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Full Length Research Paper

Prevalence of *Listeria* species in raw milk in Esfahan Province, Iran

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***Listeria* sp. is one of the most important zoonotic diseases which cause dangerous illness. It consists of six species but the most important one is *Listeria monocytogenes,* which is a major concern for the food industry. *Listeria* is ubiquitous in dairy farms and it is isolated from cows' milk. The purpose of this study is to identify *Listeria* spp. in 986 raw whole cow milk samples by cultural method and biochemical tests. From April 2010 to May 2011, totally 986 raw cow samples were collected. A total of 25 samples (2.53%) were contaminated by *Listeria* spp.: 2.02% by *Listeria monocytogenes* and 0.51% by *Listeria innocua.* In other words, 80% of infested samples was due to *Listeria monocytogenes.* Also it was seen that 92% of infested samples occurred in fall season. Our results show that contamination by *Listeria* spp. is commonly related to environmentally special food and season, because silos feeding is done in fall. Our finding suggests more studies on thermal resistance and improvement in preparing silos.**

Key words: *Listeria* species, raw milk, cow, Iran.

INTRODUCTION

Listeria sp. is a rod shaped, gram positive, facultative anaerobic, non-spore forming bacterium with a low C+G content. The genus consists of six species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria ivanovii* and *Listeria grayi*. *L.monocytogenes* is the primary human pathogen, although there are rates of illnesses caused by *L. seeligeri*, *L. ivanovii* and *L. innocua* (Jeyaletchumi et al., 2010). *Listeria* sp. is found in different places in the environment of dairy (Menendez et al., 1997), and the bacterium may also survive for a long time in a dairy (Unnerstad et al., 1996). *Listeria 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 (Scallan et al., 2011). 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 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). *L. monocytogenes* in raw milk can be killed if heated at 71.7°C for 15 s (Bradshaw et al., 1985). Although raw milk has previously been suggested as a vehicle of transmission of listeriosis, pasteurized milk is not suggested since 1980 (Gitler et al., 1980). But now we know that *L. monocytogenes* had 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 is to

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Abbreviation: *L.,* *Listeria*.

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

determine *Listeria* spp. in 986 raw whole cow milk samples using culture method and biochemical tests.

MATERIALS AND METHODS

From April 2010 to May 2011, a total of 986 raw cow milk samples were collected from bulk milk tanks in Esfahan Province, Iran. Samples were immediately sent to Central Laboratory of Islamic Azad University of Shahrekord with ice. Raw whole cow milk samples were collected from different farms in Esfahan, Iran and were analyzed based on the International Organization for Standardization (International Organization for Standardization 1995). 25 ml of each sample was aseptically added to 225 ml of Listeria Enrichment Broth (UVM, Difco 0223) and was incubated at 30 ^{0}C for 20-24 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 (Oxoid Unipath Ltd., Basingstoke, Hampshire, United Kingdom). The plates were incubated for 48 h at 37°C, and 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 are based on the principle of pH change and substrate utilization.

Listeria spp., upon 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. For catalase test, first 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 (l007). 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 *Listeria* spp. are shown in Table 2 (Janzten et al., 2006).

RESULTS

From the 986 raw milk samples, 25 (2.53%) were contaminated by *Listeria* spp. Based on biochemical observation, in 20 (2.02%) samples (80% of contaminated milk) *Listeria monocytogenes* was detected and 5 (0.51%) other samples were contaminated by *Listeria innocua* (20% of contaminated milk). This information confirms that there is a significant difference between contaminations by *Listeria* spp. with higher prevalence of *Listeria monocytogenes.* Also, it is seen that 23 contaminated samples (2.3%) were in fall season. In other words, 92% of infested samples occurred in fall season. It is noteworthy that a total of 271 samples were collected in fall, with 23 samples (8.5%) infested by *Listeria* spp. *Listeria monocytogenes* had been isolated in 86.95% of infested samples, or 7.38% of fall samples were infested by *Listeria monocytogenes.* All of *Listeria innocua* was isolated from samples which were collected in fall (13.05% of infested milk of fall season). It was only in two samples (from 255 samples of spring) that *Listeria monocytogenes* was identified in other seasons (spring).

Table 2. Identification Index of various *Listeria* spp.

Spp.	Test											
	Catalase	Nitrate reduction	Esculin hydrolysis	Voges proskauer's	Methyl red	Xylose	Lactose	glucose	α- Methyl-D mannoside	Rhamnose	Ribose	Mannitol
L. grayl	+	-	+	+	+	-	+	+	+	V	V	+
L. monocytogenes	+	-	+	+	+	-	-	+	+	+	-	-
L. innocua	+	-	+	+	+	-	+	+	+	+	-	-
L. selegeri	+	-	+	+	+	+	NR	+	-	-	-	-
L. ivanovii ivanovii	+	-	+	+	+	+	-	+	-	-	+	-
L. ivanovii londoniesis	+	-	+	+	+	+	-	+	-	-	-	-
L. welshimeri	+	NR	+	+	+	+	NR	+	-	+	-	-

+, Positive; -, negative, NR, not reported; V, variable reaction.

It shows there is significant difference between contaminations of samples in different seasons.

DISCUSSION

Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis (El-Malek et al., 2010). Listeriosis has been recognized to be 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 et al., 1997). Increasing evidence suggests that substantial portions of cases of human listeriosis are attributable to the food borne transmission of *L. monocytogenes* (Low et al., 1997). *Listeria* species are ubiquitous in the environment (Vitas et al., 2004).

According to preview studies bulk tank 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% of samples (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). Also, *Listeria monocytogenes* was found in 1.0% and *Listeria innocua* was found in 2.3% of the 294 farm bulk tank samples. The incidence of *L. monocytogenes* in dairy silo milk was 19.6% and the frequency of *L. innocua* was 8.5% (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 results are similar to that of Waak et al. (2002), who confirmed that prevalence of *Listeria* sp. is higher due to feeding with silo. Silos were fed to dairy cow in fall when other forages were rare and expensive. This information is sufficient to warn ranchers about silo preparing. The difference between our finding and others may due to method of identification, season of sampling, source of cow food (farm or silo), 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 raw milk is contaminated by numerous *Listeria* ribotypes endemic to the farm environment.

According to Waak et al. (2002), the detected level of *Listeria* spp. in raw whole milk from dairy silos varies in different studies; this may possibly be due to the age and storage conditions of the milk at sampling. Prevalence of *Listeria innocua* varies between dairy farm and dairy silos; it also varies between different studies in dairy farms or dairy silos. More studies are required in same situa-

tion. Perhaps, using some forage as feed or keeping milk for some minutes in bulks affect it, or perhaps there is a genetic resistance in some strains against lactoperoxidase system in raw milk.

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. Pasteurization eliminates any Listeria species; however, it is important this is done correctly and records can be taken to know the maximum temperature for achieving it (Doyle et al., 1987).

This provides some indication that the latter heat treatment may be sufficient to eliminate viable listeria from milk. Raw milk can be pasteurized in dairy products factories. Contamination by *Listeria* spp. is an important issue in Esfahan, and ranchers must process silos in better condition. Also dairy factories have to be sure about their product against *Listeria* spp. by using the best temperature for pasteurization. So this finding suggests that in future studies, thermal resistance of different common strains of *L. monocytogenes* and *L. innocua* should be investigated.

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