



A Survey on the Sanitary Condition and Prevalence of *Listeria monocytogenes* in Commercial Refrigerators in Khuzestan Province, Iran

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Abstract

Background: *Listeria monocytogenes* can grow in a variety of foods that are stored in refrigerators. This bacterium can adhere to various surfaces and form biofilms. Given that refrigerator temperature can support bacterial growth and proliferation, increasing the number of bacteria in refrigerator spaces endangers the consumer's health.

Objective: The purpose of this study was to investigate the prevalence of *L. monocytogenes* and the sanitary conditions of commercial refrigerators used in food supply centers in Khuzestan province, southwest Iran.

Materials and Methods: Overall, 204 swap samples were collected from the inner surfaces of various industrial refrigerators in five different zones. The samples were enriched in *Listeria* enrichment broth and cultured on PALCAM agar plates. Suspected colonies were identified by biochemical tests and the polymerase chain reaction. Additionally, the sanitary condition of the refrigerator, method and duration of refrigerator cleaning, last washing date, and type of the applied detergent were recorded, and the antibacterial susceptibility of the isolates was tested as well.

Results: The results demonstrated that four refrigerators (1.96%) located in Ahvaz (center) and Ramhormoz (east) were contaminated with *L. monocytogenes*. The internal temperatures of 67% of the refrigerators were above 5 °C, and most operators used a mixture of water and a dishwasher or water alone to clean their refrigerators. In addition, 31.9% of the refrigerators were in an unfavorable cleaning condition, and 32.8% of the refrigerators were not sealed properly. The isolates were resistant to streptomycin and represented intermediate resistance to tetracycline and penicillin.

Conclusion: The contamination of industrial refrigerators with antibiotic-resistant *L. monocytogenes* may pose a threat to public health. Authorities should increase the monitoring of industrial refrigerators and provide the necessary training for refrigerator users.

Keywords: *Listeria*, Prevalence, Commercial, Refrigerator, Khuzestan

Background

Listeria monocytogenes is a Gram-positive bacterium and one of the most important foodborne pathogens that causes listeriosis and acute problems in humans, especially in children, pregnant women, elderly people, and those with immunosuppression. The bacterium also causes gastroenteritis and flu-like illnesses in healthy individuals.¹ *L. monocytogenes* can grow at refrigerator temperatures (0-4°C) and tolerate pH between 5.4 and 9.6.² Considering that the death rate of this bacterium has been reported to be up to 30%,³ its presence in food is considered an important public health risk. Given that the dangerous form of the disease occurs in immunodeficient individuals, it is highly important to know the level of contamination of the consumed food and the antibiotic sensitivity of the strains.⁴ This bacterium has been isolated from various foods in other countries, including Iran.^{5,6} In addition, *L. monocytogenes* can adhere to various surfaces such as glass, plastic, rubber, and stainless steel to form

biofilms.⁷ Bacterial colonization in refrigerators has been reported in many cases.⁸ Considering that the temperature of the refrigerator can support the growth and proliferation of bacteria, the consumer's health is endangered by an increase in the number of bacteria in the refrigerator space.⁹ Several reports on the isolation and identification of this pathogen from household refrigerators have been published in Iran and worldwide.^{10,11}

To the best of our knowledge, there has been no investigation on the level of contamination of industrial and commercial refrigerators in Iran; therefore, this study aimed to investigate the prevalence of *L. monocytogenes* and the sanitary conditions of commercial refrigerators used in food supply centers in Khuzestan province, southwest Iran.

Materials and Methods

Collection of Samples

For sample collection, Khuzestan province was divided



into five major regions, including north, south, east, west, and center. This province is located in the southwest of Iran, and its northeastern regions have a moderate climate, but other regions have hot and humid weather in 9 months of the year. During 3 months, 204 samples were collected from the inner surfaces of the commercial refrigerators by randomly visiting retail supply centers (chicken, fish, meat, confectioneries, and dairies) and deli restaurants located in ten different cities of Khuzestan province.

