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# Prevalence and Antimicrobial Resistance of *Listeria monocytogenes* in Raw Milk in Tehran, Iran

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## Abstract

**Background and Objective:** One of the major sources of *Listeria monocytogenes*, as the causative agent of invasive listeriosis, is raw milk. The aim of the present study was to detect *Listeria monocytogenes* in raw milk samples collected from dairy stores in Tehran, Iran, 2019.

**Material and Methods:** A total of 100 raw milk samples were assessed using cultural techniques. Furthermore, antimicrobial resistance profiles of the *Listeria* isolates were assessed against eight antimicrobials using disc diffusion method.

**Results and Conclusion:** *Listeria* spp., including *Listeria grayi* (5%), *Listeria ivanovii* (3%) and *Listeria monocytogenes* (2%), were detected in 10% of the samples. *Listeria monocytogenes* isolates were susceptible to major antimicrobials used for the treatment of listeriosis with no multidrug resistances. The highest frequencies of resistance were seen against streptomycin (60%), gentamicin (50%) and tetracycline (50%). In conclusion, potential risks of listeriosis still threaten consumers of the raw milk in Tehran.

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## 1. Introduction

*Listeria* spp. are Gram-positive, short rod-shaped, non-spore forming facultative anaerobic bacteria. The optimum growth temperature and pH of the genus include 30-37 ℃ and 6-9, respectively [1]. The genus is divided into 17 species [2]. Of these species, *Listeria* (*L.*) *monocytogenes* is pathogenic to humans and the causative agent of invasive listeriosis. The *L. monocytogenes* and *L. ivanovii* are pathogenic to animals, especially sheep and goat. The disease is transmitted to humans and animals through food and feed [1]. Listeriosis may present as bacteremia, septicemia, meningitis, central nervous system infections (neurolisteriosis) [3] and rarely cutaneous infections [4]. Pregnant women [5], immunocompromised and immuno-suppressed adults [6], patients receiving biological therapies [7], neonates [5] and elderly people [8] are further vulnerable to the disease. Multistate outbreaks have been linked to raw milk and other dairy products such as cheese [9,10]. Moreover, survival and proliferation of *L. monocytogenes* at refrigeration temperatures are great concerned in refrigerated foods such as raw milk [11]. Since milk loses nutritional and health benefits during heating processes, people may prefer raw milk. However, milk is an appropriate medium for the growth of microorganisms. Hence, foodborne pathogenic bacteria may transfer to humans by the consumption of contaminated raw milk [12]. Survival of *L. monocytogenes* in products prepared from

contaminated raw milk such as cheese is a significant hazard [13,14]. Due to these reasons, monitoring of milk and milk products for *L. monocytogenes* contamination seems necessary. Although *Listeria* contamination of raw milk has frequently been studied worldwide [15-21], a few studies have been carried out in Iran [22-28] and only a study has been carried out in Tehran Province on samples from bulk milk tanks [26]. Since there are limited information on *Listeria* spp. in foods and listeriosis in Iran, it is necessary to assess the current status of the pathogen in foods. Therefore, the present study was the first study to investigate *Listeria* spp. contamination of raw milk retailed in traditional dairy stores in Tehran, Iran. In general, aims of the current study included 1) investigation of raw milk contamination retailed in traditional dairy stores of Tehran with *Listeria* spp., especially *L. monocytogenes* and 2) antimicrobial resistance assessment of the isolated *Listeria* spp.

## 2. Materials and Methods

### 2. Materials and Methods

### 2.1 Sampling

A total of 100 raw milk samples were aseptically collected from traditional dairy stores in Tehran, Iran, using randomized sampling method, March-May 2019. The study was previously approved by the Ethical Committee of Tehran University of Medical Sciences (Code No. IR.TUMS.SPH.REC.1398.029). Raw milk samples were transported to the laboratory of National Nutrition and Food Technology Research Institute (NNFTRI), Shahid Beheshti University of Medical Sciences, Tehran, Iran, under cold conditions and were analyzed immediately.

