original article

Prevalence of *Listeria* species in raw milk and traditional dairy products in Isfahan, Iran

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ABSTRACT

Aim: The study aimed to assess the prevalence of *Listeria* spp. in raw milk and traditional non-pasteurized dairy products in Isfahan, Iran.

Materials and Methods: A total of 292 samples of raw milk and traditional dairy were examined for the presence of *Listeria* spp. using a two-step selective enrichment recommended by the United States Department of Agriculture. All isolates were subjected to standard biochemical tests. *L. monocytogenes* strains were further confirmed by polymerase chain reaction (PCR) amplification.

Results: Of 292 samples, 21 (7.14%) and 4 (1.47%) were positive for *Listeria* spp. and pathogenic *L. monocytogenes*, respectively. The prevalence of *Listeria* spp. in raw milk, ice cream, cream, and freni were 5.91 (5.49%), 12.63 (19.04%), 3.27 (11.11%) and 1.25 (4%), respectively. *Listeria* was not detected from yogurt, butter, Kashk, and cheese. *Listeria innocua* at 16.21 (5.44%) was the most prevalent species isolated, followed by *L. monocytogenes* at 4.21 (19%) and *L. seeligeri* at 1.21 (4.7%). All strains of *L. monocytogenes* identified by biochemical tests were also confirmed by PCR.

Conclusion: The study shows the prevalence of *L. monocytogenes* in raw milk and traditional dairy products sold in the market. Consumption of raw milk with mild heat treatment or its usage in traditional dishes could pose serious health problems due to lack of appropriate control measures. The lack of knowledge on the risks of listeriosis transmission indicates the need for implementation of a food safety education program. In addition, the Iranian food safety authorities should urgently set up an effective standard to screen all susceptible food products for the presence of *Listeria.*

Key words: Dairy, *Listeria*, prevalence, PCR, raw milk

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INTRODUCTION

Listeria species are Gram-positive small bacilli, non-spore forming, motile, facultative, and ubiquitous bacteria that are well adapted to the environment, food, and animals.[1] While the genus *Listeria* contains seven species, only *L. monocytogenes* is considered as one of the most important food-borne bacteria that cause listeriosis in animals and humans. The disease can be life-threatening

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for newborns, elderly and in immunocompromised individuals. Listeriosis usually appears with non-invasive febrile gastroenteritis orinfluenza-like symptoms, but serious invasive listeriosis has more severe symptoms, which may lead to septicemia, meningoencephalitis, and abortion. In the case of outbreaks, mortality rate of more than 30% has been reported, and the rate could increase in vulnerable groups such as pregnant women, newborns, and the elderly.[2,3] In recent years, several outbreaks of listeriosis associated with diverse ranges of foods have been reported, especially in developed countries.[4,5] Dairy food products, non-pasteurised milk,[2,3] and soft or semi-soft cheese are major sources of outbreaks of listeriosis.

A recent fall down of reported cases of human listeriosis in the US and Europe was mainly due to good manufacturing practice implemented by the Hazard Analysis and Critical Control Point (HACCP) in the food industry as well as good veterinary inspection procedures.[4] Furthermore, to prevent human listeriosis, the World Health Organization (WHO) amended a directive that advises a surveillance system in various food products. However, the incidence of listeriosis in most developing countries is not known. There are also no reliable published data on either trend of contamination of food-borne pathogens (including *Listeria*) in food or on the implementation of HACCP in the food industry in these countries. Therefore, there is an urgent need to provide data on the risk of occurrence of food-borne pathogen *Listeria* in various food products. There are also very limited data on the prevalence of *Listeria* in food in Iran. Therefore, the present study aimed to evaluate the presence of *Listeria* species in food products with high risk of contamination, such as raw milk and traditional dairy products in Isfahan, Iran.