Sampling Method

First, the internal temperature of the refrigerator was recorded using a laser thermometer. Then, the surfaces of three different areas (each 10×10 cm²) of the inner surface of the refrigerators were sampled using three sterile swabs. All three swabs were entered into a falcon tube containing *Listeria* enrichment broth medium and considered as one sample.

The internal condition and sanitary level of the refrigerator were checked by visual observation, and the manager was asked to fill out a questionnaire about the method and time of refrigerator cleaning, last washing date, and type of applied detergent. Questionnaires were used for data analysis.

Isolation and Identification of *Listeria monocytogenes*

The tubes were transferred to the Department of Food Hygiene, Shahid Chamran University of Ahvaz under cool conditions and incubated at 30°C for 24-48 hours. An aliquot (100 µL) of the enriched culture was streaked onto PALCAM agar (Merck, Germany) supplemented with ceftazidime (25 mg/L), acriflavine (5 mg/L), and polymyxin B (15 mg/L). The plates were incubated at 30°C for another 48 hours. At least, three suspected colonies (grey-green colonies with black halo) were subcultured in trypticase soy broth (Quelab, Canada) and incubated at 30°C for 48 hours. The suspected colonies were characterized by Gram-staining, as well as catalase and oxidase tests. For confirmation, the isolates were subjected to polymerase chain reaction (PCR) analysis.

Polymerase Chain Reaction Procedures

A pure presumptive colony was cultured in 5 mL of Tryptic Soy Broth medium and incubated for 24 hours at 30°C, and then DNA was extracted by the thermal method.¹² Briefly, 1 mL of the culture was centrifuged for 5 minutes at 10 000 RPM in a microtube. The supernatant

was discarded, and the pellet was resuspended in 1 mL of distilled water. The suspension was centrifuged again, and after removing the supernatant, 100 µL of distilled water was added to the bacterial pellet and heated for 10 minutes at 100°C. The microtube was then centrifuged for 5 minutes at 10 000 RPM, and the supernatant containing the DNA was used as the PCR template.

The extracted DNA was subjected to PCR amplification. Specific primers for *L. monocytogenes* were related to the p60 protein coding gene (*iap*-P60). Table 1 lists the applied primers, product sizes, and PCR conditions.

Each PCR tube contained 6.5 µL of ddH₂O, 1 µL (each 0.5 µL) of a mixture of the two forward and revised primers, 12.5 µL of master mix (2x, Ampliqon, Denmark), and 5 µL of template extracted DNA (totally 25 µL reaction mixture). The mixture was processed in a thermocycler (Bioer Technology, China). The products were detected by electrophoresis (Paya Pajooheh, Iran), stained, and visualized using a UV light illuminator (Kiagen, Iran). *L. monocytogenes* DNA (ATCC 7644) and deionized distilled water were used as positive and negative controls, respectively.

Antimicrobial Susceptibility Testing

According to the method of the Clinical and Laboratory Standards Institute,¹³ antimicrobial susceptibility tests were conducted using the Kirby-Bauer disk diffusion method. The antimicrobial agents were erythromycin, ampicillin, streptomycin, amikacin, tetracycline, chloramphenicol, penicillin, co-trimoxazole, vancomycin, and gentamicin. Briefly, a bacterial suspension (Approximately cell density of 1.5×10⁸ cfu/mL) was prepared, and 100 µL was taken and stroked trolley on a Muller-Hinton agar (Merck, Germany) plate using a cotton swab. Antibiotic discs (Padtan Teb, Iran) were placed on agar, and the plate was incubated at 30°C for 24 hours. The diameter of the inhibition zone was measured for each antibiotic. The isolates were classified as sensitive, resistant, or intermediate (reduced susceptibility).

Data Analysis

The questionnaire data were converted into graphs using Microsoft Office Excel, version 2010.