### 2.2 Isolation and identification of *Listeria monocytogenes*

Isolation of the *Listeria* spp. was carried out based on the FDA bacteriological guidelines [29]. Briefly, 25 ml of each milk sample were aseptically added to 225 ml of buffered *Listeria* enrichment broth (BLEB) (Himedia Laboratories, Mumbai, India) and incubated at 30 ℃ for 24-48 h. A loopful (approximately 0.1 ml) of the enriched sample was streaked on Palcam *Listeria* selective agar media (Merck, Darmstadt, Germany) and incubated at 35 ℃ for 24-48 h. Gray-green colonies with black zones were reported as suspected colonies. These colonies were streaked on tryptic soy agar media (Merck, Darmstadt, Germany) and incubated at 37 ℃ for 24 h. Then, isolated colonies were tested for oxidase and catalase productions and motility at 25 and 37 ℃, respectively. Moreover, suspected colonies were assessed using rapid latex slide agglutination test (LSAT) (Microgen *Listeria* F48, South Korea) for the presumptive identification of *Listeria* spp. Confirmed colonies were identified up to the species level using biochemical tests of esculin hydrolysis, mannitol, xylose, arabitol, ribose, rhamnose, trehalose, tagatose, glucose-1-phosphate, methyl-D-glucose and methyl-D-mannose fermentations and haemolysis using Microgen *Listeria*-ID System MID-67, South Korea. All chemicals and reagents included analytical grades.

### 2.3 Antimicrobial susceptibility assessment

Antimicrobial susceptibility of the isolated *Listeria* spp. was assessed using disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) [30]. A suspension of each *Listeria* colony in sterile normal saline was prepared and the turbidity of the suspension was adjusted to 0.5 McFarland Standard ($1.5 \times 10^8$ cfu $ml^{-1}$). Suspension was spread on surface of the Mueller-Hinton agar media (Merck, Darmstadt, Germany) supplemented with 5% defibrinated sheep blood using sterile swabs. The antimicrobial discs were transferred onto the media and then incubated at 37 ℃ for 24 h. after incubation, inhibition zone diameters were measured and the isolated *Listeria* spp. were classified as resistant, intermediate or susceptible based on the breakpoints of CLSI for *Staphylococcus* and *Enterococcus* spp. [31]. The antimicrobial discs and their concentrations included ampicillin (10 µg), gentamicin (10 µg), penicillin G (10 IU), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), erythromycin (15 µg) and streptomycin (10 µg) (Padtan Teb, Tehran, Iran). Multidrug resistance was reported as the bacterial resistance to at least three antimicrobial classes [32].

### 2.4 Statistical analysis

In this study descriptive statistics were used for the statistical analysis. Frequencies were calculated as the proportion of positive samples/isolates on the total samples/isolates and expressed as percentages using SPSS Software v.17 (IBM, Chicago, USA). All assessments were carried out in duplicate.

## 3. Results and Discussion

In the present study, 100 samples of raw milk were studied for the contamination with *L. monocytogenes*. In total, ten out of 100 samples were contaminated with *Listeria* spp. The isolated *Listeria* spp. were identified as *L. monocytogenes* (2/10), *L. ivanovii* (3/10) and *L. grayi* (5/10). *L. monocytogenes* was isolated from 2% of the studied samples. (Table 1). In similar studies, prevalence of *L. monocytogenes* included 2, 2.04, 2.5 and 1.6% in Canakkale Province (Turkey) [16], Ethiopia [19] Khartoum (Sudan) [18] and Shahrekord (Iran) [33], respectively. In other studies in Kermanshah (Iran) [25], Thailand [15] and Switzerland [17], *L. monocytogenes* was not detected in raw milk samples. However, *L. monocytogenes* was isolated from 4, 4.03, 4.8, 4.39, 5, 5, 6, 18.1 and 27.2% of raw milk samples in Mashhad (Iran) [23], Yazd (Iran) [28], Finland [34], Isfahan (Iran) [27], Kerman (Iran) [24], Kars (Turkey) [35], Samsun (Turkey) [36], Estonia [20] and Jammu (India) [37], respectively. Unlike the current study, bottled raw milk was investigated in Finland [34]. In the current study, *L. monocytogenes* was only detected in samples collected from the east and center of Tehran (Table 1).