MATERIALS AND METHODS

From December 2011 to November 2012, a total of 292 samples including raw cow milk (91), ice cream (63), yogurt (12), doogh (28), butter (18), Kashk (22), cheeses (6), fereni (25), and cream (27) samples were randomly collected from 12 retail stores in Isfahan [Table 1], Iran. All samples were not pasteurized and not produced by certified companies. In addition, none of the products from which samples were taken were labeled as they were homemade or produced in traditional workshops. The samples were immediately transported to the laboratory in a cooler with ice packs and processed within an hour of collection.

Samples were analyzed using the method recommended by the United States department of agriculture (USDA).[5] Twenty-five grams of a sample was aseptically taken, blended for 2 min in 225 ml of University of Vermont media (UVM) *Listeria* enrichment broth (UVM I) (Merck, Germany) and incubated at 37°C for 24 h. One milliliter of primary enrichments was transferred to 9 ml of UVM II (Frazer broth) (Merck, Germany) and incubated at 37°C for 24-48 h. Secondary enrichments were streaked on Oxford agar (Merck, Germany) and PALCAM agar (Merck, Germany) and incubated at 37°C for 48 h. Enrichment broths and selective agars were supplemented according to the manufacturer's guidelines. The plates were examined for typical *Listeria* colonies (black colonies with black sunken centers) and at least 3-5 suspected colonies were sub-cultured on Tryptone Soy Agar supplemented with 0.6% yeast extract (TSAYE) and incubated at 37°C for 24 h. All isolates were subjected to standard biochemical tests such as Gram staining, catalase test, motility at 25°C and 37°C, acid production from glucose, mannitol, rhamnose, xylose, α-methyl-D-mannoside, and nitrate reduction, hydrolysis of esculin, and MR-VP test. For further confirmations of *Listeria* spp., other biochemical reactions, ß-hemolytic activity, and CAMP test were performed according to Bergey's Manual of Systematic Bacteriology.[6] All strains of *L. monocytogenes* were also confirmed by polymerase chain reaction (PCR) method. Bacterial strains were cultured in Brain hart Infusion Broth (BHIB) at 37°C for 18 h, and genomic DNA of bacterial strains were extracted by the method of Fitter *et al.*[7] Primer 234 (5′-CATCGACGGCAACCTCGGAGA-3′) and primer 319 (5′-ATCAATTACCGTTCTCCACCATT-3′) were selected as published by Fitter *et al.* These primers allowed amplification of a 417 bp internal fragment of the

Table 1: Prevalence of *Listeria* spp. in raw milk and traditional dairy products in Isfahan, Iran

Type of food	Number of samples	Number (%) of *Listeria* spp.	Number (%) of *L. monocytogenes*	Number (%) of *L. innocua*	Number (%) of *L. seeligeri*
Raw milk	91	5 (5.49)	4 (4.39)	0	1 (1.09)
Ice cream	63	12 (19.04)	0	12 (19.04)	0
Yogurt	12	0	0	0	0
Doogh[a]	28	0	0	0	0
Butter	18	0	0	0	0
Kashk[b]	22	0	0	0	0
Traditional Cheese	6	0	0	0	0
Fereni[c]	25	1 (4)	0	1 (4)	0
Cream	27	3 (11.11)	0	3 (11.11)	0
Total	292	21 (7.14)	4 (1.36)	16 (5.82)	1 (0.34)

a: Dairy product prepared by beating unflavored yogurt until smooth, and then diluting with water to a Consistency similar to whole milk, b: A dairy product prepared by prolonged boiling of yogurt, c: Special Iranian dairy product prepared by boiling rice powder, sugar, and rose

blyA gene.[8] PCR was achieved as described by Fitter *et al.*[7] The PCR reaction mixtures (25 µl) consisted of 2.5 µl of 10C PCR buffer (500 mmol/L KCl, 100 mmol/L Tris pH 8.3, 25 mmol/L $MgCl_2$), 3 µl of each primer (10 µmol), 0.4 µl of dNTPs (20mM), 0.2 µl Taq polymerase (5,000 U/ml), and 13.5 µl of boiled lysate. Target DNA were amplified using a Techne Thermal Sequencer (FTGENE 2D, UK). Genomic DNA was initially denatured at 94°C for 7 min, followed by 30 cycles of 1 min denaturation at 94°C, 30 sec primer annealing at 62°C, and 30 sec extension at 72°C. Tubes containing the reaction mixture were maintained at 4°C after final extension at 72°C for 1 min. The PCR amplified reaction mixture samples (16 µl aliquots) were subjected to horizontal gel electrophoresis in 1.8% agarose gels run in 1x Tris-borate (TBE). PCR products were visualized using ethidium bromide staining (1 µg/ml) and photographed under UV light.