Results

Isolation of *Listeria monocytogenes*

A total of 204 samples were collected from refrigerators in different food supply centers in various regions and cities

Table 1. List of Target Genes, Product Size (bp), Sequence of Primers, and PCR Conditions

Primer	Size (bp)	Sequence	PCR Conditions	Reference
<i>iap</i> -P60	454	F CTGGCACAAAATTACTTACAACGA R: AACTACTGGAGCTGCTTGTITTTTC	Denaturation 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 45 minutes, 72°C for 1 minute. Final extraction 72 °C for 5 minutes.	12

Note. PCR: Polymerase chain reaction.

of Khuzestan (Table 2). The cities were located in the center (Ahvaz and Bavi), west (Dezful and Andimshek), south (Mahshahr and Abadan), east (Ramhormoz and Behbahan), and north (Masjid Suleiman and Izeh) of Khuzestan Province. Out of 91 colonies formed on PALCAM agar, only 58 Gram-positive, catalase-positive, and oxidase-negative isolates had the characteristics of the *Listeria* genus. It is noticeable that enterococci are also capable of weakly hydrolysis esculin, and the product reacts with ferric ammonium citrate and creates shiny colonies with black halos (similar to the genus *Listeria* spp.).

Table 2 provides the number of suspected *Listeria* spp. by biochemical tests and the confirmed number of *L. monocytogenes* using the PCR technique. Among the suspected isolates, PCR showed the presence of the specific *iap*-P60 gene in four isolates, which was specifically located in the *L. monocytogenes* genome (Figure 1).

Two positive isolates were isolated from a deli and a butcher shop in Ahvaz located in the central area, and the other two positive *L. monocytogenes* isolates were isolated from the refrigerators of a local store supplying fresh chicken meat and a butcher shop in Ramhormoz, located in the eastern area of Khuzestan.

Sanitary Conditions of Refrigerators

The data demonstrated that half of the investigated refrigerators had a temperature range between 5 and 8°C, and 14.7% of them were higher than 8°C (Figure 2), all of which were above the permissible limit and could endanger the safety of food.

As shown in Figure 3, most operators used a mixture of water and a dishwasher or water alone to clean their refrigerators, which may not be sufficient to remove high

Table 2. Frequency Distribution of Samples Taken From Different Regions, Suspected Isolates, and Confirmed Number of *L. monocytogenes* in the Industrial Refrigerators of Khuzestan Province

Region	Number of Samples	Suspected Isolates	Confirmed <i>Listeria monocytogenes</i>
Central	44 (21.6)	18 (40.9)	2 (0.98)
North	40 (19.6)	9 (22.5)	0 (0)
South	40 (19.6)	11 (27.5)	0 (0)
East	40 (19.6)	12 (30)	2 (0.98)
West	40 (19.6)	8 (20)	0 (0)
Total	204 (100)	58 (28.4)	4 (1.96)

Note. Data are expressed as No. (%).

Table 3. Type of Shop, Temperature, Cleaning and Sanitary Status of Contaminated Refrigerators

Health Evaluation Status of Refrigerators Containing <i>Listeria monocytogenes</i>							
Type of Shop	City	Temperature (°C)	Cleaning Status	Sealing Condition	Periodic Cleaning	Cleaning Method	Proximity of Raw and Ready-to-Eat Food
Retail chicken shop	Ramhormoz	9	Unfavorable	Unfavorable	Daily	Water	No
Butchery	Ramhormoz	8	Unfavorable	Unfavorable	Weekly	Water	No
Deli	Ahvaz	5	Unfavorable	Optimal	Weekly	Water	Yes
Butchery	Ahvaz	6	Unfavorable	Optimal	Weekly	Water and dishwasher	No