**Table 1.** Geographic distribution of *Listeria* spp. isolated from raw milk in Tehran, Iran

| *District* | *No. of samples (%)* | *Listeria spp.* | *L. monocytogenes* | *L. ivanovii* | *L. grayi* |
|---|---|---|---|---|---|
| *North* | 20 (20) | 2 (10) | 0 (0) | 2 (10) | 0 (0) |
| *South* | 20 (20) | 1 (5) | 0 (0) | 0 (0) | 1 (5) |
| *West* | 20 (20) | 2 (10) | 0 (0) | 0 (0) | 2 (10) |
| *East* | 20 (20) | 3 (15) | 1 (5) | 1 (5) | 1 (5) |
| *Center* | 20 (20) | 2 (10) | 1 (5) | 0 (0) | 1 (5) |
| *Total* | 100 (100) | 10 (10) | 2 (2) | 3 (3) | 5 (5) |

L= *Listeria*

As number of the isolates was relatively small, statistical analysis was not practically possible. In Ethiopia, prevalence of *L. monocytogenes* in urban areas (3.4%) was higher than that in peri-urban areas (1.03%); however, differences were not statistically significant [19]. In Basrah (Iraq), differences between the areas with the highest and the lowest recovery rates of *L. monocytogenes* from raw milk samples were attributed to the weather condition of each area [21].

In the present study, 10% of the samples were contaminated with *Listeria* spp. Incidence rates of *Listeria* spp. in raw milk in Basrah Province (Iraq) (7.3%) [21], Khartoum State (Sudan) (7.5%) [18], and Turkey (12%) [36] were similar to those in the present study. However, contamination levels of raw milk samples with *Listeria* spp. (29%) in Egypt were three times higher than those in the present study [38]. Based on the results, the predominant species included *L. grayi* (5%), followed by *L. ivanovii* (3%) and *L. monocytogenes* (2%). In contrast, the dominant *Listeria* sp. isolated from raw milk in most previous studies was *L. innocua* [16,19,22,24,26–28,39]. Although *L. innocua* (6.4%) was the predominant species detected in raw milk samples in Ethiopia, *L. grayi* (4.4%), *L. ivanovii* (3.5%) and *L. monocytogenes* (2.04%) were isolated as well (19). In an earlier study in Isfahan, *L. monocytogenes* (4.39%) and *L. seeligeri* (1.09%) were isolated from raw milk; however, *L. innocua* was not detected in any sample [27]. In a previous study in Turkey, the predominant *Listeria* spp. isolated from raw milk belonged to *L. innocua* (8%), *L. ivanovii* (6%), *L. welshimeri* (2%) and *L. monocytogenes* (2%) [16]. In Shahrekord, *Listeria* spp. were isolated from 10.1% (21/207) of bovine mastitic milk samples, including 8.2% (17/207) *L. monocytogenes*, 1.45% (3/207) *L. innocua* and 0.48% (1/207) *L. ivanovii* [40]. In Egypt, the dominant species isolated from raw milk, milking equipment and dairy workers included *L. monocytogenes* (87.3%) [38]. Number of the isolated *Listeria* spp. may depend on the sampling seasons and methods as well as bacterial isolation techniques.

In the current study, the highest frequencies of resistance were seen against streptomycin (60%), gentamicin (50%) and tetracycline (50%), respectively (Table 2). The lowest rate of antimicrobial resistance (20%) was recorded against trimethoprim-sulfamethoxazole (Table 3).

