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Full Length Research Paper

Polymerase chain reaction (PCR4)-based detection of *hly* and *plc-A* genes in *Listeria monocytogenes* isolated from dairy and meat products in Iran

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Listeria monocytogenes **is a ubiquitous Gram positive organism causative agent of many hazardous conditions like meningitis and sepsis. To isolate *L. monocytogenes* from various food samples collected from different markets in Iran and evaluate pathogenic potential of the isolates by determining two virulence associated genes (*hly* and *plc-A*) and sequencing them, a total of 130 samples including dairy and meat products were collected. Cold enrichment method was used for the isolation of *Listeria monocytogenes*. The isolated pathogens were identified and confirmed by biochemical and polymerase chain reaction (PCR). PCR method was done to amplify two gene fragments, a 1590 and 954 bp for *hly* and *plc-A* genes, respectively by using sequencing primers. *Listeria monocytogenes* was isolated from 3.1% of all samples. The bacterium was isolated 2.5% in dairy and 4% in meat samples. All isolates were hemolytic and positive for *hly* gene. Also 100% of isolates had *plc-A* gene. The results of this study indicates that consumption of ready to eat food is hazardous specially for high risk persons .Besides that, pathogenic potential of isolated strains is important because of existences of virulence gene in all of the isolates.**

Key words: *Listeria monocytogenes*, *hly* and *plc-A* genes, polymerase chain reaction (PCR), dairy, meat.

INTRODUCTION

Listeria monocytogenes is an opportunistic intracellular pathogen that has become an important cause of human food borne infections worldwide (Liu, 2006). The bacteria can grow under a wide range of temperature and external stress such as extreme pH and salt concentration, and these are thus, ubiquitous (Indrawattana and Nibaddhasobon, 2011). In recent years different epidemiological and experimental investigations revealed that contaminated food is an important route for transmission of bacteria to humans. Also it has been shown that the main source of infections is ready-to-eat (RTE) foods with long shelf - life making *L. monocytogenes* one of the major causative agents of food-related deaths (O'Connor et al., 2010). *Listeria* species are able to survive many food manufacturing processes and with a recent increased consumption of RTE and heat to eat products, *L. monocytogenes* has emerged as a significant food borne pathogen, causing serious illness in infants, pregnant women, elderly and immunocompromised individuals (Liu et al., 2007; Lotfollahi et al., 2011). Characterization of *L. monocytogenes* relies on genotype, ideally should be based on detection of bacterial virulence genes or gene products. A marker is Listeriolysin O (LLO) required for intracellular survival of invading bacteria in mammalian host. The gene encoding LLO is *hly-A. L. monocytogenes* produces other virulence proteins beside

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Table 1. PCR primers used for *Listeria monocytogenes* detection.

Target gene	Primer sequence	Size of PCR Product (bp)	Reference
Hly	(F) 5′- ATGAAAAAAATAATGCTAG-3 (R) 5′-TTATTCGATTGGATTATCT-3′	1590	This study
Plc-A	(F) 5′-TTAGTTGAATTTATTGTTTTTTATG-3 (R) 5′-TTGTATAAGAATTATTTGC-3′	954	This study

LLO like PI-PLC and PC-PLC (Ward et al., 2010). In Iran, there is scanty information about characteristics and virulence factors of *L. monocytogenes* which could be isolated from different food stuffs. In the present study *L. monocytogenes* was isolated from various food samples collected from different markets in Tehran, Iran. Also the aim of this study was detection of two important virulence genes of *L. monocytogenes (hly and plc-A)* to evaluate pathogenic potential of the isolates.

MATERIALS AND METHODS

Sampling and isolation

A total of 130 samples were collected during December 2009 and May 2011 from different dairy and meat product shops located in Tehran, Iran. The food samples comprised meat products like sausage, calf and chicken meat concentrates and dairy products like cheese, cream and kashk (an Iranian dairy product). Twenty-five gr of each samples were aseptically added to 225 ml of Triptic Soy Broth with Yeast Extract (TSBYE) and homogenized in a stomacher. The homogenized samples were incubated at 4°C. After 1, 2 weeks and a month of incubation at 4°C, aliquots for TSBYE were streaked onto PALCAM Agar (Merck, Germany) and *Listeria* Selective Agar (Himedia, India). Five presumptive *Listeria* colonies from PALCAM and *Listeria* selective agar were purified on BHI Agar and identified using morphological, cultural and biochemical criteria. Before purification on BHI, the greenish - yellow colonies surrounded by a diffuse black zone because of esculin hydrolysis on PALCAM agar and bright yellow colonies on *Listeria* selective agar were considered to be *Listeria* spp. Biochemical tests include (catalase, oxidase, methyl-red, vogesproskauer's and urease test) and sugar fermentation (rhamnose, xylose, mannitol and α- methyl-D- mannopyranoside). The isolates were further confirmed by hemolysis on blood agar and CAMP test.