RESULTS

The obtained results are summarized in Table 1. Of the 292 samples, 21 (7.14%) and 4 (1.47%) were positive for *Listeria* spp. and *L. monocytogenes*, respectively. *L. innocua* with 16.21 (76.19%) was the most prevalent species isolated, followed by *L. monocytogenes* with 4.21 (19%) and *L. seeligeri* with 1.21 (4.7%). The prevalence of *Listeria* spp. in raw milk, ice cream, cream, and fereni was 5.91 (5.49%), 12.63 (19.04%), 3.27 (11.11%) and 1.25(4%) respectively. Ice cream was the most contaminated (19% for *L. innocua*) product tested. Of the 91 raw milk samples, 5 (5.49%), 4 (4.39%), and 1 (1.09%) were positive for *Listeria* spp., *L. monocytogenes*, and *L. seeligeri*, respectively. Of the 25 fereni samples, only 1 (4%) was positive for *L. innocua*. *L. innocua* was also isolated from 3.27 (11.11%) of cream samples [Figure 1]. *Listeria* was not detected in yogurt, butter, kashk, and cheese. All strains of *L. monocytogenes* identified by biochemical tests were also confirmed by PCR.

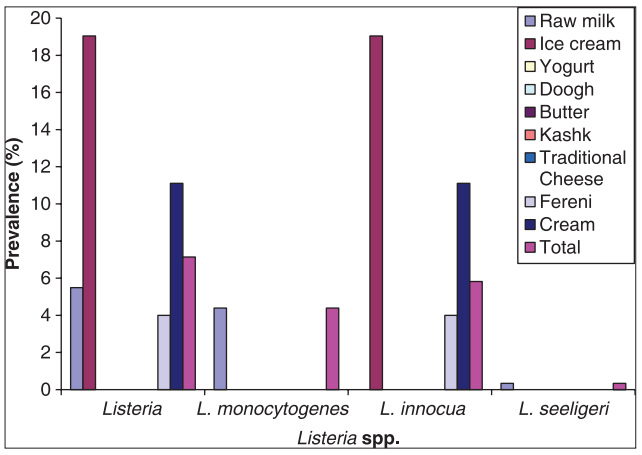

Figure 1: Prevalence of *Listeria* spp. in raw milk and traditional dairy products in Isfahan, Iran

DISCUSSION

L. monocytogenes is an important psychotropic food-borne pathogen, which may exist in milk and dairy products. The bacterium causes listeriosis with severe clinical consequences such as meningitis, septicemia, and abortion. Therefore, contamination of food stuff implies a significant health risk for humans. Minimum data on food contamination by *Listeria* are available from most developing countries. Providing such a data can convince regulatory authorities to set better codes of practice in the food industry and food distribution chain. In this case, Iran is no exception, as only few publications on the prevalence of *Listeria* in food are available; however, these data are not enough to draw a conclusion on the risk assessment of *Listeria* in an Iranian food customer.