**Table 2**. Antimicrobial resistance profiles of *Listeria* spp. isolated from raw milk in Tehran, Iran, based on the bacterial species

| Antimicrobial | *Listeria* spp. (%) (*n* = 10) | *L. monocytogenes* (%) (*n* = 2) | *L. ivanovii* (%) (*n* = 3) | *L. grayi* (%) (*n* = 5) |
|---|---|---|---|---|
| Ampicillin | 3 (30) | 0 (0) | 3 (100) | 0 (0) |
| Gentamicin | 5 (50) (*n* = 1)[I] | 0 (0) | 1 (33.3) | 4 (80) (*n* = 1)[I] |
| Penicillin | 3 (30) | 0 (0) | 3 (100) | 0 (0) |
| Tetracycline | 5 (50) | 0 (0) | 2 (66.7) | 3 (60) |
| Ciprofloxacin | 2 (20) (*n* = 5)[I] | 0 (*n* = 1)[I] | 1 (33.3) (*n* = 1)[I] | 1 (20) (*n* = 3)[I] |
| TMP-SMX | 2 (20) | 0 (0) | 2 (66.7) | 0 (0) |
| Erythromycin | 3 (30) (*n* = 6)[I] | 0 (0) (*n* = 1)[I] | 2 (66.7) (*n* = 1)[I] | 1 (20) (*n* = 4)[I] |
| Streptomycin | 6 (60) (*n* = 1)[I] | 0 (0) (*n* = 1)[I] | 2 (66.7) | 4 (80) |
| Resistance to 1 antimicrobial | 1 (10) | 0 (0) | 0 (0) | 1 (20) |
| Resistance to 2 antimicrobials | 2 (20) | 0 (0) | 1 (33.3) | 1(20) |
| Resistance to ≥ 3 antimicrobials | 5 (50) | 0 (0) | 2 (66.7) | 3 (60) |

I, intermediate resistance; TMP-SMX, trimethoprim-sulfamethoxazole; *n*, number of the isolates; L, *Listeria*

**Table 3**. Antimicrobial resistance profiles of *Listeria* spp. Isolated from raw milk in Tehran, Iran

| Status | Susceptible | | Intermediate | | Resistant | | Total | |
|---|---|---|---|---|---|---|---|---|
| Antimicrobial | No | % | No | % | No | % | No | % |
| Ampicillin | 7 | 70 | 0 | 0 | 3 | 30 | 10 | 100 |
| Gentamicin | 4 | 40 | 1 | 10 | 5 | 50 | 10 | 100 |
| Penicillin | 7 | 70 | 0 | 0 | 3 | 30 | 10 | 100 |
| Tetracycline | 5 | 50 | 0 | 0 | 5 | 50 | 10 | 100 |
| Ciprofloxacin | 3 | 30 | 5 | 50 | 2 | 20 | 10 | 100 |
| TMP-SMX | 8 | 80 | 0 | 0 | 2 | 20 | 10 | 100 |
| Erythromycin | 1 | 10 | 6 | 60 | 3 | 30 | 10 | 100 |
| Streptomycin | 3 | 30 | 1 | 10 | 6 | 60 | 10 | 100 |

TMP-SMX, trimethoprim-sulfamethoxazole

Although 80% of the isolated *Listeria* spp. were resistant to at least one antimicrobial agent, 10% of them showed resistance to one and 20% to two antimicrobial agents. The isolated *L. monocytogenes* strains showed resistance to none of the tested antimicrobials. However, *L. monocytogenes* isolates were intermediately resistant to erytromycin or streptomycin and ciprofloxacin (Table 2). Intermediate resistance to ciprofloxacin and erythromycin were demonstrated in 50 and 60% of the isolates, respectively (Table 3). As shown in Table 4, two multidrug resistant *Listeria* isolates showed resistance to eight and six tested antimicrobials, respectively. In a study in Isfahan Province, most of the isolated *Listeria* spp. (96.4%) showed resistance to nalidixic acid, followed by penicillin (34.5%) and tetracycline (27.3%) [22]. According to our results half of the isolated *Listeria* spp. (50%) were resistant to tetracycline; similar to previous studies (49.4%) [26] and (70.3%) [28]. In Isfahan Province, a lower rate of tetracycline resistance (27.3%) was observed [22]. In the present study, none of *L. monocytogenes* isolates were resistant to tetracycline. Similarly, strains of *L. monocytogenes* isolated over a period of 20 years in Argentina were susceptible to tetracycline [41]. All 15 *L. monocytogenes* isolated from raw milk and dairy products in Kars (Turkey) were also susceptible to tetracycline [35]. In contrast, tetracycline resistance was reported in 22.8% of 259 *L. monocytogenes* isolates from food, food processing environment and patient samples in Germany [42]. In Turkey, 34.6% of *L. monocytogenes* isolates were resistant to tetracycline, followed by resistances to chloramphenicol (25%) and penicillin G (23%) [36]. In Egypt, the highest antimicrobial resistance schemes of *L. monocytogenes* isolates were against tetracycline and clindamycin (81% each) [38]. Uses of tetracycline in veterinary medicine and animal feed for the prevention of infectious diseases, especially in Iran, are possibly important reasons for the high rates of resistance to tetracycline in *Listeria* spp. [26,43].