Polymerase chain reaction (PCR)

Genomic DNA was extracted from the bacterial cells grown at 37°C overnight in Brain Heart Infusion broth (BHI) using a DNA extraction Kit (Roche Co, New York, USA).The DNA extracted from *L. monocytogenes* was applied as templates for PCR assay. *L. monocytogenes*(ATCC7644) was used as a positive control and sterile distilled water as negative control. For PCR assay, sequence of primers is shown in Table 1.

The reaction mixture consisted of 2µl template DNA (0.4 µg), 3 µl of 1xPCR buffer, 1.5 mM magnesium chloride, 0.15 mMdNTP, 20 pmol of each forward and reverse primer, 1.25 U Taq DNA polymerase and deionized sterile water to a final volume of 30 µl. The reaction mixture was amplified in a thermocycler (Corbeit, Sydney, Australia) with the following PCR conditions: Denaturation at 94°C for 5 min, 30 cycles with denaturation at 94°C for 30 s, annealing at 50°C for *hly* gene and 45°C for *plc-A* gene for 30 s, extension at 72°C 45 s and final extension at 72°C for 5 min. The PCR products were separated by electrophoresis in 1.5% agarose gel for 40 min in Tris-acetate buffer, visualized by cyber green staining, illuminated by UV- transilluminator and documented by a gel documentation apparatus. A 100 bp DNA ladder (fermentase) was used as a size reference for PCR assay.

Gene sequencing

The PCR products for both genes were confirmed further by sequencing. The nucleotide sequence accession number for *hly* and *plc-A* genes was GU395204 and GU395203, respectively.

RESULTS

L. monocytogenes was isolated from four tested samples. The pathogen was isolated from cheese with highest frequency (2/85%). However no *L. monocytogenes* was isolated from cream, kashk and sausage (Tables 2 and 3). The isolated *L. monocytogenes* were hemolytic and CAMP positive with *S. aureus*. The identified *L. monocytogenes* isolates included both *hly* (Figure 1) and *plc-A* genes (Figure 2). Positive control yielded PCR products of respective base pairs. Negative controls produced no detectable product.

DISCUSSION

Food-borne diseases present a growing health problem worldwide and over 200 different diseases are known to be transmitted by food (Adzitey et al., 2013). Among these food-borne pathogens, *L. monocytogenes* has special importance because of high death rates and ability to growth in refrigerator temperature. In Iran, there is no completely organized information about prevalence, characteristics and virulence factors of *L. monocytogenes* which could be isolated from various foods. Moreover, listeriosis is not a reportable disease in Iranian health system and there are no criteria for listeriosis in food industry in the country. In Iran a variety of locally produced foods and traditional foods are consumed and knowledge of industrial stuff about prevalence importance of *Listeria* in food is essential (Jalali and Abedi., 2008). *L. monocytogenes* has been reported from several countries like Spain, New zeland, Brazil. The incidence rate of *L. monocytogenes* in all food samples in this study

Table 2. Incidence of *L. monocytogenes* in dairy products.

Sample (Number of samples)	Number of positive isolates	Percentage of positive isolates
Cheese (70)	2	2.85
Cream (5)	0	0
Kashk (5)	0	0
Total (80)	2	2.5

Table 3. Incidence of *L. monocytogenes* in meat products.

Sample (Number of samples)	Number of positive isolates	Percentage of positive isolates
Sausage (30)	0	0
Chicken concentrate (10)	1	10
Calf meat concentrate (10)	1	10
Total (50)	2	4

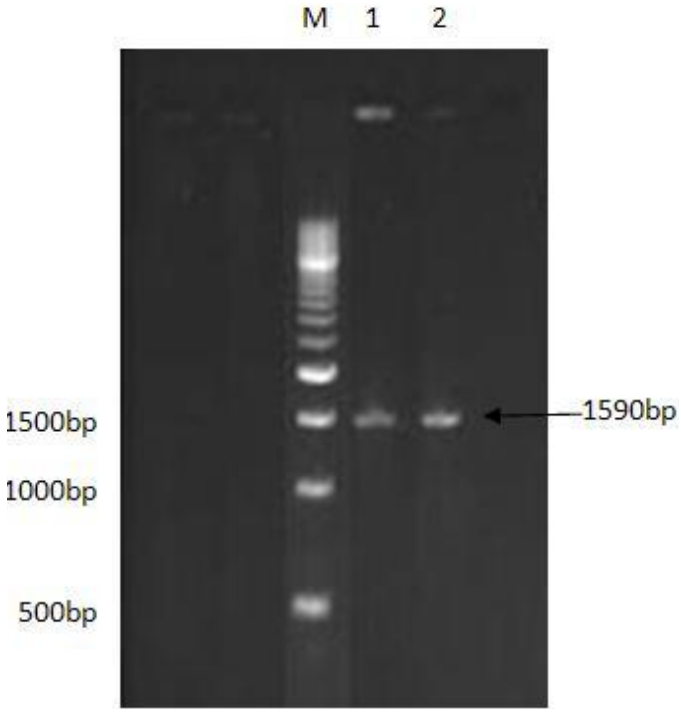

Figure 1. PCR profiles of *Listeria monocytogenes* isolate from food. Lane- M- PCR Marker, Lane 1 and 2 *hly*gene (1590bp) from various food samples.