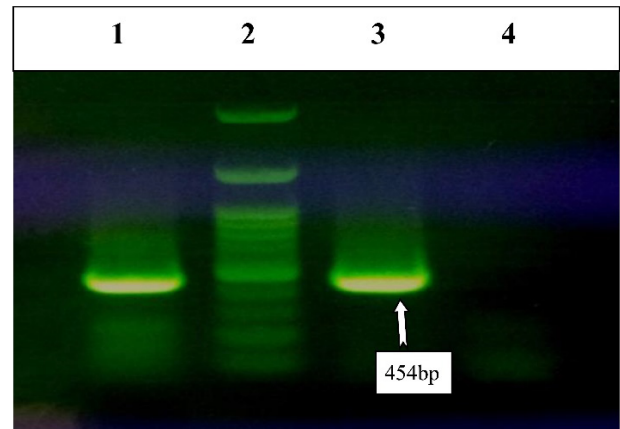


Figure 1. Polymerase Chain Reaction Results on Gel Electrophoresis. Note. *L. monocytogenes*: *Listeria monocytogenes*; Line 1: Positive control (*L. monocytogenes*, ATCC7644); Line 2: Ladder 100 bp; Line 3: A positive isolate; Line 4: Negative control (deionized distilled water).

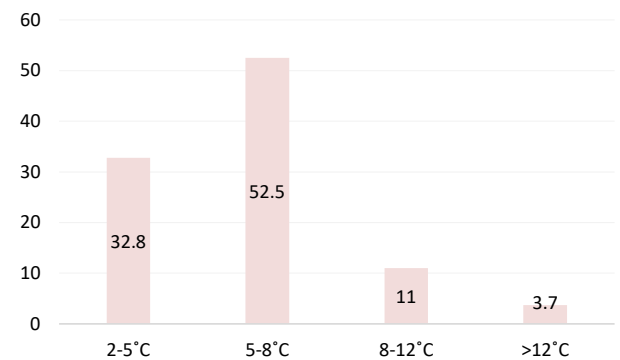


Figure 2. The Temperature Percentage of Investigated Refrigerators.

levels of molds and bacterial contamination from these types of refrigerators.

The questionnaire results showed that 48% of the investigated refrigerators were cleaned within a time interval of more than one day, and more than half of them were daily washed according to the owner of the guild (Figure 4). The presence of high bacterial contamination and *L. monocytogenes* in these refrigerators indicated an inadequate cleaning process.

Our observations revealed that 31.9% of the refrigerators were in an unfavorable cleaning condition, and 32.8% of the refrigerators were not sealed properly. In addition, observations represented that in 22% of the refrigerators, raw or unwashed food materials and ready-to-eat food were stored adjacent to each other. Table 3 presents the temperature, washing method, and sanitary conditions

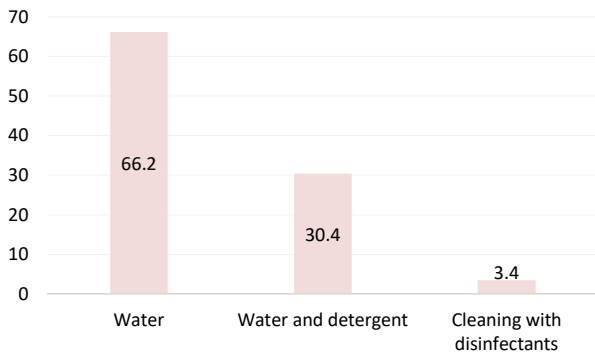


Figure 3. Refrigerator Cleaning Methods.

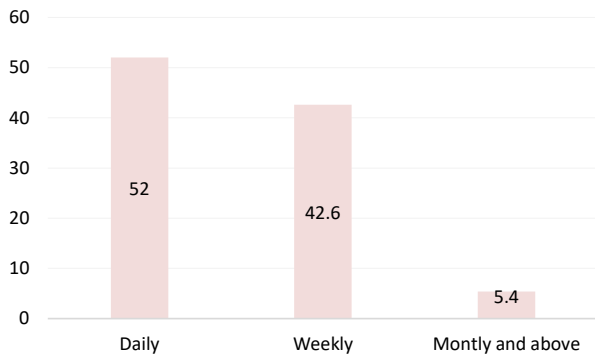


Figure 4. Periodic Cleaning of Refrigerators.

of four refrigerators from which *L. monocytogenes* was isolated.

