World Applied Sciences Journal 10 (2): 249-253, 2010
ISSN 1818-4952

The Presence of *Listeria* spp In Raw Milk Samples in Mashhad, Iran

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Abstract: The purpose of this preliminary study was to determine the prevalence of raw milk contamination with *Listeria* genus. In this study number of 100 bulk tank milk samples which were delivered to Pegah pasteurization factory in Mashhad collected randomly. For isolation and identification of *Listeria* spp. samples were firstly enriched using cold enrichment method in *Listeria* enrichment broth, followed by plating onto supplemented Oxford agar. For final identification of suspected colonies a PCR assay, was employed. The *prs* primers are specific for Putative phophoribosyl pyrophosphate synthetase (*prs*) gene of *Listeria* spp. Using this method, the contamination of raw milk with *Listeria* spp. determined as 34%. The sensitivity of the primers was 3.5×10^3 cfu ml^{-1} and the specificity determined as 100%. Considering the high specificity and sensitivity of the employed PCR assay, it is recommendable to use this method for identification of suspected colonies of *Listeria* spp.

Key words: *Listeria* spp · Bulk tank milk · 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* [1]. *Listeria* spp. can be found in dairy products, meat and poultry, as well as in vegetables [2]. Among the genus of *Listeria*, which cause the infection of listeriosis in both animals and man, *Listeria monocytogenes* is a major pathogenic microorganism [3] and is associated with septicemia, meningoencephalitis and abortion in humans and animals, primarily affecting pregnant, new-born and immunocompromised individuals [4-5]. 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 [2]. The recovery of *Listeria* spp. 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 [6]. Suspected colonies are usually classified as *Listeria* if the bacteria display the characteristics such as gram-positive rods, aerobic and facultative anaerobic, non-spore forming, catalase-positive ,oxidase-negative, fermentative in sugars and producing acid without gas [1]. Biochemical standard methods are laborious and time-consuming, requiring a minimum of five days to recognize *Listeria* spp. [2].

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 [1-6]. Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of food-borne pathogens [7].

PCR-based detection of *Listeria* spp. is more sensitive than culture-based methods for detecting the pathogen in contaminated food samples [8-9]. Among the target genes for PCR detection of *Listeria* spp are the *iap* gene [10], *16SrRNA* gene [11] and *prs* gene [12].

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

MATERIAL AND METHODS

Sample Collection: During the June and July of 2008, a total of 100 bulk tank milk samples, obtained randomly from 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-suspended in 10 ml of *Listeria* enrichment broth (LEB), in a sterile screw-cap tube, followed by incubation at 4 °C for 10 days. Amount of 0.1 ml of the enriched culture was surface plated on Oxford agar, supplemented with Natamycin 25 mg/litre, Colistin sulphate 20 mg/litre, Acriflavine 5mg/litre, Cefotetan 2mg/litre and Fosfomycin 10 mg/litre (Listeria Selectavial-SV33 Series- MAST International), followed by incubation at 30°C for 48 hours. Typical suspected colonies were considered for DNA extraction.

PCR Assay: In order to confirm the suspected colonies as *listeria* spp. 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 [13].

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

For the PCR assay, the sequences of the primers are shown in Table 1. The *prs* primers are specific for Putative phophoribosyl pyrophosphate synthetase gene of *Listeria* spp.

The reaction mixture consisted of 2µl of extracted DNA, 2.5µl of 10× PCR buffer , 1.5µl $MgCl_2$ (50 mM), 0.5µl dNTP (10m M), 1.25µl of each primer, 0.4µl 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 100V 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 cultured BHI broth were prepared and 200 µ l from each dilution were considered for DNA extraction and consequently the 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 template for the PCR assay.

RESULTS

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

Table 1: PCR primers used for *Listeria spp* detection

Target gene	Sequence (5→3')	PCR product (bp)
Putative phophoribosyl	(F) GCT GAA GAG ATT GCG AAA GAA G	370
pyrophosphate synthetase	(R) CAA AGA AAC CTT GGA TTT GCG G	

