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Full Length Research Paper

Comparative contamination of *Listeria monocytogenes* in traditional dairy products in Esfahan Province, Iran

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***Listeria monocytogenes,* a primary human pathogen, has been found in different places in the environment of dairy, and the bacterium may survive for a long time in a dairy. *L. monocytogenes* is a major concern for the food industry, as it can cause listeriosis in humans. Listeriosis is one of the most important infections in Europe and the United States. It may cause fever, muscle aches and gastroenteritis; but does not usually cause septicaemia in healthy non-pregnant individuals. In pregnant women, it may cause abortion or neonatal death. From June 2010 to April 2012, a total of 420 samples were collected from different places in Esfahan Province. Samples were collected and analyzed based on the International Organization for Standardization using cultural method and biochemical test. Totally, in 57 samples (13.57%) of 420 dairy product samples *L. monocytogenes* was isolated. Based on biochemical observation, out of 210 raw milk samples, 42 (20%) were contaminated by *Listeria monocytogenes.* In traditional butter samples, 9 samples were contaminated by *L. monocytogenes* (12.85%); in traditional cheese samples, 5 (7.14%) samples were contaminated by *L. monocytogenes* and in traditional curd samples, 1 sample was contaminated by *L. monocytogenes* (1.43%). According to our finding, using traditional dairy products is dangerous, and it must be controlled by the ministry of health organization or other related organizations.**

Key words: *Listeria monocytogenes*, dairy products, Esfahan, Iran.

INTRODUCTION

Listeria monocytogenes is a rod shaped, gram positive, facultative anaerobic, non-spore forming bacterium with a low C+G content. *L. monocytogenes* is the primary human pathogen, although there have been rates of illnesses caused by *Listeria selegeri*, *Listeria ivanovii* and *Listeria innocua* (Jeyaletchumi et al., 2010). *L. monocytogenes* has been found in different places in the environment of dairy plants (Menendez et al., 1997), and the bacterium may survive for a long time in a dairy (Unnerstad et al., 1996). *L. monocytogenes* is a major concern for the food industry, as it can cause listeriosis in humans (Kathariou 2002). Listeriosis is one of the most important infections in Europe (European Food Safety Authority-European Centre for Disease Prevention and Control, 2007) and United States (Mead et al., 1999). Exposure to food borne *L. monocytogenes* may cause fever, muscle aches and gastroenteritis Riedo et al., 1994), (but does not usually cause septicaemia in healthy

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Abbreviations: *Listeria monocytogenes, Listeria innocua.*

Table 1. Result interpretation.

Well	Test	Principle	Positive reaction
1	Catalase	Detects catalase activity	Effervescence when treated with 3% H_2O_2
2	Nitrate reduction	Detects Nitrate reduction	Pinkish red
3	Esculin hydrolysis	Detects Esculin hydrolysis	Black
4	Voges proskauer's	Detects acetoin production	Pinkish red
5	Methyl red	Detects acid production	Red
6	Xylose	Carbohydrate utilization	Yellow
7	Lactose	Carbohydrate utilization	Yellow
8	glucose	Carbohydrate utilization	Yellow
9	α- methyl-D mannoside	Carbohydrate utilization	Yellow
10	Rhamnose	Carbohydrate utilization	Yellow
11	Ribose	Carbohydrate utilization	Yellow
12	Mannitol	Carbohydrate utilization	Yellow

non-pregnant individuals (Riedo et al., 1994). In pregnant women, it may cause abortion (Linnan et al., 1988; Riedo et al., 1994) or neonatal death (Linnan et al., 1988). *Listeria* is ubiquitous in dairy farms (Nightingale et al., 2004), and it is isolated from milk of cows (Jayarao and Henning 2001; Van Kessel et al., 2004; Arimi et al., 1197; Margolles and Reyes-Gavilan 1998; Unnerstad et al., 1996). *L. monocytogenes* in raw milk can be killed if heated at 71.7°C for 15 s (Bradshaw et al., 1985). *L. monocytogenes* has been detected in pasteurized whole milk, non-fat milk and chocolate milk produced in the United States (Frye and Donnelly 2005; Jayarao et al., 2006). The objective of this study was to determine contamination rate of *Listeria* in 420 different dairy product samples using cultural method and biochemical tests.