As previously stated, intermediate resistance to erythromycin and ciprofloxacin were observed in *L. monocyto-genes* isolates of the current study.

In Jamali et al. study, two out of 18 *L. monocytogenes* isolates from raw milk samples collected from bulk milk tanks in Tehran farms were resistant to erythromycin [26]; similar to the present study. Although *L. monocytogenes* rarely shows resistance to erythromycin and ciprofloxacin, 1.9 and 9.7% of 259 *L. monocytogenes* isolated from food, food processing and clinical samples in Germany were resistant to erythromycin and ciprofloxacin, respectively [42]. In contrast, findings of a similar study from Kars (Turkey) showed that all of *L. monocytogenes* isolates were susceptible to erythromycin. However, one out of 15 (6.7%) *L. monocytogenes* isolates similarly showed intermediate resistance to ciprofloxacin [35].In the current study, 50% of the isolated *Listeria* spp. were resistant to three or more antimicrobials. Multidrug resistance was observed in *L. ivanovii* (66.7%) and *L. grayi* isolates (60%). However, none of the *L. monocytogenes* isolates showed multidrug resistance.

**Table 4.** Multidrug-resistant *Listeria* spp. isolated from raw milk in Tehran

| Sum | *Listeria* spp. | | *L. ivanovii* | | *L. grayi* | | Patterns of the antimicrobial combinations |
|---|---|---|---|---|---|---|---|
| | No. | %* | No. | % | No. | % | |
| 8 | 1 | 10 | 1 | 10 | 0 | 0 | ampicillin/gentamicin/penicillin/tetracycline/ciprofloxacin/thrimethoprim -sulfametoxazole /erythromycin/ streptomycin |
| 6 | 1 | 10 | 1 | 10 | 0 | 0 | ampicillin/penicillin/tetracycline/thrimethoprim-sulfametoxazole/erythromycin/streptomycin/ ciprofloxacin** |
| 4 | 1 | 10 | 0 | 0 | 1 | 10 | Gentamicin/tetracycline/ciprofloxacin/erythromycin |
| 3 | 2 | 20 | 0 | 0 | 2 | 20 | Gentamicin/tetracycline/streptomycin/ erythromycin**/ ciprofloxacin** |
| | | | | | | | Gentamicin/tetracycline/streptomycin/erythromycin** |
| Total | 5 | 50 | 2 | 20 | 3 | 30 | |

Sum, sum of various antimicrobial resistances; *percentages are calculated on the basis of total *Listeria* spp. isolated from raw milk samples ($n$ = 10); **intermediate resistance; L, *Listeria*

In a previous study, multidrug-resistant *Listeria* spp. (8.4%) including *L. monocytogenes* (71.4%) and *L. innocua* (28.6%) were isolated from milk samples of farm bulk milk tanks in Tehran Province [26]. In Isfahan Province, 16.4% of *Listeria* spp., including *L. monocytogenes* (22.2%), *L. innocua* (66.7%) and *L. seeligeri* (11.1%) isolated from milk and dairy products, showed multidrug resistance [22]. In Samsun (Turkey), 36.5% (19 of 52) *L. monocytogenes* isolates were resistant to multiple drugs [36].

