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# VIRULOTYPING OF LISTERIA MONOCYTOGENES ISOLATED FROM HUMAN AND FOOD PRODUCTS SAMPLES USING MULTIPLEX PCR IN DUHOK PROVINCE, IRAQ

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**ABSTRACT**

*Listeria monocytogenes* is a facultative intracellular, food-borne zoonotic-bacterium causing life-threatening infections worldwide with a significant mortality rate in both humans and animals. The aim of the study was to examine the existence of five crucial virulence-associated genes (*inIA, inIB, inIC, inIJ* and *prfA*) in 48 *L. monocytogenes* isolates that were obtained from human and different food products using multiplex Polymerase Chain Reaction (PCR). The most frequent genes among isolates were *inIC* (93.8%), *inIB* (91.7%) and *inIJ* (83.3) followed by *prfA* (81.2%) and *inIA* (79.2%). Regardless of the source of bacteria, all tested isolates showed the prevalence of multiple virulence-associated genes. The presence of three virulence genes was detected in 20.8%, four of five virulence genes were found in 33.3% of isolates, and 45.8% of isolates carried all five virulence genes. In conclusion, the presence of potential virulence genes increases the pathogenicity of *L. monocyogenes.* Furthermore, the occurrence of multiple virulence related genes in a single isolate may imply the presence of highly virulent isolates. To investigate the connection between those genes and clinical outcomes, more research is needed.

**KEY WORDS**: *Listeria monocytogenes*, virulence genes, Food products, Duhok, multiplex PCR

## INTRODUCTION

*Listeria monocytogenes* is a Gram positive bacillus, facultative intracellular microbe with a unique life cycle that may enter, persist, grow in phagocytic and non-phagocytic cells, and disseminate across tissues and remain in host cells. It is a foodborne bacteria that cause listeriosis (Farber & Canada, 2015; Lamond & Freitag, 2018). It is transmitted via consumption of contaminated food such as vegetables, cheese, ice-cream, meat and dairy products (FDA, 2019a; CDC, 2016). *L. monocytogenes is* able to cause aggressive diseases in human with weak immunity (such as elderly, neonates, and pregnant-women) resulting in gastroenteritis, meningitis, septicemia, stillbirth, miscarriage, or serious contagions to baby with a 20%–60% mortality rate (Lamond & Freitag, 2018; Schlech III, 2019; Ireton et al., 2021). It is worth mentioning that antimicrobial resistance is a challenge in our community because of the high resistance rates among various bacteria (Assafi et al., 2015; Rasheed & Hussein, 2020 a &b; Hussein et al., 2019) including *Listeria* (Al-Brefkani & Mammani, 2019). Studying the virulence genes in pathogenic bacteria can give a better insight about the pathogenicity of bacteria and the severity of infections caused by microorganisms (Hussein et al., 2008; Rasheed & Hussein, 2020). Thus, the virulency of *L. monocytogenes* is mostly controlled by some virulence related genes that are associated with severe human infections (Kose & Yakupogullari, 2015). Such genes play an sentential role in the pathogenicity and evasion from hosts immune responses because they involve in the essential phases of pathogenicity such as adhesion, invasion, multiplication, and distribute intracellularly (Campuzano-Maya, 2014). The most common virulence-associated genes of *L. monocytogenes* are internalins that are encoded by (*inlA, inIB, inlC* and *inlJ genes*), listeriolysin which is encoded by *hlyA* gene, actin encoded by *actA* gene and the invasion associated protein encoded by *iap* gene (Ward et al., 2004). The expression of these virulent associated factors are involved in the pathogenesis of *L. monocytogenes*, which is operated and coincide by the regulatory *prfA* gene (Orsi & Wiedmann, 2016; Vasanthakrishnan et al., 2015). It has been suggested that the shift of *L. monocytogenes* from a non-pathogen to sever pathogenic bacteria was related to the prevalence of virulence associated genes (Tan et al., 2015; Schlech III, 2019). Therefore, this study was aimed to investigate the presence of five crucial *L. monocytogenes* virulence genes in human and food product samples by using multiplex Polymerase Chain Reaction (PCR).

