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Original Article

Prevalence of *Listeria Monocytogenes* in Raw Milk of the Healthy Cattle in Lorestan Province (Iran) by PCR

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Abstract

Background & Objective: The prevalence of *Listeria monocytogenes (L. monocytogene)* in milk and dairy products, the high complications and damage associated with zoonotic Listeriosis have led to this bacterium being considered as a risky infection for public health.

Materials & Methods: In the present descriptive and cross-sectional study, to investigate the prevalence of *L. monocytogene*, 100 samples of raw milk obtained from industrial farms in Lorestan province in winter and spring of 2019-2020 were collected and *16SrRNA* and *hlyA* genes were detected using PCR reaction to identify *Listeria* genus and *L. monocytogene* species, respectively.

Results: Based on the observed results, three samples (3%) were positive for *Listeria* genus, while no report of *L. monocytogens* species was observed.

Conclusion: The results of this study did not show evidence of the presence of *L. monocytogenes* in raw cow milk, which indicates the sanitation and desirable nutritional conditions in industrial farms. Therefore, due to the presence of *Listeria* (other species of *Listeria*) in raw milk, if you consume raw and unpasteurized milk, the risk of human infection with listeriosis should not be ignored.

Keywords: *Listeria monocytogenes*, Milk, Zoonosis, Cattle, Lorestan, PCR

Introduction

L. monocytogene is one of the most important bacterial infections transmitted through raw milk and dairy products that causes listeriosis in humans and animals (1). According to the Centers for Disease Control and Prevention (CDC), about 1,600 people are diagnosed with listeriosis and about 260 die each year (2). Due to the ability of this microorganism to grow at low temperatures and its transmission through food and dairy, in countries with low levels of health standards, high reports of listeriosis have been recorded (1). Intermittent excretion of *L. monocytogenes* in raw milk of ruminants has been reported without clinical signs of listeriosis (3, 4). Therefore, diagnosis of infected animals is

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essential to prevent the spread of listeriosis (5).

Using the common and valid PCR molecular detection method to identify *L. monocytogenes* in raw milk is more favorable compared to isolation and detection of bacteria by culture and biochemical methods, due to the time consuming nature of these methods and the increasing use of antibiotics in the diet of livestock and the probability of reporting false results. Listerio lysine O (LLO) is a secretory protein and a major causative agent of *L. monocytogenes* that is present in all pathogenic *L. monocytogenes* and is encoded by the *hlyA* gene, so its detection in food is an indicator of the presence of *L. monocytogenes* (6, 7). There are few studies related to the prevalence of *L. monocytogenes* in cow's milk and no such study has been performed on livestock in Lorestan province (Iran). Therefore, considering the importance of cow's milk as the main source of dairy production and the important role of milk in the possible transmission of infection to humans, in the present study, PCR method was used to directly detect *L. monocytogenes* to investigate the prevalence of this bacterium in cow's milk in Lorestan province.

Materials & Methods

Milk sampling

Milk samples were randomly collected from 100 cows from industrial farms in Lorestan province (Iran) from September 2019 to March 2020. 50 milliliters of each sample was gathered in tubes under aseptic conditions and transferred to laboratory on an icebox. The samples were stored at –20 °C for further studies.

DNA extraction

Frozen milk samples were thawed at room temperature and 10 mL of each sample was transferred to a 10 mL tube. The tubes were centrifuged at 13,000 × g for 10 min. Then, using a sterile swab, the top layer of fat was removed and the liquid was poured around. Next, 200 µL of precipitate were transferred to a new tube for DNA extraction using DNA Purification Mini Kit (GeneAll, Korea) according to the manufacturer instructions. The quality and quantity of extracted DNA was assessed by 1% agarose gel and Nanodrop spectrophotometer, respectively (Thermo Scientific, Waltham, USA). Finally, the extracted DNA was stored at -20°C for use in PCR reaction.