In the present study, 5.49% of raw milk samples were contaminated with *Listeria* spp. *L. monocytogenes* was the most frequent (4.39%) species identified in raw milk. Unlike most previous studies, no *L. Innocua* was discovered in raw milk.[9,10] In several Iranian studies, the prevalence of *L. monocytogenes* in raw milk is reported to be 1-4%.[9-12] The prevalence rate of *L. monocytogenes* in the current study is in agreement with a previous investigation in Iran. In the study by Jalali and Abedi (2008), unlike the current study, only 1 of the 88 samples of dairy products was positive for *L. innocua*.[13] In another study by Rahimi *et al.* in Iran, of the 90 raw milk samples collected, 10 were positive for *Listeria* spp., from which 1.1% was related to *L. monocytogenes* and 1.1% to *L. seeligeri*.[10] In contrast to the present study, the highest rate of contamination (8.9%) was from *L. innocua*.[10] In the present study, *Listeria* was not isolated from traditional cheese, while Rahimi *et al.* reported a relatively high rate (15%) of contamination.[10] Lower prevalence rate (1.7-3.3%) of *L. monocytogenes* was reported by Mahmoodi (2010), in two milk processing facilities in South of Iran.[11] Similar to the results of present study, none of the samples of yogurt and yogurt drink was found to contain *L. monocytogenes* in previous studies.[10,11] The reason could be low PH in these products.[11] In another study by Moshtaghi and Mohamadpour (2007) in Shahrekord,[9] the prevalence of *Listeria* spp. in 500 samples of raw milk was reported to be 2.2%, of which, 1.6% was contaminated with *L. monocytogenes* and 0.16% with *L. innocua*. In these studies, the rate of prevalence of *L. monocytogenes* was less than that found in the current study.[9] In general, it seems that the findings of the current study are aligned with those of other Iranian investigation.

Results of the current study are in agreement with most previous investigations; however, it is in contradiction to some reports. For instance, in studies conducted in Brazil, Portugal, Turkey, and Mexico, higher rates of contamination of raw milk have been reported. In a study by Nero *et al.*, a much higher rate of 25.3% contamination of *L. monocytogenes* in

raw milk has been reported in Brazil.[14] In another study by Mena *et al*. in Portugal, contamination with *L. monocytogenes* was determined to be 16.7% in raw milk.[15] In a study done in Turkey, 10% of raw milk samples were contaminated with *Listeria* spp., of which 5% were related to *L. monocytogenes*.[16] In Mexico, of 1,300 raw milk samples collected from tanks delivering the raw milk, 23% were positive for *Listeria* spp., of which 13%, 6%, 4%, and 1% were *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, and *L. innocua*, respectively.[17]

There are also some studies, which have reported similar or lower rates of contamination of *Listeria* in non-pasteurized dairy products. For example, a study on contamination of raw milk in Costa Rica (2003) indicated that 3% of the samples were contaminated with *L. monocytogenes*.[18] In another study done in Turkey, a lower prevalence of 2.2% in raw milk compared to the present study was reported to be positive for *L. monocytogenes*. In contrast to the current study, 8.23% of white cheese samples were also contaminated with *L. monocytogenes*. Similar to the present study, no contamination was discovered in butter and yogurt.[19] The absence of *Listeria* in yogurt in the current study and other studies can be attributed to the low pH (>3.5) of the product.[10,16,20] In a study by Erol and Sireli (2002), 15% of the samples of unpasteurized butter contained *Listeria* spp. of which 5% were *L. monocytogenes*.[21] This finding is not in agreement with the results of the current study. The results obtained in the present study are almost also similar to those of Arsalan and Azdemir, El-Sharef *et al*., and Rahimi *et al*.[12,16,22]

The results of the present study are congruent with those of most other studies. However, care must be taken when comparing and interpreting currently available studies. Only a limited number of studies on contamination of traditional dairy products have been published, and these have involved various numbers of samples with different sampling methods and culture techniques. At present, *L. monocytogenes* is identified as an important pathogen transmitted from food products, especially raw milk and dairy products.[16,23-25] Although pasteurization of raw milk is effective in destruction of *Listeria*, there are reports of listeriosis caused by the consumption of pasteurized dairy products.[26,27] However, this contamination is usually related to post-pasteurization contamination. Traditional dairy products in Iran are generally non-pasteurized and produced in small workshops and homes. In addition, these producers normally do not hold any license from health authorities. There are also no reliable data available on the level of hygienic practice in these small workshops, but, ironically, they are known to have poor food safety culture. Pasteurization was not done during production of dairy products investigated in the current study. Therefore, these products can be considered to be of very high risk in terms of the presence of *Listeria* and other food-borne pathogens.

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