MATERIALS AND METHODS

From June 2010 to April 2012, 420 samples were collected from different places in Esfahan Province. They consist of 70 samples of raw cow milk, 70 samples of raw ewe milk, 70 samples of raw nanny goat milk, 70 samples of traditional cheese, 70 samples of traditional butter and 70 samples of traditional curd. Milk samples (1,000 ml) and others samples (500 g) were collected as finished packaged. Samples were immediately sent to Central Laboratory of Islamic Azad University of ShahreKord with ice. Samples were collected and analyzed based on the International Organization for Standardization (International Organization for Standardization 1995). 25 ml of each raw milk sample was aseptically added to 225 ml of Listeria enrichment broth (UVM, Difco 0223) and was incubated at 30°C for 20 to 24 h; and then 0.1 ml of this pre-enriched culture was added to Fraser Broth (Difco 0219) and incubated at 35°C for 24 to 48 h. After selective enrichment, samples were cultured into the PALCAM Listeria selective agar (Oxford Unipath Ltd., Basingstoke, Hampshire, United Kingdom). The plates were incubated for 48 h at 37°C. Twenty-five gram (25 g) of each cheese and butter sample was removed and transferred to a bag containing 225 ml of 2% sodium citrate solution, and then homogenized for 2 min at room temperature in a stomacher. Each sample was serially diluted in 0.1% sterile peptone water and a total of 333 µl of the diluted sample was spread plated onto Oxford agar plates. Presumptive *Listeria* colonies were counted. Presumptive *Listeria* spp. were confirmed by picking five colonies or all when fewer, and investigating the colonies for biochemical tests such as the presence of catalase, hemolysis, fermentation of xylose and rhamnose, oxidase, and umbrella-shaped growth in motility in SIM medium (sulfur reduction test, indole production, motility) using identification kit (Himedia, KB012 HiLiteria, India). Identification kit can also be used for validating known laboratory strain. Each kb012 is a standardized colorometric test system based on motility carbohydrate utilization and other biochemical tests specific for the identification of listeria species. The tests were based on the principle of PH change and substrate utilization. *Listeria* spp., on incubation, exhibit metabolic changes which are indicated by a color change in the media that can be either interpreted visually or after addition of reagent wherever required. All tests were done based on the guidance of identification kit. For catalase test, firstly, a loopful of growth was well scraped from the surface of the third well. Then the loop was dipped in a small clean test tube with 3% H_2O_2. Positive catalase test was seen as effervescence coming out from the surface of the loop. No effervescence was observed in negative catalase test. The samples that were brown-greenish and surrounded by a black halo were transferred to trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE, Difco) and incubated at 30°C for 24 to 48 h. For nitrate reduction in second well, one to two drops of sulphanilic acid (R015) were added as well as one to two drops of N, N-dimethyle-1-Napthylamine reagent (R009). Immediate development of pinkish red color upon addition of reagent indicates positive reaction and no change in color indicates negative reaction. Esculin hydrolysis in third well was indicated by blackening in the third well. For Voges proskauer`s test in fourth well, three to four drops of Barritt reagent A (5% A-napthol in absolute ethanol. R029) and one to two drops of barritt reagent B (40% potassium hydroxide, R030) were added. Upon addition of reagent, pinkish red color is observed within 10 minutes. No change in color or a slight copper color (due to reaction of barritt reagent A and barritt reagent B) denotes a negative reaction. Methyl red test was done in fifth well by adding one to two drops of methyl red reagent (I007). Reagent remains distinct red if the test is positive. Reagent decolorizes and becomes yellow if the test is negative. Carbohydrate fermentation test was done in sixth to twelfth: well color of the medium changes from red to yellow color due to acid production if the test is positive. Medium remains red in color if the test is negative (Table 1). Gram staining was also performed on the doubtful colonies. Main laboratory tests for the differentiation of

Table 2. Identification Index of *Listeria monocytogenes*

Tests	*L. monocytogenes*
Catalase	+
Nitrate reduction	-
Esculin hydrolysis	+
Voges proskauer's	+
Methyl red	+
Xylose	-
Lactose	-
glucose	+
α- methyl-D mannoside	+
Rhamnose	+
Ribose	-
Mannitol	-

+, Positive; -, negative.

L. monocytogenes are shown in Table 2 (Janzten et al., 2006). All statistical analyses were performed using SPSS software, version 16 (SPSS Chicago, IL, USA).

RESULTS

Totally, 57 samples (13.57%) of 420 dairy product samples *L., monocytogenes* was isolated. Based on biochemical observation, out of 210 raw milk samples, 42 milk samples (20%) were contaminated by *L. monocytogenes:* 15 (21.43%) raw ewe milk samples (35.70% of contaminated milk samples), 14 (20%) raw cow milk samples (33.35% of contaminated milk samples) and 13 (18.57%) raw nanny goat milk samples (30.95% of contaminated milk samples). From 70 traditional butter samples, nine samples were contaminated by *L. monocytogenes* (12.85%); from 70 traditional cheese samples, five samples were contaminated by *L. monocytogenes* (7.14%) and from 70 traditional curd samples, one sample was contaminated by *L. monocytogenes* (1.43%). In other words, in contaminated samples, 73.7% isolated cases belong to raw milk samples (24.57% in cow milk samples, 26.32% in ewe milk samples and 22.81% in nanny goat milk samples). In three other dairy product samples, contamination was found in 26.3% of samples, so that contamination rate in traditional butter samples was 15.78%; in traditional cheese samples, 8.77% and in traditional crud samples, 1.75%. This information confirms that there is a significant difference between contaminations of raw milk and dairy products. In addition, it is seen that there is no significant difference between various kinds of raw milk samples. There are significant differences between various kinds of dairy products: each three kinds of dairy products have significant difference between each other.

