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Original Article

Prevalence and Characterization of *Listeria* Species in Domestic and Industrial Cheeses of Isfahan Region

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Abstract

Background: *Listeria monocytogenes* is of major concern to the food industry in general and the dairy industry in particular. Little is known about incidence of this pathogenic bacterium in dairy products in Iran.

Methods: A survey was made from 23 September 2006 to 22 June 2007 for *Listeria* species in ninety samples of traditional and industrial cheeses, in milk and surface where the cheeses were manufactured from unpasteurized raw milk in the province of Isfahan (Iran).

Results: *Listeria murrayi*, *L. grayi* and *L. ivanovi*i, were detected in nine traditional cheeses and one raw milk sample. None of the different *Listeria* species were isolated from the industrial cheeses and their environment.

Conclusion: There are almost good hygienic conditions in domestic cheese manufacturing farmhouses in Isfahan area, but we should try to improve hygienic levels until we have none of the *Listeria* spp. in our samples.

Keywords: Listeria, *Cheese, Milk, Iran*

Introduction

Listeria spp. *(L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. murrayi* and *L. grayi)* are psychrotrophic, grow well in media with pH levels between 4.4-9.4 over a temperature range of 0-45˚ C and water activity above 0.92 (1). Among all species of *Listeria*, *L. monocytogenes* causes human listeriosis. Mild symptoms of listeriosis including diarrhea, fever, headache and myalgia are developed (2) but in the case of invasive listeriosis, severe symptoms including septicemia, meningoencephalitis, abortion and stillbirth especially in pregnant women, neonates, adults with underlying disease (cancer, AIDS, diabetes, chronic hepatic disorder, transplant recipients), the elderly (>65 yr old) and the immunocompromised individuals are seen (1, 3, 4). Studies have shown that the first major amplification source of food product contamination with *L. monocytogenes* might be cross contamination which occurs in the environment of the food processing such as cheese making areas (5-8). This organism has been involved in several outbreaks and sporadically cases of disease associated with the consumption of pasteurized milk, cheeses made from unpasteurized milk and other dairy products.

Prevalence rate of *Listeria* spp. is different in different countries. In a study in Spain, *L. monocytogenes* and *L. innocua* were detected in 3.6% and 2.7% of raw milk samples (9). In Abou-Eleinin *et al.*, study 35 of 450 raw goat milk samples (7.8%) were positive for *Listeria* spp., in which *L. innocua* was detected in 26 samples (5.8%) and *L. monocytogenes* was detected in 17 samples (3.8%) (10). In Iran, little data exist on the prevalence of *L. monocytogenes* in foods. In Rahimi *et al.* (2010) study, one of the 95 commercial (1.1%) and 25 of the 168 traditional (14.9%) dairy product samples were positive for *Listeria*

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spp. and the difference was statistically significant (3). Also in Jalali and abedi (2008) study 617 food samples were examined. The incidence of *Listeria* spp. was 4.6% in all food samples. *L. monocytogenes* was found in 1.2% of food samples. It was found that *Listeria* spp. was present in 6.7% of meat and meat product samples, 1.3% of diary samples, 1.2% of vegetable samples, and 12% ready to eat samples (11).

The presence of *Listeria monocytogenes* in food products is a very important and complex issue for microbiological risk assessment and may result in drastic healthy problems and economic losses for the industry (8). The distribution of species recovered may vary according to the type of food and detection methodology utilized, although the most frequent isolates are *L. innocua* and *L. monocytogenes*. Since *Listeria* spp. generally occurs in low numbers in foods both conventional and rapid detection methods for food samples require one or more enrichment steps in selective broth. The aim of this study was determining the percentage of *Listeria* spp. in domestic and industrial cheeses and raw milk in the province of Isfahan to evaluate the quality of sanitation in retail and cheese manufacturing industries and manufacturing area.