(3.1%) is in agreement with the earlier reports. In Gaborone (Botswana), Morobe (2009), isolated *L. monocytogenes* in 4.3% of various food products. Souza et al. (2008) isolated the pathogen in 4.3% of food samples in refrigerator of pregnant women under investigation for contamination with *L. monocytogenes*. *L. monocytogenes* were isolated from 4% of total meat products. The results of our study show much lower incidence in comparison with some earlier studies by Hudson et al. (1992), Simon et al. (1992) and Ismaiel et al. (2013) where *L. monocytogenes* was isolated 12.5, 17.3 and 13.3% (frozen beaf) respectively in New Zealand and Spain and Egypt (Ismaiel et al.,, 2013). The lower incidence in our study may be because of low number of meat samples tested. The results of present study are in agreement with the report of Wang et al. (2012) where the pathogen was detected in 4.3% of meat samples and Ismaiel et al. (2013), that reported the pathogen incidence in frozen chicken samples as 3.33%. In dairy products our isolation results were in agreement with Morobe (2009) who isolated *L. monocytogenes* in 2.75% of cheese samples tested. Jalali and Abedi (2008) and Ismaiel et al. (2013) did not isolate any *L. monocytogenes* from tested cheese, kashk and Zabady cheese samples, respectively. Kargar and Ghasemi isolated *L. monocytogenes* from 13.1% of cheese samples investigated and that was much higher than our reported results (Kargar and Ghasemi., 2009). The reason for difference between these two studies in iran may be different geographical area (Tehran is in the center and Jahrom is in south of Iran). In 2006, Burton et al., investigated the frequency of *hly* gene in *L. monocytogenes* isolated from foods, environmental and clinical samples by PCR (Burton and Blais, 2006). At present study, frequency of *hly* gene was 100% that is in agreement with study by Shakuntala et al. (2006). At the mentioned study, all *L. monocytogenes* isolates were hemolytic and positive for *hly* gene. Kargar and Ghasemi (2009) reported *hly* gene in 91.7% of cheese samples investigated. All of *L. monocytogenes* isolates in our study were positive for *plc-A* gene that is in agreement with result of Shakuntala et al. (2006) study; these authors detected *plc-A* gene in all of investigated isolates. These results were higher than Karegar and Ghasemi

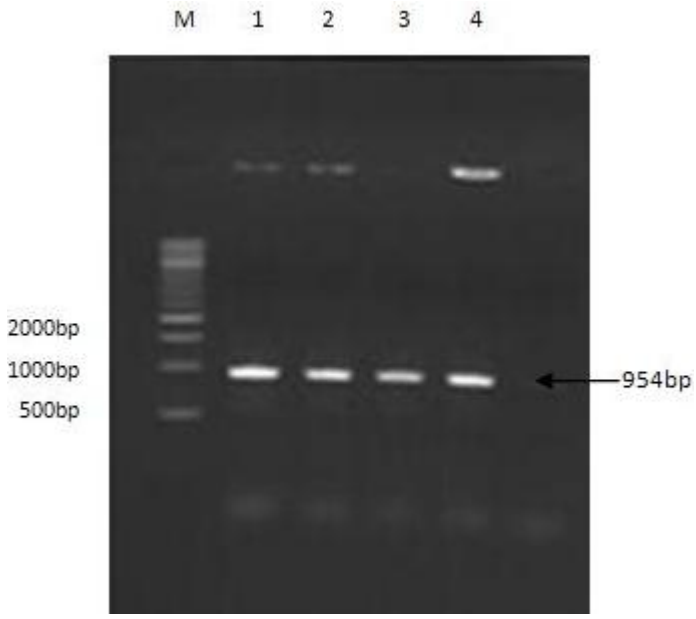

Figure 2. PCR profiles of *Listeria monocytogenes* isolated from food. Lane- M-PCR Marker, Lane 1, 2, 3 and 4- *plcA*gene (954bp) from various food samples.

(2009) results that detected this gene in 90% of strains analyzed. In conclusion, the results of this study indicate the potential risk of "ready to eat" food consumption, eating undercooked or raw and unpasteurized foods in Iran.

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