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Short Paper

The presence of *Listeria monocytogenes* in raw milk samples in Mashhad, Iran

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Summary

The purpose of this preliminary study was to determine the prevalence of raw milk contamination with *Listeria monocytogenes*. In this study, 100 bulk tank milk samples were collected randomly and delivered to Pegah Pasteurization Factory in Mashhad. For isolation and identification of *L. monocytogenes*, the samples were first enriched using cold enrichment method in *Listeria* enrichment broth, followed by plating onto supplemented Oxford agar. For final identification of suspected colonies a multiplex-PCR assay, using two pair of primers was employed. The *prs* primers are specific for putative phophoribosyl pyrophosphate synthetase (*prs*) gene of *Listeria* spp. and the *LM lip1* primers are specific for *prf* A gene of its *monocytogenes* serovar. Using this method, the contamination of raw milk with *L. monocytogenes* was determined to be 4% and the sensitivity of the primers was 3.5×10^3 cfu ml^{-1}, and the specificity was determined to be 100%. Considering the high specificity and sensitivity of the employed multiplex-PCR assay, it is recommended to use this method for the identification of suspected colonies of *Listeria* spp. and *L. monocytogenes*.

Key words: *Listeria* spp., *Listeria monocytogenes*, Bulk tank milk, Multiplex PCR

Introduction

There are six species currently recognized in the genus of *Listeria*, including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (Gasanov *et al*., 2005). *Listeria* spp. can be found in dairy products, meat and poultry, as well as in vegetables (Amagliani *et al*., 2004). Among the genus of *Listeria*, which cause the infection of listeriosis in both animals and man, *Listeria monocytogenes* is a major pathogenic microorganism (Aygun and Pehlivanlar, 2006). Listeriosis caused by *Listeria monocytogenes* has increased drastically in recent years (Choi and Hong, 2003). *Listeria monocytogenes* is associated with septicemia, meningoencephalitis and abortion in humans and animals, primarily affecting pregnant, new-born, and immunocompromised individuals (Choi and Hong, 2003; Rossmanith *et al*., 2006). Several outbreaks of listeriosis were proven to be associated with the consumption of milk and are causing great concern in the dairy industry due to the number of cases and the nearly 30% overall mortality rate of these outbreaks (Amagliani *et al*., 2004). The recovery of *L. monocytogenes* from foods and environmental samples in conventional methods requires the use of enrichment cultures followed by selective plating. Currently, PALCAM, Oxford and LPM are the most frequently used plating media (Reissbrodt, 2004).

Significant developments have occurred not only in selective culture enrichment procedures, but also in the availability of many new and rapid detection methods based on antibody and molecular technologies (Reissbrodt, 2004; Gasanov *et*

al., 2005). Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of foodborne pathogens (Olsen *et al*., 1995).

The aim of this preliminary study was to determine the prevalence of *L. monocytogenese* contamination in raw milk samples which were delivered to a pasteurization factory in Mashhad using a combination of conventional culture method and multiplex-PCR assay.

Materials and Methods

Sample collection

During June and July of 2008, a total of 100 bulk tank milk samples were obtained randomly from the Pegah Milk Pasteurization Plant in Mashhad. The samples were transported to the laboratory in sterile plastic falcon tubes (50 ml) under refrigerated conditions.

Bacteriological methods

Raw milk samples were centrifuged at 6000 × g for 15 min at 4°C to pellet the bacterial cells, then the supernatants was discarded and the pellet was re-suspend in 10 ml of *listeria* enrichment broth (LEB), in a sterile screw-cap tube, followed by incubation at 4°C for 10 days. 0.1 ml of the enriched culture was surface plated on Oxford agar, supplemented with Natamycin 25 mg/L, Colistin sulphate 20 mg/L, Acriflavine 5 mg/L, Cefotetan 2 mg/L and Fosfomycin 10 mg/L (*Listeria* Selectavial-SV33 Series-MAST International), followed by incubation at 30°C for 48 h. Typical suspected colonies were considered for DNA extraction.