## MATERIALS AND METHODS

### Study isolates

A total of 48 frozen *L. monocytogenes* isolates that were obtained by Al- Brefkani and Mammani (Al-Brefkani & Mammani, 2019a) were used that were isolated from various foods (41 isolates) and human clinical samples (7 isolates) from June 2021 to July 2022 in Duhok Province, Iraq. Human samples included blood (3), high vaginal swabs (HVS) (3) and urine (1), while, foods were from meat products; frozen chicken meat (27), fresh red meat (10), and dairy products; raw milk (3), white soft cheese (1). Blood samples were collected from septicemia and meningitis cases at Duhok Kidney and Diseases Transplantation Center and Hevi Pediatric Teaching Hospital, while, the urine and HSV samples were collected from women with poor obstetric history such as miscarriage and stillbirth attended the Duhok Obstetrics and Gynecology Teaching Hospital. Meat and dairy products were obtained from the Food Testing Laboratory of the Directorate of Prevention Affairs in Duhok.

### Laboratory procedures

All isolates were microbiologically detected by using Gram staining, catalase test, haemolysis test on sheep blood agar and Harlequin™ Listeria Chromogenic Agar (ISO) subculture

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(Neogen®Company-UK) that was used to discriminate *L. monocytogenes* from other *Listeria* spp (Al-Brefkani & Mammani, 2019a). Microgen Listeria-ID microwell test strip (Microgen-UK) was used for biochemical confirmation which includes eleven desiccated carbohydrate components (Dominguez Rodriguez et al., 1986). All phenotypically identified isolates were confirmed by PCR technique using species specific 16s rRNA primers (Macrogen, South Korea). The DNA extraction and PCR conduction were performed according to Al-Brefkani and Mammani (Al-Brefkani & Mammani, 2019a). The molecular work was conducted at Duhok Research Center (DRC) at College of Veterinary Medicine, University of Duhok, Duhok, Iraq.

**Multiplex PCR for the identification of virulence-associated genes**

For amplification of *inIA*, *inIB*, *inIC*, *inIJ* and *prfA* genes, specific primers were used as shown in Table 1 (Klein & Juneja, 1997; Liu et al., 2007) with minor modifications using thermocycler machine (Applied Biosystems 9700, USA). The final volume of master mix reaction was 25 with following contents: 12.5 µL of ready-to-use Prime Taq Premix (GeNet Bio- South Korea), 2 µL of DNA (10 ng) template from each *L. monocytogenes* isolate and 1.0 µL of each primer (GeNet Bio- South Korea), (stock concentration, 10 µM), together with 5.5 µL of dd$H_2O$ (Ambion-USA) was prepared for each reaction. For *L. monocytogenes* internalin (*InIA*, *InIB*, *InIC* and *InIJ*) genes, the cyclic program was adjusted as following: initial heat denaturation at 94˚C for 2 min, 30 cycles of denaturation at 94˚C for 20 sec, annealing at 55°C for 20 sec, and extension at 72°C for 50 sec. The final extension set upped at 72˚C for 2 min, then incubated at 4°C (Liu et al., 2007). The PCR settings were made up for *prfA* gene as follows: initial denaturation at 95°C for 4 min, 35 cycles at denaturation at 94°C for 30 sec, annealing at 63°C for 60 sec and extension at 72°C for 60 sec. Final extension was at 72°C for 5 min (Klein & Juneja, 1997). The gel electrophoresis conditions were conducted following the instructions defined by Liu et al (Liu et al., 2007). The DNA ladder (100 bp) (Promega) was used as a marker for measuring the amplicon size of amplified genes. A previously sequenced *L. monocytogenes* strain (access number: MK968366) was used as a positive control (Al-Brefkani & Mammani, 2019b).

