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Short Communication

Prevalence, and virulence determination of *Listeria monocytogenes* strains isolated from clinical and non-clinical samples by multiplex polymerase chain reaction

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Abstract

Introduction: This study aimed to determine the prevalence, and virulence factors of *Listeria monocytogenes* isolated from various samples by multiplex polymerase chain reaction (MPCR). **Methods:** A total of 617 isolates were obtained and MPCR was employed for detection of the *inlA*, *inlC,* and *inlJ* genes. **Results:** *L. monocytogenes* was detected in 46 (7.45%) of the 617 specimens. *inlA*, *inlC,* and *inlJ* were detected in 100%, 76.26%, and 71% isolates, respectively. **Conclusions:** This study validated MPCR in the analysis and rapid detection of *L. monocytogenes*. The role of the genes in pathogenesis of the strains can also be affirmed.

Keywords: *Listeria monocytogenes.* Virulence genes. Multiplex-PCR.

Listeria monocytogenes is a gram positive, non- sporulating and facultative intracellular bacteria that caused infection in both humans and animals. *L. monocytogenes* infection in humans, listeriosis, is a serious illness that affects mostly immunosuppressed individuals, newborns, and the elderly, with symptoms ranging from septicemia, meningitis, encephalitis, abortions, to occasional death[1]. In recent years, many outbreaks of listeriosis have been implicated in the contamination of trading nutriments such as vegetables, milk, and meat foodstuffs[2]. *Listeria monocytogenes* detection from specimens based on selective enrichment media followed by biochemical studies is arduous and requires at least 5 days for a positive diagnosis. Polymerase chain reaction (PCR) is a rapid method with high sensitivity and specificity for specific deoxyribonucleic acid (DNA) sequences and permits direct detection of the pathogens[2]. A PCR employing amplification of the *iap*, *prfA*, *hly*, *inl,* and *plcA* gene sequences was recently reported for *L. monocytogenes* detection[1]. Liu et al. showed that the *inlA* gene is species-specific and the *inlC* and *inlJ* genes are virulence-specific for *L. monocytogenes*[3]. The *inlA* gene is present in all *L. monocytogenes* isolates irrespective of the source and serovar, but not in other Listeria spp. and other bacteria[3]. The InlA protein facilitates interaction between *L. monocytogenes* and the host cell receptor E-cadherin and leads to *L. monocytogenes* adhesion and invasion of epithelial cells in the human intestine[3]. InlC (also known as *IrpA*) is a virulence marker responsible for the post-intestinal dissemination of *L. monocytogenes* infection[4]. Only four proteins of the internalin family are part of the repertoire of secreted proteins, and among these, *inlC* is the most important. The gene encoding the inlC protein is present in pathogenic *L. monocytogenes* and *L. ivanovii* species, but not in other *Listeria* species[1]. The *InlJ* (or *lmo2821*) gene is responsible for passage of *L. monocytogenes* through the intestinal barrier and can be used for evaluating virulence of *L. monocytogenes*[5]. Detection of virulent genes is necessary as it decreases the time and labor required for diagnosis and will be useful in a large-scale investigation for detecting virulent strain of *Listeria monocytogenes* species includes a range of strains

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with varying virulence and pathogenicity, and while numerous *L. monocytogenes* strains are naturally virulent and capable of produce major illness and death, others are avirulent[2]. Key virulence-related proteins and the genes encoding them can be targeted to better evaluate virulent strains of *L. monocytogenes*[5] [6]. Therefore, it is imperative to develop a rapid and precise laboratory method that can easily discriminate between virulent and avirulent strains. A rapid method that provides information about epidemiological feature of microorganism can helpful in the control and prevention of listeriosis. In addition, it will also provide insights regarding the outbreak and prevalence of naturally both virulent and avirulent strains. This study aimed to determine the prevalence, and virulence factors of *L. monocytogenes* isolated from clinical and non-clinical samples by using multiplex PCR.