Multiplex PCR assay

In order to confirm the suspected colonies as *L. monocytogenes* on agar plates, five suspected colonies from each plate were separately suspended in 1 ml of 0.01 M Tris-HCl and subjected to phenol/chloroform DNA extraction method (Simon *et al*., 1996).

The DNA extracted from suspected *Listeria* colonies on selective media were employed as templates for m-PCR assay. *L. monocytogenese* (ATCC-7644) were used as a positive controls and sterile distilled water as negative control.

For the multiplex PCR assay, the sequences of the two pairs of primers are shown in Table 1. The *prs* primers are specific for the putative phophoribosyl pyrophosphate synthetase gene of *Listeria* spp. and the *LM lip1* primers are specific for the *prf* A gene of *monocytogenes* serovar.

The reaction mixture consisted of 2 µl of extracted DNA, 2.5 µl of 10 × PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 m M), 1.25 µl of each primer, 0.4 µl of Taq DNA polymerase (5 U/µl) and deionized water to a final volume of 25 µl.

The reaction mixture was amplified in a thermocycler (Bio-Rad iCycler) with the following PCR conditions: denaturation at 94°C for 5 min, 33 cycles with denaturation at 94°C for 45 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR product was separated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in Tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. A 100 bp DNA ladder (Fermentas) was used as a size reference for PCR assay.

Primer sensitivity and specificity test

To assess the sensitivity of the primers, the overnight culture of *L. monocytogenese* (ATCC-7644) in BHI broth was prepared and enumerated by surface plating from serial dilutions of inoculated media on Oxford agar plates. Serial dilutions from the

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

Target gene	Sequence (5′→3′)	PCR product (bp)
Putative phophoribosyl pyrophosphate synthetase	(F) GCT GAA GAG ATT GCG AAA GAA G (R) CAA AGA AAC CTT GGA TTT GCG G	370
prf A	(F) GAT ACA GAA ACA TCG GTT GGC (R) GTG TAA TCT TGA TGC CAT CAG	274

cultured BHI broth were prepared and 200 µl from each dilution were considered for DNA extraction and consequently m-PCR assay.

To determine the specificity of the primers, extracted DNA of different bacterial colonies including *Staphylococcus aureus* (ATCC-25923), *Salmonella typhymorium* (ATCC-14028), *Campylobacter jejuni* (ATCC-33291), *Bacillus cereus* (ATCC-10876), *Clostridium perfringenes* (ATCC-13124), and *Escherichia coli* O157:H7 (ATCC-35150) and *L. monocytogenes* (ATCC-7644) were considered as the template for the m-PCR assay.

Results

In this study from 100 bulk tank milk samples, 37 of the (37%) 100 samples showed suspected colonies on selective agar plates. Multiplex PCR assay was employed after DNA extraction from presumptive *Listeria* colonies.

The multiplex-PCR assay, using *prs* primers that amplify a 370 bp fragment of the putative phosphoribosyl pyrophosphate synthetase gene of *Listeria* spp. and *LM lip1* primers that amplify a 274 bp fragment of the *prf* A gene of *L. monocytogenes* was performed. Using this method, the contamination rates of the raw milk samples with *L. monocytogenes* were determined as 4% (Fig. 1).

In a sensitivity test the results showed that these primers can detect at least 3.5×10^3 cfu ml^{-1} of bacterial cells, and in specificity test the results clearly indicated that the primers have a high affinity for the correct target sequence and are specific only for *Listeria* spp.