Antimicrobial Susceptibility Test Results

The isolates were resistant to streptomycin and showed intermediate resistance to tetracycline and penicillin. Further, they were sensitive to the other tested antibiotics.

Discussion

There are many reports regarding the isolation and identification of *L. monocytogenes* from foodstuffs in Khuzestan Province. For example, the bacterium has been reported in 4% of warm-water farmed fish,¹⁴ 1.4% of shrimp samples,¹⁵ and 0.66% of retail mince beef samples.¹⁶ To the best of our knowledge, there is little information about the prevalence of *L. monocytogenes* in the household, commercial, and industrial refrigerators in Iran and the world. For instance, *L. monocytogenes* was presented in three out of 86 domestic refrigerators in Portugal. Moreover, *Listeria grayi* and *Listeria innocua* were isolated from four and one refrigerators, respectively.¹¹ In another study, *L. monocytogenes* was obtained from 1.2% of the examined household refrigerators (n = 342) in Northern Ireland, United Kingdom.¹⁷ *L. monocytogenes* and *Listeria seeligeri*, *Listeria innocua*, *Listeria ivanovii*, and *Listeria welshimeri* were recovered from 1%, 13%, 21%, and 25% of domestic refrigerators investigated in university student hostels in Lagos, Nigeria, respectively.¹⁸ *L. monocytogenes* was also found to be present in 4 out of the 100 sampled household refrigerators, and *Listeria*

innocua was observed in 22 out of the 100 refrigerators investigated in Uruguay.¹⁹

The presence of *Listeria* spp. in household and student accommodation refrigerators was investigated in the southwest of Iran. Based on these results, one refrigerator out of 180 (0.5%) was contaminated with *L. monocytogenes*, and two refrigerators (1.2%) were contaminated with *Listeria innocua*.⁶ A high prevalence of *L. monocytogenes* (41.6%) was also reported in Tehran, where 104 household refrigerators located in 10 health centers in five areas were sampled.¹⁰ The results obtained from the present study showed that the contamination rate of *L. monocytogenes* in commercial and industrial refrigerators in Khuzestan province was approximately 2%. Although there are no data regarding contamination of commercial refrigerators in Iran and the world for comparison, the current findings are relatively in line with the results of other surveys conducted on household refrigerators.^{6,17}

All the above-mentioned data indicate that household and commercial refrigerators could be contaminated with pathogenic bacteria such as *L. monocytogenes* and pose a risk to consumer health. It should be noted that *L. monocytogenes* is a psychrophilic bacterium and can grow at refrigerator temperatures. Furthermore, it can rapidly multiply under these conditions and transfer to ready-to-eat or stored foods.²⁰ The presence of *L. monocytogenes* in domestic or commercial refrigerators, even in small numbers, is an important public health concern.

Moreover, in a study, it was emphasized that the isolation of other species of *Listeria*, because of their similar ecological characteristics, in the environment can prove the possible presence of *L. monocytogenes*.²¹ In the present study, although the isolation and identification of other *Listeria* spp. were not considered, the characteristics of many colonies grown on the PALCAM agar medium were very similar to those of the *Listeria* genus. Therefore, according to the isolation of four strains of *L. monocytogenes*, it can be assumed that many of these isolates are related to the other species of the *Listeria* genus; thus, the presence of *L. monocytogenes* in negative samples is possible. This indicates that a large number of food supply center refrigerators in Khuzestan province may be contaminated with *Listeria*; as a result, health officials should consider more monitoring and control of food supply centers.