Materials and Methods

Samples of cheese, milk, and surfaces

Among twenty one different types of domestic cheeses, three (each in 5 replication) were obtained from special regions of Isfahan including Alavijeh, Meymeh and Najafabad as the main samples (Table 1) and 18 (in different replication) samples from other regions of Isfahan and their countryside, two different industrial cheeses each in 5 replication, three milk samples and three environment samples obtained from the animals hide and their watering places, walls and ground from 23 September, 2006 to 22 June, 2007.

Isolation of Listeria

Twenty five gram of each sample was transferred aseptically to a stomacher bag, 225 ml of LEB broth (*Listeria* enrichment broth, Liofilchem, Italy) (1) at temperature of 25˚ C was added to the sample. The mixture was homogenized in the stomacher (Seaward 400, England) for 60 s until the cheese was thoroughly dispersed. The enrichment medium containing cheese sample was then transferred to a 250 ml sterile flask and incubated at 30˚ C for 2 d. The growth of *Listeria* was checked every 24 h by taking a loop of sample on the Palcam agar (Liofilchem, Italy) as duplicate plates and incubated at 37˚ C for 48 h (5, 12). For food processing facility environment, swabs were randomly collected from the animals hide and their watering places, walls and ground. The swabs were subjected for bacterial isolation following each bacterial standard protocol (13). All the developed colonies on the plates were tested using Gram and spore staining, catalase, motility, MR/VP; haemolysis on blood agar and fermentation of sugars (14).

Physicochemical tests performed on the cheese samples

At first the appearance of the cheese samples including color, surface properties and texture were checked, then Chemical properties of cheese samples including pH (AOAC 2002, 981.12), acidity (AOAC 2002, 920.124), moisture (AOAC 2002, 977.11) and salt content (AOAC 2002, 975.20) were determined (15).

An Indicator strain of Listeria

To compare the results of the biochemical tests performed on the strains isolated from the cheese and other samples it was decided to use a known *Listeria* strain for the experiments as an indicator *Listeria*. For this purpose *L. monocytogenes* RITCC 1293, serotype4a, was obtained from the department of Microbiology, Faculty of Medical Sciences, Isfahan University of Medical Sciences, Iran. The identity of the microorganism was confirmed using morphological and biochemical tests according to the Bergey's manual (16).

Results

In this study, all traditional cheeses had flat surfaces some with very fine holes with depth of 1

mm on them. However, the pasteurized samples had only flat surfaces without any hole on them. Color of all cheeses was white and opaque.

The result of pH, acidity, moisture, and salt content of some samples were shown in Table 1. Another samples that are not shown in Table had pH value of 5-6.5, moisture content of 59-66%, acidity of 0.2-2 (lactic acid %) and salt content of 4-6%. Domestic cheese samples obtained from the local farmhouses were assessed for the presence of *Listeria* genus using traditional techniques. Among all 90 samples (cheese, milk and surface) *Listeria* was isolated from 10 samples using Palcam agar. This means that almost 11% of the samples were contaminated with genus of *Listeria*. The result of the biochemical properties of the isolates revealed that they were belonged to the species of *L. murrayi*, *L. grayi* and *L. ivanovii*. (Table 2 and 3).

Nine out of ten isolates were detected in the cheeses with pH 5-5.5 and only one isolate was detected in the cheese with pH value of 3.5.

After isolation of *Listeria* species from some cheese samples, it was tried to isolate *Listeria* species from the milk and surface area of the cheese manufacturing locations related to the contaminated cheeses. *Listeria* was only detected in one milk samples and none of the examined surfaces was contaminated with *Listeria* spp. In addition, none of the *Listeria* species were isolated from the pasteurize cheeses.