Between December 2012 and November 2015, 617 samples, including both clinical and non-clinical specimens, were collected from Tehran hospitals, Iran. Five milliliters of fluid specimens (including blood and urine), fecal and vaginal swabs, and 25g of the placental tissue were inoculated into 50, 10, and 225ml of TSYBE broth (tryptic soy broth positive 0.6% yeast extract) (Merck Co., Germany), respectively. Solid specimens (including dairy products and processed meat) were inoculated into 225ml of TSYBE. After inoculation in TSYBE, the samples were incubated for 7-16 days. In addition, samples stored at 4°C for 6 months were inoculated into PALKAM agar (Merck Co., Germany) supplemented with 5mg polymixin B, 2.50mg acriflavin, and 10mg ceftazidime and incubated at 35°C for 48h. Approximately 5-6 suspend grown colonies from both culture media were inoculated into Brain Heart Infusion (BHI) agar (Merck, Germany) and analyzed using standard microbiological and biochemical tests such as catalase, CAMP test, beta hemolysis on blood agar, bile esculin, hydrolysis of sodium hippurate, and motility at 4ºC. Bacterial DNA was extracted from colonies grown overnight in BHI broth at 37°C by using a DNA extraction Kit (Roche Co, New York, USA) according to the manufacturer's protocol. Primers used in the M-PCR assay were from a previously published study by Liu et al.[3]. The reaction mixture consisted of 2µl extracted DNA, 2.5µl 10× PCR buffer, 1.5µl $MgCl_2$ (50mM), 0.5µl dNTP (10mM), 1.25µl each primer (10pmol/uL), 0.4µl Taq DNA polymerase (5U/µl) and deionized water to a final volume of 25µl. The reaction mixture was amplified in a thermo cycler (Eppendorf, Germany) with the following PCR conditions: denaturation at 94°C for 5 min, 31 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s, and final extension at 72°C for 5 min. The PCR products were further analyzed by electrophoresis in 1.50% agarose gel for 40 min in Tris-acetate buffer, visualized by ethidium bromide staining, illuminated by a UV-Trans illuminator, and the images were captured by a gel documentation apparatus (UVP Gel Seq Software, England). *L. monocytogenes* ATCC-764 was used as a positive control and *L. ivanovii* ATCC 19119*, Staphylococcus aureus* ATCC-25923, *Streptococcus pyogenic* ATCC-19615, *Enterococcus feacalis* ATCC-29212, *Escherichia coli* ATCC 25922, and *Acinetobacter baumannii* ATCC 19606 were used as negative controls. Data analysis was conducted by employing descriptive statistics, *t*-test, and chi square test, using SPSS-21 software and a 5% significance level was set in all analyses.

Of the 617 specimens, 170 and 447 samples were collected from clinical and non-clinical sources, respectively. Clinical samples including blood, urine, placenta tissue, rectal swabs, and vaginal swabs were obtained from patients with spontaneous abortions hospitalized in Shariati hospital, Tehran, Iran. Non-clinical samples were obtained from dairy products, processed meat, and animals. Dairy products and processed meat samples were obtained from supermarkets and retail market from Tehran, Iran. Animal specimens were obtained from the Tehran university veterinary clinic (**Table 1**). The multiplex PCR method was performed using the *inlA, inlC,* and *inlJ* primers that amplify regions of 800, 517, and 237 bp, respectively. As indicated in **Figure 1**, the distribution of species and virulence-specific genes in the clinical samples (n; 170, 27.4%) showed that 14 (8.2%), 9 (5.3%), and 8 (4.7%) strains harbored the *inlA*, *inlC,* and *inlJ* genes, respectively. Of the 447 (72.6%) non-clinical samples, 32 (7.11%), 27 (6%), and 25 (5.60 isolates were positive for the *inlA*, *inlC* and *inlJ* genes, respectively. As shown in **Table 2**, there were no significant association between the bacterial isolation and non-clinical sources ($p = 0.65$). In addition, the prevalence of the *nlA*, *inlC,* and *InlJ* genes was not statistically significant between the clinical group and non-clinical group ($p > 0.05$).

