-stage enrichment techniques.

**Materials and Methods**

**Raw milk samples**

They were obtained from the Foundation of Milk Industry, three private dairy companies, individual dairy farms in Sivas during April and May. We collected raw milk samples into glass bottles (100ml) from containers of which capacity changed from 2 to 10 liters. All samples were kept refrigerated (4°C) prior to testing. Analysis was always begun on the same day of sampling.

**Enrichment procedures**

**a-Food and Drug Administration Enrichment Procedure (FDA)**

Raw milk sample was shaken thoroughly and 25 ml of each sample was added to 225 ml of Enrichment Broth (EB) and incubated at 30°C. At intervals of 2 and 7 days, 0.1 ml of each culture was directly plated onto Modified McBride Agar (MMA) containing 5% sheep blood. In addition, 1.0 ml of EB culture was diluted with 9 ml of 0.5 % KOH, mixed briefly, and streaked onto MMA as described by Lovett *et. al.*, (11). Plates were incubated at 35°C for 48 h in 5-10% CO<sub>2</sub>.

**b- Two-stage enrichment procedure**

Ten ml of each raw milk sample were inoculated into 90 ml of Tryptose Broth (TB) which was kept at 4°C (5). Cold enrichment broths were subcultured at the end of 7, 14, 21st days as previously described (12). Briefly, 1 ml of TB culture were inoculated into 9 ml of EB. After incubation of the subculture at 35°C for 24 h in 5-10% CO<sub>2</sub>, 1 ml of the subculture was diluted to 9 ml with Sterile Ringer solution containing Lactate and 0.1 ml of this culture was plated onto MMA. The inoculated plates were incubated at 37°C for 24 h in 5-10% CO<sub>2</sub>.

**Isolation procedure and confirmatory tests**

MMA plates were examined for suspect colonies typical of those formed by *Listeria* species (translucent, slightly raised, bluish gray, 0.5-1.5 mm in diameter and/or weakly β-hemolysis) according to Rocourt (2). These colonies were examined by gram-stain reaction and in morphology (gram-positive, coccoid to diphtheroid-like rods). These colonies were streaked onto translucent tryptose-nalidixic agar and incubated for 48 h at 35°C. Plates were examined by the technique of Henry (1933) using obliquely transmitted light and Olympus S2 stereoscopic zoom microscope (10). Colonies of *Listeria* spp. were recognized by characteristic blue-green coloration. The suspect colonies were plated onto MMA plates and incubated at 35°C for 48 h in 5-10% CO<sub>2</sub>. The colonies were checked for motility, catalase production, oxidase production, metil red, Voges-Proskauer reaction, indole production, esculin hydrolysis, urease production, nitrate reduction, H<sub>2</sub>S production (TSI), citrate utilization, hemolytic activity, CAMP test, and ability to ferment glucose, rhamnase and mannitol, xylose with acid production. Isolates biochemically confirmed as *L. monocytogenes* and *L. innocua* were serotyped by the Reference Laboratory of Institut Pasteur, France and phagetyped in *Institut Fur Hygiene und Mikrobiologie der Universitat Wurzburg, Germany.*

**Results and Discussion**

Isolation of *Listeria* spp. from raw milk were performed using the Food and Drug Administration (FDA) and two-stage enrichment procedures. The results are shown in Table 1. Of the 100 raw milk samples, 4 (4%) were positive for *L. monocytogenes* and 2 (2%) for *L. innocua*. The use of two-stage enrichment procedure gave more isolates than FDA. Most of the positive isolations by the two-stage enrichment procedure were from end of 21 st day sampling of cold enrichment at 4°C. One of the samples yielded two serotypes of *L. monocytogenes* (1/2b and 4b). Serotypes and phagetypes of isolated *Listeria* species are summarized in Table 2.

A lot of case reports suggested that foodborne transmission of listeriosis to human beings can occur by raw and pasteurized milk (3, 8, 11, 13). There was

Table 1. Isolation of *Listeria ssp* by Food and Administration (FDA) and two-stage enrichment procedures.

Listeria spp	FDA		Two-stage enrichment		
	1. day	7. day	7. day	14. day	21. day
<i>L. monocytogenes</i> (4b)	-	1	2	2	2
<i>L. monocytogenes</i> (1/2b)	1	1	1	1	3
<i>L. innocua</i> (6a)	1	2	2	2	2
Total	2	4	5	5	7

Table 2. Serotypes and phagetypes of isolated *Listeria* spp.

<i>Listeria</i> spp	Serotype	Phagetype
<i>L. monocytogenes</i>	4b	NT*
<i>L. monocytogenes</i>	4b	NT
<i>L. monocytogenes</i>	1/2b	475
<i>L. monocytogenes</i>	1/2b	NT
<i>L. monocytogenes</i>	1/2b	NT
<i>L. innocua</i>	6a	4286:3528
<i>L. innocua</i>	6a	4286:3528

\*NT: nontyped

considerable variability in the efficacy of two enrichment procedures for isolating *L. monocytogenes* and other species. In this study, two-stage enrichment procedure was much more productive than FDA enrichment. Dilutions of the samples improve isolation of *L. monocytogenes* effecting of further reducing the concentrations of antagonistic microflora during course of cold enrichment (12). The number of isolates of *L. monocytogenes* and *L. innocua* increased from the samples incubated for 21 days, indicating that the organism may not survive well once, it has been resuscitated and/or grows among competitive microflora at 4°C. Using FDA enrichment procedure, prolonged incubation of samples for 7 days did not consistently improve recoveries over the initial 2 days incubation time of medium.

*Listeria monocytogenes* was found in 4% of the raw milk from Sivas. Our results are similar to the other investigators from United States and Canada (11,13, 14). However, other investigators from Spain found a much higher incidence of *L. monocytogenes* in the milk, 45.3

% and 44.7 % respectively (6, 7).

In Ankara, Turkey, 14 (18.2%) of all raw milk contained *L. monocytogenes* (15). In this study, all of tested pasteurized milk samples were found to be negative for *L. monocytogenes*. Other investigators from Diyarbakir, Turkey, reported that 1.1 % *L. monocytogenes*, 8.2 % *L. innocua*, 1.3 % *L. welshimeri* and 0.4% *L. ivanovii*. were found in raw milk samples (16). The overall incidence of *Listeria* spp. in cheese samples was found to be 5.8% according to Tümbay *et al.* (17). The contamination of cheese with *L. monocytogenes* was 2.1%. Results of this investigation have revealed that raw milk which is used for making cheese in Turkey could serve as vector for the transmission of human listeriosis. The specific role of milk could be defined by isolating the bacteria after pasteurization or production of cheese from raw milk. Conditions of prolonged cold storage give advantage to proliferation of *L. monocytogenes*. Consequently, all raw milk should be assumed to be contaminated with the organism, therefore, adequate measures to ensure proper pasteurization and to prevent postpasteurization contamination of processed dairy products must be adhered to by dairy manufacturers to ensure a safe product.

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