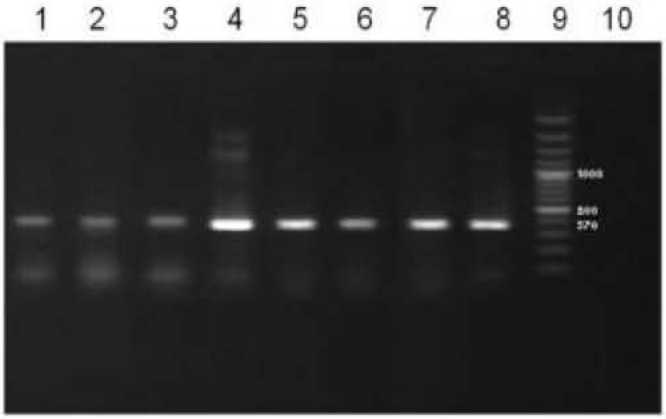

Fig. 1: Detection of *Listeria* spp in raw milk samples by PCR assay, amplifying 370 bp segment of Putative phophoribosyl pyrophosphate synthetase gene, specific for *Listeria spp*. Lane (8) positive control (*L. monocytogenes* ATCC: 7644), Lane(10) negative control, Lane(9) 100bp markers, Lanes 1-7 positive samples for *Listeria* spp.

The PCR assay, using prs primers that amplifies a 370 bp fragment of the Putative phosphoribosyl pyrophosphate synthetase gene of *Listeria* spp. was performed. Using this method, the contamination rate of raw milk samples with *Listeria* spp were determined as 34 (34 %) (Fig. 1).

In 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

Many food borne illness outbreaks are due to the consumption of raw milk and raw milk products [14-15]. Even though pasteurization is an effective control method for bacterial pathogens, it is important to maintain high preprocessing standards. Additionally, raw milk contaminated with zoonotic pathogens might provide a reservoir for recontamination at milk processing plants. In this study, the presence of *Listeria* spp were investigated in total of 100 samples from bulk tanks milk which were collected from different dairy herds, located in Mashhad suburb and were delivering to a pasteurization factory. In this study, the prevalence of *Listeria* spp. in raw milk samples was found to be 34%. The prevalence of *Listeria* spp. in raw milk samples found in this study (34%) is comparable with the results reported from Mexico City in Mexico (23%) [16] and Ankara in Turkey (10%) [17], but it has been reported from 1.3 to 2.2 in other areas in Iran [18-19]. 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* spp. further investigations should be carried out in dairy farms with large number of samples. Since the recognition of *Listeria* spp. specially *Listera monocytogenes* as a food-borne pathogen has an important health concern, there have been rapid advances in the development of suitable methods for isolation and identification of this bacteria. 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 molecular technologies [1]. An acceptable specimen should be free of inhibitory substances that could produce a false-negative result. Some clinical samples may contain substances which are not always removed by the extraction process and which may inhibit the PCR amplification. Because of the presence of calcium ions as a PCR inhibitor in raw milk [20], it seems that direct PCR is not a recommendable procedure. In this study we used enrichment step because injured organisms are likely to be present and because of limitation in sensitivity of primers which determined about 3.5×10^3 cfu ml^{-1}. In order to eliminate the PCR inhibitors which may interfere with PCR assay, after enrichment step we used selective plating to isolate presumptive colonies. In this study we used cold enrichment method and Listeria selective enrichment broth was used as cold storage medium. It has been reported that the sensitivity of cold enrichment at 4°C in Listeria selective enrichment broth for isolation of this bacteria was 94% [21]. And preference of cold

enrichment method or rapid methods like as FDA procedure is discussable [22].

For selective plating we used oxford agar medium which is recommended by the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA) and in the ISO detection and enumeration methods [6-23-24]. Growing of *Listeria* spp. on this media is detected by the activity of the h-D-glucosidase enzyme (esculinase), which occurs in both pathogenic and non-pathogenic species. This enzyme cleaves esculin, resulting in grayish-green colonies and by reaction of the breakdown product esculetin with ferric iron, gives brown-black halos with colonies of all *Listeria* spp [6]. Although the specificity of primers determined 100%, but it should be noticed that the test has performed by the available strains and it would be better, if the test had been performed with a large number of different bacterial strains. Comparing with other reports with the sensitivity ranging from 10^4-10^5 [25] to 4×10 cfu ml^{-} [26] in detection of *Listeria* spp. 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 *Listeria* spp. 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 this country. Considering the risk factors associated with contamination of raw milk by *Listeria* spp. 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 [27], new standard detection techniques, in addition with new safety programs should be used and developed in order to isolate, detect and control *L. monocytogenes* in milk.

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