Table 1: Chemical properties of collected cheese and milk samples

Place of sample collection	Farm	Type of sample	Replication	Moisture	pH	Salt	Acidity
Alavijeh	1	Farmhouse cheese	5	62.10	5	6.44	0.38
	2	Farmhouse cheese	3	61.20	5.30	5.90	0.33
Najafabad	1	Farmhouse cheese	5	58.91	5.08	5.6	0.67
	2	Farmhouse cheese	3	60.12	6.30	6.20	0.22
Meymeh	1	Farmhouse cheese	5	61.20	4.68	5.04	0.75
Kushk	1	Farmhouse cheese	3	59.27	5.7	4.50	0.30
	2	Milk	1		6.20		0.22
Mihan	-	Industrial cheese	5	66.13	4.78	1.11	4.50
Ruzaneh	-	Industrial cheese	5	64.38	4.26	1.21	4.18

Table 2: *Listeria* positive samples among the total replication related to each sample

Place of sample collection	Type of sample	Farm	Total number of samples	Isolated *Listeria* species
Alavijeh	Cheese	1	5	*L. murrayi* , *L. grayi*
	Cheese	2	3	*L. ivanovii*
	Animal body surfaces	1	3	-
	Environmental surfaces	1	3	-
Najafabad	Cheese	1	5	*L. murrayi*
	Cheese	2	3	*L. murrayi*
Meymeh	Cheese	1	5	*L. grayi*
Kushk	Cheese	1	3	*L. ivanovii*
	Milk	1	2	*L. grayi*
	Animal body surfaces	1	3	-
	Environmental surfaces	1	3	-

* All the tests were done as duplicate

Table 3: Biological properties of *Listeria* spp. isolated from each sample

	Gram	Spore	Catalase	Motility	MR/VP	Manitol	Xylose	Rhamnose
L. monocytogenes RITCC 1293 (Control)	+	-	+	+	+	-	-	+
L. grayi	+	-	+	+	+	+	-	-
L. murrayi	+	-	+	+	+	+	-	+
L. ivanovii	+	-	+	+	+	-	+	-

+ Positive reaction
- Negative reaction
*All the tests were done as duplicate
*The result of each test was compared to the results presented in the Bergey's manual (Seeliger and Jones, 1984)

Table 4: Sensitivity of *Listeria monocytogenes* serotypes 1/2 and 4a (commonly isolated from food) to inhibition by bacteriocin like inhibitory peptides produced by *Listeria innocua* and *L. monocytogenes* strains (Kalmokoff et al., 1999)

Serotype	Isolates tested	*L. innocua* 743	*L. innocua* 755	*L. innocua* 228	*L. monocytogenes* 538
1/2a	4	+	+	+	+
1/2b	1	+	+	+	+
1/2c	2	1/2	+	1/2	1/2
3a	2	+	+	+	+
3b	3	+	+	+	+
3c	1	+	+	-	-
4a	1	+	+	+	+
4ab	1	+	+	+	+
4b	13	+	+	9/13	9/13
4b(x)	3	+	+	+	+
4c	1	+	+	+	+
4d	1	+	+	+	+
4e	1	+	+	+	+
6a	1	+	+	+	+
6b	2	+	+	-	-

Discussion

As previously mentioned, most of *Listeria* spp. were isolated from samples with pH range from 5 to 5.5. The pH range 5-5.5, may allow growth of *Listeria* during production, maturation, or storage, depending on other factors including the temperature of storage, the salt content, type, and concentration of organic acids present in the cheese. This result corresponds to the results of other investigators who mentioned that genus of *Listeria* is capable of growing at a pH range between 5.2- 9.6 and its optimum pH is neutral or few alkaline (14, 17). Also in this study *Listeria* was isolated from one cheese sample with pH of 3.5. Similarly, Faleiro (2003) and Gahan (1996) isolated *Listeria* from the cheeses with the same pH (18, 19). Gahan (1996) showed that tolerance to severe acid stress (pH 3.5) could be induced in *Listeria* following hour incubation in the mild acid condition (pH 5.5) (19); this phenomenon termed the acid tolerance response. As previ-

ously said all the samples had not passed ripening period. In the farmhouse cheeses after adding rennet enzyme to the warmed milk, it is necessary to keep it warm for 1-1.5 h to form curd. It is possible that during this period pH reaches to 3.5.