DNA amplification and detection of polymerase chain reaction (PCR) products

To identify the isolates, the PCR method was employed and specific primers of *16S rRNA* (553 bp) and *hlyA* (702 bp) genes for identification *of Listeria* spp were used *L. monocytogenes,* respectively. PCR amplification was conducted in 25 µL total volume using 12.5 µL of 2X master mix (Ampliqon Taq DNA Polymerase Master Mix RED), 0.5 µL of each specific forward and reverse primers (10nM) (provided by Takapou Zist Company, Tehran, Iran) (Table 1), and 4 µL of the extracted DNA. Genomic DNA isolated from reference strain *L. monocytogenes* (PTCC 1294) (Iran Scientific and Industrial Research Center) and sterile water were considered as positive and negative controls, respectively. These amplifications were conducted by Bio-Rad thermocycler (Model T- 100, USA) under the following conditions: Program A is for *Listeria spp* and program B is for *L. monocytogenes*. A: The initial step of 94°C for 5 min was followed by 30 cycles of 94°C for 30 Second as denaturation, annealing at 60°C for 45 Second, extension at 72°C for 45 Second, and followed by final extension at 72°C for 10 min. B: The initial step of denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 45 Second, extension at 72°C for 2 min, and final extension at 72°C for 8 min. The PCR products were separated in a 1.2% (w/v) agarose gel (Merck, Germany) containing 2.5 µg/mL DNA safe *stain* (Cinnagen, Iran). Electrophoresis was performed in 0.5x Tris/Borate/EDTA (TBE) buffer for one hour at 100 V. The resulting PCR products were visualized under a UV transilluminator (E-Box, Iran) and the 100 bp DNA ladder (Smobio, Taiwan) plus was used as the molecular size marker.

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Table 1. PCR primers used for *Listeria* spp. and *L. monocytogenes* detection

Target gene	Primer name	Primer sequence	Amplified size	References
16S rRNA	Forward	5'-CCT TTG ACC ACT CTG GAG ACA GAG C -3'	553(bp)	(8)
	Reverse	5'- AAG GAG GTG ATC CAA CCG CAC CTT C -3'		
hlyA	Forward	5'-CCTAAGACGCCAATCGAA-3'	702(bp)	(9)
	Reverse	5'-AAGCGCTTGCAACTGCTC-3'		

Results

In the present study, by examining 100 samples of raw milk collected from industrial farms in Lorestan province, based on PCR results of *16s rRNA* gene, the results showed that 3 samples (3%) were infected with *Listeria spp* (Figure 1). But, none of the samples of *L. monocytogenes* was detected by PCR results of *hlyA* gene (Figure 1).

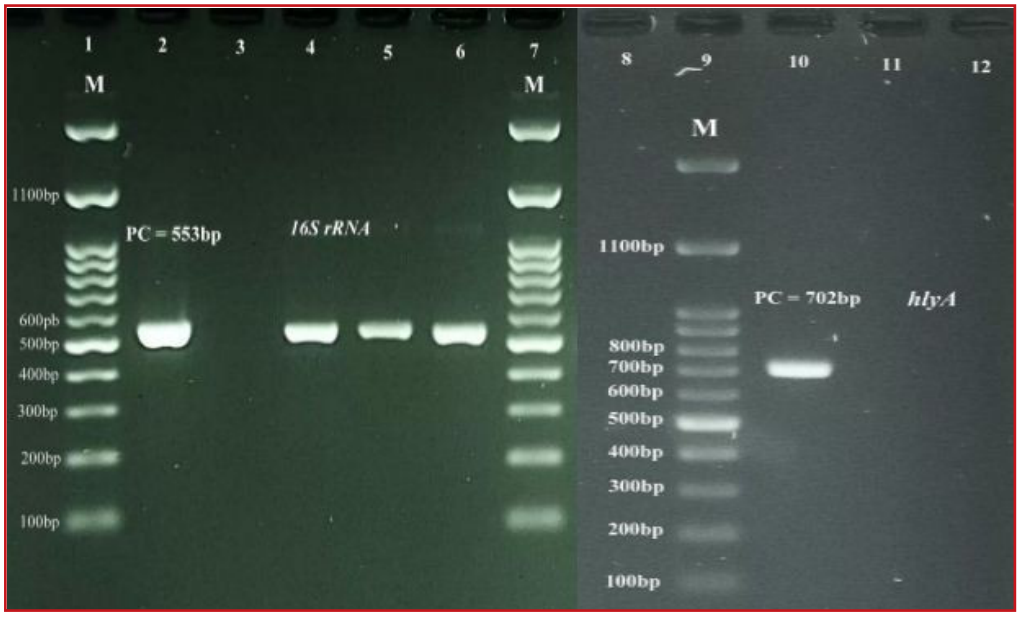

Figure1. PCR assay for the detection of *Listeria spp. and L. monocytogenes* in raw milk samples. Lane 1, 7, 9 (M) Standard DNA marker; Lane 2: Positive control for *Listeria spp*; Lane 3: Negative control; Lanes 4–6: Positive samples for *Listeria spp*; Lane 8: empty; Lane 10: Positive control for *L. monocytogenes*; Lane 11: Negative control; Lanes 12: Negative samples for *L. monocytogenes*. (PC: Positive control)