DISCUSSION

Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis (EI-Malek et al., 2010). Listeriosis has been recognized as one of the emerging zoonotic diseases during the last two decades and is contracted mainly from the consumption of contaminated foods and food products (Farber, 2000; Low and Donachie, 1997). Increasing evidence suggests that substantial portions of cases of human listeriosis are attributable to the food borne transmission of *L. monocytogenes* (Low and Donachie, 1997). According to preview studies, milk was contaminated by *Listeria* spp., especially *L. monocytogenes* in different rates; for example, 23% of 172 samples contain *Listeria* spp. in which *L. monocytogenes* was in 19.7% (Latorre et al., 2009). *L. monocytogenes* was isolated in 4.6% and 6.5% of bulk tank milk samples (Jayarao and Henning 2001; Van Kessel et al., 2004) and also found in 1.0%. The incidence of *L. monocytogenes* in the dairy silo milk was 19.6% (Waak et al., 2002); also incidence of *L. monocytogenes* was reported as 33.3% (54.0% for *Listeria* spp.), by Harvey and Gilmour (1992). Our result is similar to that of Waak et al. (2002), who confirmed that prevalence of *L. monocytogenes* is high in raw milk samples. This information is sufficient to warn ranchers about their farming. The difference between our finding in raw milk samples and others may be due to method of identification, season of sampling, source of food, geographic location, kinds of media employed, cross contamination and hygiene during milking. Cross contamination and hygiene during milking means that workers during milking have to clean the teats carefully so that the feces attached to the teats do not transfer to the milk and milking machine. There are some studies which showed *Listeria* spp. in fecal sample that may infest milk, leading to septicaemia. For example, Lattore et al. (2009) showed that 25% of fecal samples were infested by *Listeria* spp. and in 7.1% samples, *L. monocytogenes* was isolated. In addition, they reported that approximately the source of infestation is environmental and fecal. Arimi et al. (1997) stated that diversity of *Listeria* ribotypes is isolated from different farm and dairy-related environments. They suggested that the raw milk is contaminated by numerous *Listeria* ribotypes endemic to the farm environment.

Our findings show varied range of contaminations in traditional dairy products from 1.43 to 12.85%. This difference may be due to their nature and processing. It means that curd samples enriched with salt and low moisture have lowest contamination rate between different products. This product is commonly used by women, because it is rich in calcium and other mineral elements. According to our finding, widespread usage of traditional curd of Iran cannot be a serious problem, but we suggest that pregnant women should the commercial type because it is prepared in a hygienic condition; and there is not any literature about contamination of commercial curd. This obtained information confirms previous study of Mojtahedi et al. (2004) on curd samples, where they mentioned there was no contamination of curd sample. Perhaps the little difference between our results and theirs is the size of the samples.

In this study, we isolated 12.85% of contamination in traditional butter samples. This almost high prevalence is due lack of heating during processing stages or due to contamination of instruments used for *L. monocytogenes*. According to Dole et al. (1987), *L. monocytogenes* was isolated from milk heated at 72.2°C for 16.4 s. The organism was not detected in the few trials of milk heated at 76.4 to 77.8°C for 15.4 s. In another study, Beckers et al. (1987) found out that *L. monocytogenes* inoculated at a level of 1.8×10^4/ml did not survive heating at 67°C for 20 s or more. This information and our finding showed that heating in a good manner can reduce the contamination rate of *L. monocytogenes*, because in the traditional processing of dairy products, heating is not in the main stage.

Beckers et al. (1987) stated that soft cheese samples (10.14 5%) were contaminated by *L. monocytogenes*. In other studies, Mojtahedi et al. (2004) isolated 2.5% and Delgado da Silva et al. (1998) recovered *L. monocytogenes* from seven out of 17 samples (41%) of cheese made from raw milk and from one of 33 samples (3%) of cheese made from pasteurized milk. In another example, Carvalho et al. (2007) recovered *L. monocytogenes* from three out of 93 samples (3%) of cheese made with pasteurized milk. However, according to our findings, *L. monocytogenes* was isolated in five out of 70 samples (7.14%).

Traditional cheese was prepared from raw milk, so we expected more contamination rate of *L. monocytogenes* in cheese samples. As a result, processing stages and storing of traditional cheese in Iran need much salty solution, which may lead to low prevalence than expected. It is noteworthy that contamination in cheese prepared from raw milk is almost equal in different studies.

According to our finding, using of traditional dairy products is dangerous and it must be controlled by Ministry of Health Organization or other related organizations.

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