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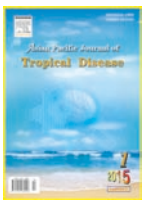

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Presence of *Listeria monocytogenes* in silage products of Shahrekord city

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ABSTRACT

Objective: To investigate the presence of *Listeria monocytogenes* in the silage samples.
Methods: Silage samples obtained from 150 different farms in Shahrekord city (Iran) and after DNA extraction, all samples were analyzed by PCR technique using one pair of primers for presence of this pathogen. The amplified products were detected on 1.5% agarose gel electrophoresis.
Results: *Listeria monocytogenes* was isolated in 4 (2%) of the 150 samples. The detection of this bacterium from silage samples in Shahrekord city indicated that these products could create a serious risk in public health of animal and human. The findings showed that in positive silage samples for *Listeria monocytogenes,* the pH value was about five and it was due to bacterial activity in these products.
Conclusions: The quality of silage and hygiene parameters and good herd health management play an important role in the microbiological quality of herd and farm. Considering the high specificity and sensitivity of the employed PCR technique, it is recommended to be useful technique for identification of *Listeria monocytogenes*.

1. Introduction

Listeria spp. are widely spread bacteria in nature. They could be found in silage, soil, decaying vegetation, sewage water, animal feces, and other environmental sources. They have ability to multiply in diverse habitats and are tolerant to extreme conditions such as high salt, low temperature and low pH conditions[1,2]. *Listeria monocytogenes* (*L. monocytogenes*) is one of the ubiquitous Gram-positive bacterium that exists as a saprophyte in soil[3,4]. They have same characteristics such as rod-shaped morphology, facultative anaerobic, non-spore-forming, with a low G+C content[2]. *L. monocytogenes* exists in the Corynebacteriaceae's family, order Eubacteriales[5-7]. It is one of the opportunistic pathogen that plays an important role in human food borne infections[2,6,7]. *L. monocytogenes* is facultative intracellular bacterium and can cause acute listerial gastroenteritis in human across populations[3,8]. It is capable of causing invasive disease in both livestock and human which may result in death[3,8]. The genus *Listeria* contains 6 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi*[1]. *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* may be pathogenic in human and *L. monocytogenes* can be found in unpasteurized milk and milk products[1]. It is capable of producing life threatening and severe infections, such as septic abortion, septicemia, and meningoencephalitis that is related to central nervous system[3,9]. *L. monocytogenes* pathogen is causing foodborne listeriosis. In comparison with other food borne microbial pathogens it is a relatively rare but serious disease with high fatality rates (20%–30%)[2,10].

One of the causative agents of human listeriosis and serious threats for the health of immunocompromised individuals is *L. monocytogenes*[11]. It is clear that cattle farms have an important role in the spread of *Listeria* between populations such as

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domestic and wild animals including farm ruminants (cattle, sheep, and goats) and human. Ruminant farm animals have a critical position in the persistence of *Listeria* spp. via a continuous faecal-oral cycle[12-15]. Most wild and domestic animals are *L. monocytogenes* asymptomatic carriers and have an essential role in the dissemination of this microorganism[16]. Listeriosis is an often fatal illness and severe with clinical manifestations such as meningitis or sepsis in immunocompromised patients or neonatal babies and abortion during pregnancy in women or flu-like illness[2,4,7]. The poor quality fermented feeding increases the risk of listeriosis. For example, when dairy cattle are fed with ensilage foods they will be contaminated by *L. monocytogenes*. Furthermore, *L. monocytogenes* may also contaminate milk from animals with mastitis and may contaminate raw and pasteurized milk, chocolate milk, butter, soft cheeses, and processed meat and poultry products[12,15]. The quality of silage, hygiene parameters and good herd health management play an important role in the microbiological quality of the milk[12,17]. Silage is formed in specific situations. Harvesting a forage crop and grass with a subsequently fermenting and high moisture content (greater than 50%) results in a silage product[1,16]. Eliminated air, achieved low pH and no changes in heat or composition of silage, prepare a good silage that remains stable[1,9]. Improperly fermented silage will result in *Listeria* spp.[1]. Macrophages have innate immune responses against *L. monocytogenes* and it is known that cytosolic invasion is catalyzed by the bacterial toxin listeriolysin[18]. Serotyping and phage typing are phenotypic methods to determine the existence of non-typable[12]. *L. monocytogenes* primarily causes uterine infections and encephalitis in ruminants. Uterine infections are characterized by septicemia or late-term abortions in neonates. The encephalitic form of animal listeriosis is characterized by neurological signs, including excessive salivation, circling and unilateral facial paralysis. Eye infections and keratitis in ruminants are caused by *L. monocytogenes*, and these symptoms have been related to direct inoculation of the eye with *L. monocytogenes* presented in feeds, especially silage[15]. Relation between listeriosis and silage consumption in ruminants was first recognized in 1922, the disease was known in Iceland as silage sickness or "votheysveiki". This relationship between listeriosis and the feeding of silage to dairy cattle, sheep, and goats has been well documented[19,20]. We can isolate enterococci-derived bacteriocins from many fermented foods. They have a strong biological activity against *Listeria* spp. by their bacteriocins[21]. Killing *L. monocytogenes* is due to the production of bacteriocin. Bacteriocins are products of bacteria ribosomally synthesized polypeptides with capability to inhibit or kill the growth of similar bacterial strain(s) like *L. monocytogenes*. Nisin is one of the most commercially used bacteriocins due to its relatively long history of safe usage[22]. Genotypic methods include ribotyping, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and random ampli fied polymorphic DNA (RAPD) techniques[12]. RAPD-PCR and PFGE are most often used to determine the type of *L. monocytogenes* strains[12]. *L. monocytogenes* is a unique microorganism because it can exist both as a pathogen in animals and human and as a saprophyte in natural environments among foodborne pathogens[23-25]. So, the aim of present study was to determine the prevalence of *L. monocytogenes* in silages samples collected from Shahrekord city in Southwest Iran.