In this study, all *L. monocytogenes* were susceptible to the major antimicrobials such as ampicillin, penicillin G, gentamicin and thrimethoprim-sulfamethoxazole clinically administered for the treatment of listeriosis. Earlier reports from Germany [42], Morocco [39] and Argentina [41] revealed that *L. monocytogenes* isolates showed no resistance against reference antimicrobials used for the treatment of listeriosis. In contrast, 66.7% of *L. monocytogenes* isolated from raw milk in Egypt were gentamicin resistant [38]. Moreover, 26.7% of *L. monocytogenes* isolates showed resistance against trimethoprim-sulfamethoxazole in Kars (Turkey) [35]. Considering that 50 and 60% of *Listeria* isolates respectively showed intermediate resistance to ciprofloxacin and erythromycin, the actual rates of ciprofloxacin and erythromycin resistance reached 70 and 90%. Hence, resistance to quinolones should carefully be considered because of their uses in treatment of infections. Since erythromycin and tetracycline are bacteriostatic agents and antimicrobial resistance genes to these agents are located on plasmids, these chemicals are not recommended for the treatment of listeriosis [44].

## 4. Conclusion

Based on the current results, there are still potential risks of listeriosis through consumption of raw milk in Tehran. Although the *L. monocytogenes* isolates did not show multidrug resistant and were susceptible to the highlighted antimicrobials used for the clinical treatment of listeriosis, 50% of the *Listeria* isolates were multidrug resistant. Since dissemination risks of antimicrobial resistance from resistant to susceptible bacteria are frequently reported, continuous monitoring of the antimicrobial resistance emergence is highly necessary. Moreover, use of antimicrobials in veterinary medicine, especially in animal feed, must strictly be limited. Due to the short shelf-life of raw milk, storage time, temperature and packaging type and condition are considered as the most important factors affecting the risk of human listeriosis; thus, food safety control systems of *L. monocytogenes* in retail markets should correctly be established by the national authorities. Therefore, further studies with larger sample sizes for the continuous surveillance of *Listeria* spp. especially *L. monocytogenes*, in raw milk and antimicrobial susceptibility assessments of the bacteria are highly recommended.

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## 6. Conflict of Interest

The authors report no conflicts of interest.

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# شیوع و مقاومت ضدمیکروبی *لیستریا مونوسایتوژنز* در شیر خام در تهران، ایران

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## چکیده

**سابقه و هدف:** شیر خام یکی از منابع عمده *لیستریا مونوسایتوژنز*، عامل ایجاد کننده لیستریوزیس تهاجمی می-باشد. هدف مطالعه حاضر، شناسایی *لیستریا مونوسایتوژنز* در نمونه‌های شیر خام جمع‌آوری شده از فروشگاه‌های لبنی شهر تهران، ایران در سال ۲۰۱۹ بود.

**مواد و روش ها:** در مجموع یکصد نمونه شیر خام به‌روش کشت مورد ارزیابی قرار گرفت. متعاقبا، مقاومت ضدمیکروبی جدایه‌های *لیستریا* در برابر ۸ نوع آنتی‌بیوتیک به‌روش انتشار دیسک تعیین شد.

**یافته‌ها و نتیجه‌گیری:** گونه‌های *لیستریا*، شامل *لیستریا گریی* (۵٪)، *لیستریا ایوانووی* (۳٪) و *لیستریا مونوسیتوژنز* (۲٪) در ده درصد نمونه‌ها شناسایی شدند. جدایه‌های *لیستریا مونوسیتوژنز* به مواد ضد میکروبی اصلی مورد استفاده در درمان لیستریوزیس حساس بودند اما مقاومت چند دارویی نشان ندادند. بیشترین فراوانی مقاومت آنتی بیوتیکی در برابر استرپتومایسین (۶۰٪)، جنتامایسین (۵۰٪) و تتراسایکلین (۵۰٪) مشاهده شد. در مجموع، خطرات بالقوه لیستریوز هنوز مصرف کنندگان شیر خام را در تهران تهدید می‌کند.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- گونه های لیستریا
- شیر خام
- مقاومت ضد میکروبی
- تهران

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