In total, forty-six (7.5%) *L. monocytogenes* isolates were recovered from the 617 specimens tested in the present study, which is comparable with the results of the study conducted by Lotfollahi *et al*[2]. Four (7.5%), 2 (5.7%), 5 (14.2%), 3 (8.5%), and 0 (0%) *L. monocytogenes* isolates were obtained from the placental tissue, urine, vaginal swabs, rectal swabs, and blood, respectively. In the present study, vaginal swabs and blood exhibited the highest and lowest prevalence, respectively, amongst the clinical samples. In the current study, *L. monocytogenes* was isolated from the urine and rectal-vaginal swabs of two women. Of the two cases, *L. monocytogenes* was isolated from the vaginal and rectal swabs of one patient and in the other case, *L. monocytogenes* was isolated from placental tissue and rectal and vaginal swabs of the same patient. These results highlight the role of *L. monocytogenes* in spontaneous abortions in these women. The results of this study are in agreement with previous reports in which the bacterium was isolated 7% of 100 vaginal samples[5], 22 of 428 women with a poor obstetric history[6], 3 of 100 women[7] and 9 of 670 women[8] and therefore, implicate *L. monocytogenes* as a causative agent of human abortions. Of the 107 different dairy products, 9 (7.4%) *L. monocytogenes* were isolated from 5 (7.1%) cheese samples, 2 (7%) cream samples (10%) and 2 kashk samples. Kargar and Ghasemi reported a higher prevalence of *L. monocytogenes* (13.1%) than that reported in our study[7]. This is probably due to differences in geographical regions in which the two studies were conducted. *L. monocytogenes* was recovered from 5.2% of processed meat product samples tested in the present study. Our results showed a much lower prevalence in contrast to those observed in studies conducted by Hudson *et al.* (New Zealand), Simon *et al.* (Spain) and Ismaiel *et al.* (Egypt), where

TABLE 1

Bacterial isolation.

Samples	Origin sample	Samples		Listeria monocytogenes isolates	
		n	%	n	%
clinical	placenta	53	31.2	4	7.5
	blood	12	7.0	0	0.0
	urine	35	20.6	2	5.7
	vaginal swab	35	20. 6	5	14.2
	rectal swab	35	20. 6	3	8.5
	Total	170	100.0	14	8.2
Non-clinical	**Dairy products**				
	cheese	70	65.4	5	7.1
	cream	20	18.7	2	10.0
	kashk	17	15.9	2	11.7
	Total	107	100.0	9	8.4
	Processed meat				
	sausage	90	42.9	5	5.5
	beef extract	55	26.1	4	7.2
	poultry extract	65	31.0	2	3.0
	Total	210	100.0	11	5.2
	Domestic animals				
	goat	60	46.1	6	10.0
	sheep	50	38.5	4	8.0
	cattle	20	15.4	2	10.0
	Total	130	100.0	12	9.2
	Standard strain			ATCC 7644	

the prevalence of *L. monocytogenes* was 12.5%, 17.3%, and 13.3% (frozen beef), respectively[8][9][10]. This discrepancy may be due to the lower number of meat specimens that investigated in our study. However, our findings are consistent with those of studies conducted by Wang *et al.* and Ismaiel *et al.,* where the prevalence of the pathogen was 4.7% (in meat samples) and 5.3% (in frozen chicken samples)[8][9].

In the present study, the *inlA* gene was detected in all *L. monocytogenes* isolates (100%); these results were similar to those obtained by Liu *et al*. (USA) and Almeida *et al*. (Brazil) [3][11]. Studies conducted by Poyart *et al*. and Jung *et al*. showed that the *inlA* gene is species-specific, thereby suggesting their species-wide sequence conservation[12][13]. Rawool *et al*. showed that multiple virulence factors, including phosphatidylinositol phospholipase C (*plcA*), hemolysin (*hlyA*), actin polymerization protein (*actA*), invasive associated protein (*iap*), and internalin A (*inlA*), are necessary for the pathogenesis of *L. monocytogenes*.