2. Materials and methods

In fifteen different farms of Shahrekord city in Chaharmahal Va Bakhtiari province located in southwest of Iran, 150 silage samples were collected between October 2012 and May 2013. The samples were analyzed according to the microbiological protocol previously optimized for the detection of *L. monocytogenes*, according to the NF EN ISO 11290-1.2 standard (M.M. Guerra *et al*, dados não publicados). The samples were collected in sterile container and transported to the Biotechnology Research Center with ice containing boxes. Each sample (25 g/mL) was taken and placed in a stomacher bag to which 225 mL of sterile *Listeria* Selective Enrichment Broth (Oxoid) was added and homogenized with a stomacher (Masticator, IUL Instruments-Spain) and incubated at 30 ℃ for 48 h. A loopful of homogenate was surface streaked in duplicate on Palcam agar (Oxoid). The Palcam plates were incubated at 37 ℃ for 48 h under microaerophilic conditions. All colonies surrounded by a brownish green and/or black halo were taken as possible *Listeria* spp. One suspected *Listeria* spp. colony from each plate was purified on Tryptic Soy Agar (Oxoid CM 131) and was incubated at 30 ℃ for 24-48 h with 0.6% yeast extract (Oxoid L 21) for further biochemical characterization. Presumptive *Listeria* isolates were confirmed and identified at the species level based on Gram staining, typical umbrella motility in SIM medium (Oxoid CM 435), H2S production, urease, indole oxidase, reaction, catalase, nitrate reduction, methyl-red/voges-proskauer, β-hemolysis (Oxoid CM 43), CAMP tests and fermentation of mannitol, sorbitol, D-ksilose, L-rhamnose, dextrose, dulcitol, esculin, salicin, and maltose. In order to verify the suspected colonies as *L. monocytogenes* on agar plates, 4 suspected colonies were chosen from each colony and genomic DNA was extracted using DNA Extraction Kit (Qiagen, Germany), according to manufacturer's instructions, and assayed on 2% agarose gel electrophoresis and measured by NanoDrop ND-1000 (PeqLab) at a wavelength of 260 nm according to the method described by Sambrook and Russell (2001)[26]. The *Listeria* primers are specific for the *hlyA* gene of *L. monocytogenes*. The primer sequences were: Lis-F: 5′-TGTTAATGAACCTACAAGACCTTC-3′ and Lis-R: 5′-TAGTTCTACATCACCTGAGACAGA-3′ that amplified 224 bp fragment in PCR[24]. The amplification was done using a thermal cycler (Palm Cycler Gradiant, Australia), and PCR was carried out in 25 μL total reaction volumes, each containing 2.5 μL of 10× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 μL dNTPs, 1 unit of *Taq* DNA polymerase (all Fermentas, Germany), 100 ng of template DNA, and 0.2 pM of each primer. *L. monocytogenes* ATCC number 7644 strain and sample without DNA was used as positive and

negative controls, respectively. The amplification reaction consisted of 5 min of pre-denaturing at 95 ℃, followed by 30 cycles of 1 min denaturation at 94 ℃, 1 min annealing at 62 ℃ and 1 min extension at 72 ℃, and final extension at 72 ℃ for 5 min. The PCR amplification products (10 μL) was separated by electrophoresis on 1.5% agarose gel at 100 V for 30 min in Tris-borate-EDTA (TBE) buffer, and were visualized by ethidium bromide staining, that illuminated by UV transilluminator and images were obtained in UVIdoc gel documentation systems (Uvitec, UK). A 100 bp DNA ladder (Fermentas, Germany) was used as a size reference for PCR assay.

3. Results

L. monocytogenes was detected in 2% (4 specimens) of the 150 silage samples. However, in present study, silage samples contaminated with *L. monocytogenes* were obtained only from damped silage. In the clarification of this result, we concluded that rainy and humid weather conditions were the cause of *L. monocytogenes* proliferation in silages. In our study, *L. monocytogenes* were not found from cow's milk samples not fed silage. In current study, the pH value of the silage samples that contaminated with *L. monocytogenes* was greater than 5.3 in positive samples that determined by PCR method. Figure 1 shows amplified fragments of *hlyA* gene of *L. monocytogenes* that isolated from silage products.