The results of the current study represented that the temperature of the three refrigerators, where *L. monocytogenes* was isolated, was above the permissible limit. One study highlighted the importance of adequate refrigerator temperature control to ensure food safety.¹⁷ In contrast, other studies reported that there are no significant correlations between the visual inspection scores, temperature, and frequency of isolation of specific pathogens in domestic refrigerators, while a significant

correlation was found between contamination and education of owners.⁴ However, it should be noted that in many studies conducted in Iran and the world, it has been emphasized that failure to maintain the optimal degree of cold in refrigerators can generally lead to the contamination of food stored in the refrigerator, leading to food poisoning.^{6,11,18}

As shown in Figure 3, in the current study, most operators used a mixture of water and dishwasher or water alone to clean their refrigerators. According to the results, it seems that the use of these types of liquids, owing to the high level of the contamination of these refrigerators, is insufficient. Although refrigerator manufacturers recommend cleaning the interior of plastic refrigerators with bicarbonate solutions, it has been shown that some bacteria, especially *L. monocytogenes*, are sensitive to anionic detergent products. Therefore, it is undesirable to use water or dishwashing solutions to disinfect and remove bacteria and molds from refrigerators.²²

Based on data in Table 3, in all four positive refrigerators, only water or water containing the dishwashing liquid was used to clean the refrigerator. This issue is not an assured hygienic method for cleaning the interior surfaces of refrigerators, especially in the case of the refrigerators of butchers and chicken shops, where an extremely high load of pollution enters the refrigerator every day, or in the case of refrigerators that are used for the simultaneous storage of raw or sitting food and ready-to-eat. In parallel with the current study, a correlation was reported between microbial contamination and refrigerator cleaning methods in previous investigations.²⁰

The examination of the cleaning and sealing of the refrigerators in the current study revealed that 31.9% and 32.8% of the refrigerators were not in a favorable cleaning condition and were not properly sealed, respectively. This defect may affect the inside temperature of the refrigerator and cause easy contamination of the refrigerator with environmental microbes. It was also observed that in 22% of the refrigerators, raw materials and ready-to-eat foods were stored together, which is clearly contrary to the health inspection regulations and can lead to the cross-contamination of foodstuffs. This could be a serious factor in the contamination of both foodstuffs and the refrigerator itself.¹⁹ In another study, no significant correlation was observed between temperature, isolation of specific pathogens, and visual inspection scores in domestic refrigerators. Contrarily, a significant correlation was found between contamination and the education of users.¹⁰ This finding clearly represents that compliance with hygiene principles by refrigerator users, who are familiar with and follow hygiene principles, can play an important role in controlling refrigerator contamination.

In the current study, the isolated strains of *L. monocytogenes* were resistant to streptomycin and demonstrated an intermediate susceptibility to tetracycline

and penicillin. The isolates also showed susceptibility reactions to other tested antibiotics. These findings are in conformity with those obtained in a previous study in Khuzestan Province.⁶ Similarly, these findings are in agreement with those of worldwide researchers, reporting that *L. monocytogenes* isolated from meat products were resistant to tetracycline, streptomycin, and penicillin.²³⁻²⁵

Interestingly, resistance to antibiotics that are commonly prescribed to treat listeriosis in humans, including cotrimoxazole, amoxicillin, and ampicillin, has rarely been observed in *L. monocytogenes* strains isolated from the foodstuff source.²⁶ However, the increasing number of antibiotic-resistant strains of the bacterium in foods and refrigerators may indicate that *Listeria* spp. are rapidly acquiring a wide variety of antibiotic resistance genes, which can be dangerous for the public health of human societies.²⁷

Conclusion

The contamination of industrial refrigerators in food supply centers in Khuzestan, which provide food for a large number of people every day, is a potential risk to antibiotic-resistant *L. monocytogenes*. Health authorities should increase the monitoring of these centers and provide the necessary training for refrigerator users.

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Conceptualization: Siavash Maktabi.

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Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Approval

The experiments were conducted according to the protocol approved by the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz.

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