Table 1: Oligonucleotide primers sequences

| | | Virulence associated genes | | |
|---|---|---|---|---|
| Target gene | Primer | Primer sequence (5' →3') | bp | Reference |
| *prf A* | ELMPRFF | 5′-CGGGATAAAACCAAAACAATTT-3′ | 508 | (Klein & Juneja, 1997) |
| | ELMPRFR | 5′-TGAGCTATGTGCGATGCCACTT-3′ | | |
| *inlA* | Internalin AF | 5'-ACTATCTAGTAACACGATTAGTGA-3' | 250 | (Hudson et al., 2001) |
| | Internalin AR | 5'-CAAATTTGTTAAAATCCCAAG TGG-3' | | |
| *inlB* | Internalin BF | 5'-AAGCACAACCCAAGAAGGAA-3' | 1107 | (Johnson et al., 2004) |
| | Internalin BR | 5'-AAAATTCCACTCATGCCCAC-3' | | |
| *InlC* | Internalin CF | 5'-AATTCCCACAGGACACAACC-3' | 517 | (Liu et al., 2007) |
| | Internalin CR | 5'-CGGGAATGCAATTTTTCACTA-3' | | |
| *inlJ* | Internalin JF | 5'-TGTAACCCCGCTTACACAGTT-3' | 238 | (Liu et al., 2007) |
| | Internalin JR | 5'-AGCGGCTTGGCAGTCTAATA-3' | | |

## STATISTICS

IBM SPSS Statistics 21 software was used for conducting the data curation. Chi-Square analysis was used to study the significant variations among isolates. *P*-value <0.05 was considered significant.

## ETHICAL APPROVAL

For the ethical approval, the study was conducted according to the guidelines of the Ethics Committee and current legislation on research and ethical approval of the College of Health and Medical Technology-Shekhan (issued no.100520174), Duhok Polytechnic University- Iraq.

Table 2: Relationship between Virulence factors and various sample sources.

| Sample Source | Positivity of virulence associated genes (no=48) | | | | | |
|---|---|---|---|---|---|---|
| | *prfA* no. (%) | *InIA* no. (%) | *InIB* no. (%) | *InIC* no. (%) | *InIJ* no. (%) | Total no. |
| HVS | 2(66.7%) | 2(66.7%) | 2(66.7%) | 3(100%) | 3(100%) | 3 |
| Blood | 3(100%) | 2(66.7%) | 3(100%) | 3(100%) | 3(100%) | 3 |
| Urine | 0(00.0%) | 1(100%) | 1(100%) | 1(100%) | 1(100%) | 1 |
| Frozen chicken meat | 22(81.5%) | 22(81.5%) | 26(96.3%) | 24(88.9%) | 22(81.5%) | 27 |
| Fresh red meat | 10(100%) | 8(80%) | 9(90%) | 10(100%) | 8(80%) | 10 |
| White soft cheese | 1(100%) | 1(100%) | 1(100%) | 1(100%) | 1(100%) | 1 |
| Raw milk | 1(33/3% | 2(66.7%) | 1(100%) | 3(100%) | 2(66.7%) | 3 |
| Total | 39(81.2%) | 38(79.2%) | 44(91.7%) | 45(93.8%) | 40(83.3%) | 48 |
| Chi-Square | 12.505 | 1.471 | 6.158 | 2.489 | 2.347 | |
| P value | 0.052 | 0.961 | 0.406 | 0.870 | 0.885 | |

## RESULTS

This study screened for five potential virulence-associated genes in *L. monocytogenes* isolates. The results showed that *inIC* 45(93.8%), *inIB* 44(91.7%), and *inIJ* 40(83.3%) were the most frequent genes in *L. monocytogenes* isolates. However, the prevalent of *prfA* and *inIA* were noticed in 39(81.2%) and 38(79.2%) isolates, respectively (Table 2 and Figure 1). The

virulence genes of *inIC* and *inIJ* were identified in all human and dairy product samples, whereas, *prfA*, *InIA* and *InIB* were detected in different frequencies among the same samples. Furthermore, the prevalence of *inIA*, *inIJ*, *inIB*, inIC, *inIJ* and *prfA* virulence genes were found to be highest among frozen chicken meat and followed by fresh red (Table 2). There was a significant difference between the prevalence of *prfA* gene and source of *L. monocytogenes* isolates ($p<0.05$), whereas, no significant relationship was detected between the presence of *InIA, InIB, InIC, InIJ* genes and source of bacterial isolates ($p>0.05$) (Table 2).