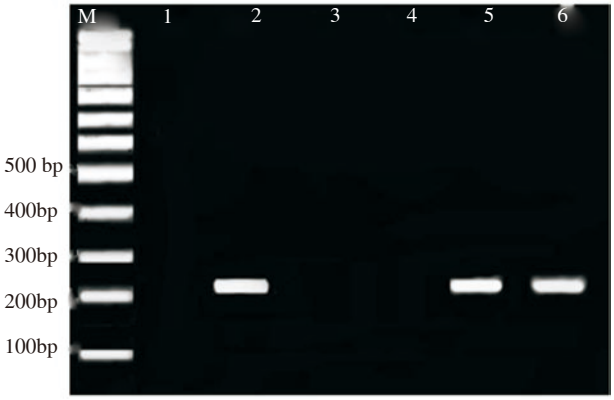

Figure 1. PCR products of *hlyA* gene (224 bp) on 1.5% agaros gel electrophoresis stained with ethidium bromide.
Lane M: 100 bp ladder; lane 1: negative control (no DNA); lane 2: positive control; lanes 3 and 4: negative samples; lanes 5 and 6: amplified samples.

4. Discussion

The aim of this work was to investigate the presence and prevalence of *L. monocytogenes*in in silage products of Shahrekord city and vicinity in Iran by tracing the *hlyA* gene of this bacterium using molecular technique. This pathogen was detected in 2% of collected silage samples. The results showed low presence of *L. monocytogenes* in silage products in this city and outskirt of Iran. Moreover, in infected silage samples with *L. monocytogenes* the pH rate was about 5 and it was due to bacterial activity in silage products.

Certainly, the use of quantitative microbiology tools such as the growth/no growth kinetics and interface models in combination with a systematic application procedure can make an effective modeling approach to evaluating the compliance of silage products. *L. monocytogenes* is one of the contaminant in various kinds of foodstuffs, including raw vegetables, raw milk, fish, poultry and both processed and fresh meat and silage products. In contaminated stuff, the organism's colony counts may exceed 109 colony– forming unit (CFU)/g[24]. In studies that performed for determination the presence of this bacterium, Vilar *et al.* detected *Listeria* spp. in 33.7% of silage samples. Donnelly observed that 8 of 44 Holstein cows fed by *Listeria*-contaminated silage shed the organism in their milk. Furthermore, milk from these animals was free of *L. monocytogenes* one month after feeding of contaminated silage ceased[1,12]. Some report indicates that rates of contamination increase during the winter months, while others showed increasing rates during summer. Two factors may explain the increased isolation rate during winter in our study. The winter season is usually very rainy in Shahrekord city in Southwest Iran, and water is moisturized silage. Then, the quality of silage is changed. Dairy cattle usually give birth to their young in early spring or late winter. During winter gestation, as a direct result of pregnancy the dairy cattle develop a weakened immune system, which, in turn, makes these animals more susceptible to abortions and listerial infections. The pH value in infected silage samples by *L. monocytogenes* was from 5.28 to 7.3. Various studies have verified that contamination of *L. monocytogenes* is most frequently associated with poor-quality silage. Poorly fermented silage, which has a pH greater than 5.5, is ideal for *Listeria* growth. We believe that the contamination sources of *L. monocytogenes* are the using up of bad-quality silage, subjected to insufficient fermentation, with pH values higher than 4.8, which allows the multiplication of *L. monocytogenes*. Isolation of *L. monocytogenes* from silage samples in Shahrekord city shows that these yields could have a potential risk for animals. Silage production sustenance is necessary for preventing introduction of *Listeria* into the herd, its spread within the herd, and its entry into milk. When animals were fed by low-quality silage, it may increase the risk of contamination of milk by *Listeria* spp.[1]. *L. monocytogenes* ingested through silage are reintroduced and amplified into the environment of the farm by cattle. Some researchers found evidence that the prevalence of *L. monocytogenes* in soil was higher than that seen in feed, and it means that soil may function as a source of animal feed contamination by *L. monocytogenes*[15]. The ecology and epidemiology characteristics of *L. monocytogenes* change between small-ruminant and bovine farms. *L. monocytogenes* population's diversity on bovine farms was more than that observed in small-ruminant farms. Ecosystems of ruminant farm keep a high prevalence of *L. monocytogenes*, including subtypes linked to human listeriosis cases and outbreaks[15]. In conclusion, the results of the present study suggest that the poor quality fermented feeds increase the *L. monocytogenes* propagation that

increases the risk of listeriosis in the farm animals. Furthermore, pH, humidity, temperature, seasonal variations, air condition and cattle's farm are the most important factors of silage contamination with *L. monocytogenes*. In addition, PCR technique is one of the most sensitive methods for investigating the presence of *L. monocytogenes* in silages products.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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