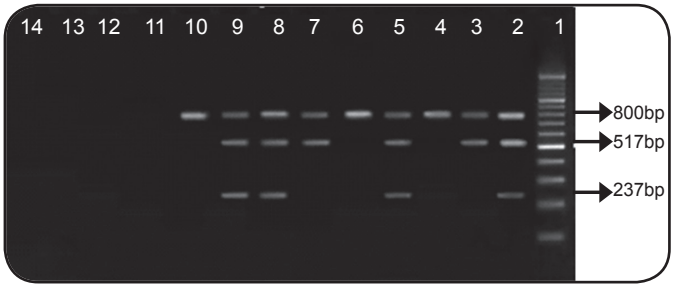

FIGURE 1. Multiplex-PCR amplification of the *InlA*, *InlC* and *InlJ* genes in the eight selected isolates of *Listeria monocytogenes*. Lane 1: DNA Ladder 1kb; **Lane 2:** positive control **(***Listeria monocytogenes* **ATCC7644); Lane 3-4:** strains isolated from clinical samples; **Lane 5-6:** strains isolated from dairy samples; **Lane 7-8:** strains isolated from meat samples; **Lane 9:** strain isolated from animal samples; **Lane 10:** *L*isteria *ivanovii* ATCC 19119; **Lane 11:** *Staphylococcus aureus* ATCC 25923; **Lane 12:** *Streptococcus pyogenes* ATCC 19615; **Lane 13:** *Enterococcus faecalis* ATCC 29212; **Lane 14:** *Escherichia coli* ATCC 25922. (band 800 bp: *inlA*, band 517 bp: *inlC,* and band 237bp: *inlJ*). **PCR:** polymerase chain reaction; **DNA**: deoxyribonucleic acid.

TABLE 2

Characteristics of bacterial isolation based on clinical and non-clinical samples.

	Bacterial isolation		
Samples	**Yes**	**No**	**Total**
Clinical	14 (2.3%)	156 (25.3%)	170 (27.5%)
Expected count	12.72	157.31	170.0
Non-clinical	32 (5.2%)	415 (67.3%)	447 (72.5%)
Expected count	33.30	413.71	447.03
Total	**46 (7.5%)**	**571 (92.6%)**	**617(100.0%)**

*P = 0.65

Therefore, detection of just one virulence factor by PCR is not always sufficient to identify *L. monocytogenes* strains. In addition, it is plausible that spontaneous mutations enable the removal of one or more virulence determinants in some *L. monocytogenes* strains. Thus, simultaneous detection of multiple virulent genes in a single assay is desirable as it reduces the time and labor involved and will be useful for large-scale investigations for detection of pathogenic strains of *Listeria*[14]. According to our study, the prevalence of *inlC* and *inlJ* virulence genes was 9 (64%) and 8 (57%) in clinical samples and 27 (84%) and 25 (78%) in non-clinical specimens, respectively. Similarly, Liu *et al*. reported that the *inlC* and *inlJ* genes are present in 80.5% and 77.81% of *L. monocytogenes* strains, respectively[10]. These findings implicate a role of the virulence genes *inlA*, *inlC* and *inlJ* in the pathogenesis of *L. monocytogenes* isolates. Accordingly, the present report describes the development of a multiplex PCR that incorporates the *inlA* gene primers for confirming species-specificity and the *inlC* and *inlJ* gene primers for virulence determination of *L. monocytogenes*.

In conclusion, a multiplex PCR incorporating the *inlA* gene primers for *L. monocytogenes* species-specific recognition and the *inlC* and *inlJ* gene primers for virulence determination was developed that enables the rapid and simultaneous confirmation of the *L. monocytogenes* species and its virulence.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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