In evaluation of moisture and salt content of the samples all the cheeses used in this study were soft and semi-soft and because of the low density of these samples, more the oxygen level is convenient for growing the microaerophil *Listeria*. According to Rodulf and Scherer (2001) semi-soft cheeses were more capable of getting contaminated to *Listeria* species than the hard samples (20).

It is known that *Listeria* grows optimally in the low salt concentration and it can tolerate high salt content (10%) and grows very slowly in such conditions. All the samples in this study had the salt concentration below 10% that is suitable for *Listeria* growth; this was according to Pintado et al study (2005) which *Listeria* was isolated from soft cheeses containing 3.79-5.77 salt (14).

The first reason for the low isolation rate of *Listeria* from the cheeses could be attributed to the presence of undesirable conditions in cheeses for *Listeria* to grow. The high concentration of salt and the low acidity and moisture content of cheeses can prevent *Listeria* growth while in this study the amount of chemical factors tested were suitable for *Listeria* growth. To answer why in such conditions *Listeria* could not grow, it could be said that the presence of certain conditions during cheese manufacturing process selected some species compatible effectively against *L. monocytogenes* strains. Another possible hypothesis is that the isolation procedure based on the enrichment and subsequent plating could have suppressed *Listeria* growth or favored the growth of other bacteria rather than *Listeria*. Considering the heterogeneous isolation sources in terms of both number of cheese plants and geographical locations, a possible pressure based on the common technology applied in this plants could explain the reason for selection of few dominating *Listeria spp.* strains among the domestic cheeses instead of the species of *L. monocytogenes*.

The third effective factor in the appearance of *Listeria* spp. is production of inhibitors including bacteriophages and monocins by *Listeria* species that are effective on the *Listeria* spp. growth rather than the producer (21).

According to the literatures, the production of bactericidal substances is widespread throughout the eubacteria (22, 23). Kalmokoff et al. (1999) proved the existence of these inhibitory substances produced by *Listeria* spp. In their study the heat-stable, protease sensitive, peptide inhibitors were produced by four isolates of *Listeria* demonstrated a broad spectrum of activity against most *L. monocytogenes* serotypes tested, including those falling within the 1/2 and 4b serogroups, the most common *Listeria* agents of the foodborne outbreak (Table 4).

Bacteriocins produced by other bacterial species like *Enterococcus* and *Lactobacillus* can also suppress *Listeria* growth. It seems that inhibitory effect of LAB bacteriocins on the *Listeria* is because of familiarity of these two groups in classification (24, 25).

The forth reason is competition between *Listeria* species and natural flora of the cheese and milk on the growth or nutritional requirement. Bess et al. (2005) showed that the natural flora of contaminated foods has no effect on *Listeria* spp. in each enrichment steps and final changes of species are due to the nutritional competitions (26).

As the fifth reason, factors like Lauric, Linoleic and Linolenic fatty acids in high concentrations have strong effect on the *Listeria* growth. Bovinlactoferrin, peroxidase, lysosyme and antibiotics were also found effective on *Listeria* (27).

In conclusion, the results of present study demonstrated that there are almost good hygienic conditions in the domestic cheese manufacturing farms in Isfahan region, but it is necessary to try to improve hygienic levels until there is none of the *Listeria* spp. in the samples.

The presence of *Listeria* should trigger a review of the production process in order to improve the control of hygiene at critical control points and prevent contamination. Also a quantitative risk assessment relevant to the soft cheeses studied in

the present investigation is needed on individual farms, which will obtain further information about the animals, the incidence of *Listeria* in the raw milk, the process of production and sale of the cheese. *Listeria* spp. are ubiquitous in the farm and industrial environments and therefore the control of *L. monocytogenes* during food process is extremely difficult. Thus, it is suggested that the hygienic conditions described in the HACCP programs should still be enforced in order to minimize the count of *Listeria* species in dairy products during the manufacturing, handling and storage process in dairy plants and retail stores.

Ethical Considerations

Ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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