Discussion

In this study, a low prevalence (4%) of *L. monocytogenes* was found in the raw milk samples. Similar frequency findings of *L. monocytogenes* (0-5%) in bulk tank milk samples have been reported from different countries such as Austria 1.5% (Deutz *et al*., 1999), Spain 3.6% (Gaya *et al*., 1998), India 1.7% (Adesiyun *et al*., 1996), USA 4.1%

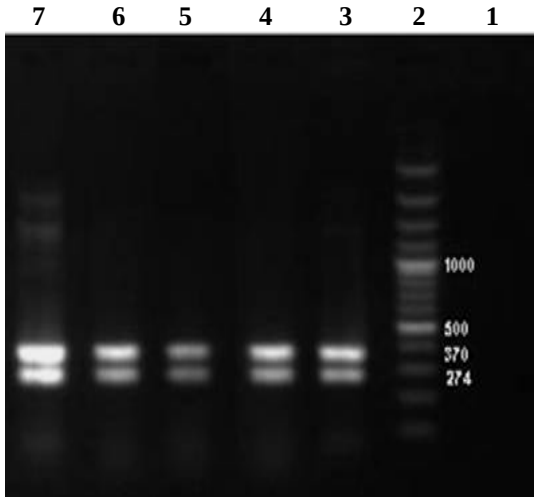

Fig. 1: Detection of *listeria monocytogenes* in raw milk samples by multiplex PCR assay, amplifying 370 bp segment of putative phophoribosyl pyrophosphate synthetase gene, specific for *Listeria* spp. and 274 bp segment of *prf* A gene, specific for *L. monocytogenes*. Lane 7: positive control (*L. monocytogenes* ATCC: 7644), Lane 1: negative control, Lane 2: 100 bp markers, Lanes 3, 4, 5, 6: positive samples for *L. monocytogenes*

(Rohrbach *et al*., 1992), Canada 1.9% (Fedio and Jackson, 1990) and Iran 1.6% (Moshtaghi and Mohammadpour, 2007). The disparate levels of contamination which have been reported from localized studies might have been due to variations in regions or to variations in sampling and detection techniques. Therefore, to determine the accurate prevalence of *Listeria monocytogenes*, further investigations should be carried out in dairy farms using a large number of samples. Because of the presence of calcium ions as a PCR inhibitor in raw milk (Bickley *et al*., 1996), it seems that direct PCR is not a recommended procedure. In this study the enrichment step was used, as injured organisms are likely to be present and because of the limitation in the sensitivity of the primers which was determined to be about 3.5×10^3 cfu ml^{-1}. In order to eliminate the PCR inhibitors which may interfere with the PCR assay, after the enrichment step we used selective plating to isolate presumptive colonies. In this study we used the cold enrichment method, and *Listeria* selective enrichment broth was used as the cold storage medium, but this method

is time-consuming. It has been reported that the sensitivity of cold enrichment at 4°C in *Listeria* selective enrichment broth for *L. monocytogenes* isolation was 94% (Erdogan *et al*., 2002), and the preference of the cold enrichment method or FDA procedure is debatable (Pini and Gilbert, 1988).

Although the specificity of the primers was determined to be 100%, it should be noticed that the test was performed by the available strains and it would be better if the test had been performed with a larger number of different bacterial strains. Compared with other reports with sensitivity ranging from 10^4-10^5 (Herman *et al*., 1995) to 4×10^1 cfu ml^{-1} (Kirkan *et al*., 2006) in the detection of *Listeria* spp. and *L. monocytogenes*, it seems that the sensitivity of the applied test in this study (3.5×10^3 cfu ml^{-1}) is acceptable. The combined conventional culture and PCR method allows accurate detection of *L. monocytogenes* in various food samples and could serve as a rapid screening method. The results of this study also suggest that there is a potential hazard for the population of this area of the country. Considering the risk factors associated with contamination of raw milk by *L. monocytogenes*, which includes, inadequate frequency of cleaning the exercise area, poor cow cleanliness, insufficient lighting of milking barns and parlors, and incorrect disinfection of towels between milkings (Sanaa *et al*., 1993), new standard detection techniques, and, in addition, new safety programs should be used and developed in order to isolate, detect and control *L. monocytogenes* in milk.

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