Discussion

Listeriosis is a zoonotic disease with food originality. Due to the widespread prevalence of *Listeria* species in the environment and the ability of this bacterium to grow at temperatures of 0 to 4 ° C and its high resistance to osmotic pressure, raw milk and contaminated dairy products are the most important route of Listeriosis transmission, so that in many studies, more emphasis has been placed on identifying *listeria* isolates from raw milk samples (4, 10, 11). In the present study, the prevalence of Listeria in milk samples isolated from industrial dairies in Lorestan province was determined to be 3%. While, *L. monocytogenes* was not detected in any of the samples. Numerous studies in different countries have examined the prevalence of *L. monocytogenes* in cow's raw milk. This prevalence was reported 4% In the United States (12), 2% in Turkey (13), 4% in Netherlands (14), 3.6% in Ethiopia (15), 1.7% in Italy (16), 1.1% in Brazil (17), 11% in Iraq (18) and 1.7% in Iran (19) and 1/1% in Iran (11). However, the present study is in line with the studies of Rahimi et al (11) and Mohammadi (19) which show a low prevalence of *L. monocytogenes* in cow's milk and indicates the low prevalence of *L. monocytogenes* in cattle population of Iranian livestock.

According to studies, one of the important sources of milk contamination with *Listeria isolates*, especially *L. monocytogenes*, is the feeding with low quality silage and lack of sanitation during milking (20, 21). However, in the present study, the selected cows not only had a history of feeding on silage, but during milking from the cow's udder, the nipples were disinfected with 70% alcohol. Therefore, the amount of detected infections by *Listeria* and *L. monocytogenes* in this study can confirm the observance of sanitation and nutritional conditions in the target farms.

The present study, consistent with other studies conducted in Iran, Rahimi et al. (11) and Jamali et al. (22), shows that the prevalence of *listeria* in cow's milk is lower than sheep's and goat's milk by comparison of the prevalence of *listeria* in cow's milk with the prevalence in sheep and goat milk in these studies. Rahimi et al reported that the prevalence of *listeria* in cow, sheep and goat were 11.1%, 22.6% and 6.7%, respectively (11). In the study by Jamali et al, these rates of prevalence were determined to be 1.1%, 6.5% and 1.7%, respectively (22). It seems that this condition can be affected by the conditions of dense husbandry.

The present study is in agreement with the study of Iran (23) and other studies conducted in the United States (24), Brazil (25 - 27) Australia (28) and Egypt (29) which have reported 0% prevalence of *L. monocytogenes* in raw cow milk. Although good nutrition and sanitation are effective, studies have shown that high levels of natural microbiota in raw foods can reduce the survival of pathogens such as *L. monocytogenes* in food (raw milk). Because pathogens need specific conditions to grow. Therefore, the presence of high levels of natural microbiota in raw milk prevents the growth of pathogens (30 - 34). Numerous studies have shown that depending on the *listeria* strain and the amount of its presence in milk, this bacterium can survive in the long periods of pasteurization and even freezing at very low temperatures (35- 38). Thus, despite the low prevalence and concentration of pathogens, the presence of bacteria is an important risk factor for infection caused by eating contaminated food, especially when consuming unpasteurized dairy products (39).

Conclusion

Due to the importance of cow's milk in the preparation of raw dairy products such as local cheese, which is made from raw milk in most rural areas, the potential risk of *Listeria* infection in people, especially for pregnant women, the elderly and people with defective immune systems is worrying. Therefore, high-risk groups should avoid unpasteurized dairy products. Also, due to enzymes secreted such as lipase and

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proteinase from *Listeria* bacteria in sterilized milk, it is very important to observe hygienic standards, identify and treat infected animals.

Conflict of interest

The authors declare that no conflict of interest exists.

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