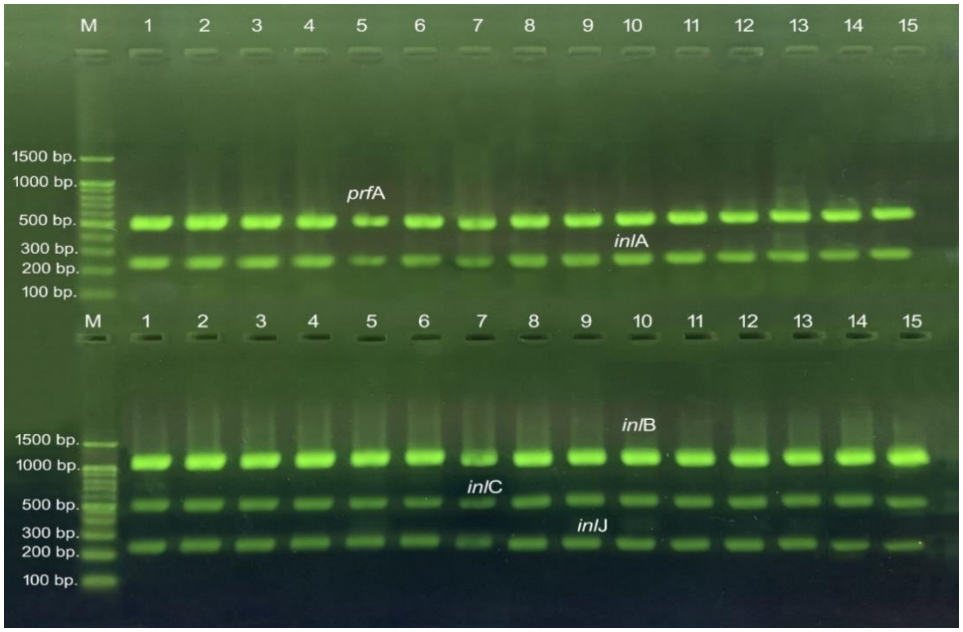


Figure 1: Gel electrophoresis for the multiplex PCR amplification of *prfA, inLA, inIB, inIC* and *inIJ* genes of *L. monocytogenes* isolates at 508bp, 250bp, 1107bp, 517bp and 238bp, respectively. Lane M: 100 bp DNA ladder; Lane 1 (HVS), 2 (Blood), 3 (Urine), 4-8 (Frozen chicken meat), 9–12 (Fresh red meat), 13–14 (raw milk), 15 (White soft cheese).

## DISCUSSION

The high virulence of *L. monocytogenes* lies in its ability to penetrate intestinal wall, placenta and blood–brain barriers to cause severe CNS infections (Disson & Lecuit, 2013; Vimentin et al., 2018; Schlech III, 2019). Furthermore, the capability of *L. monocytogenes* to live in harsh circumstances such as growing in up to 45 °C, resistance to cold, resistance to various disinfectants and could stay at surfaces for long time, increases its ability to cause infections (CDC, 2016). *Listeria* is considered one of the bacteria of interest for the health sector due to the severity of its infections and its complications among infected individuals especially individuals with weak immunity (Lamond & Freitag, 2018; Schlech III, 2019; Ireton et al., 2021). The severity of these infections is controlled by various virulence genes. Several surface related proteins of the family "internalins" (*InIA, InIB* and *InIC*) of *L. monocytogenes* bacterium interact with specific host receptors to facilitate entry and cross human cells (Ireton et al., 2021). The present study investigated the presence of some of *L. monocytogenes* virulence genes (*InIA, InIB, InIC, InIJ* and *prfA*), that enhance the bacteria to transit between human cells and increase its pathogenicity.

The regulatory *prfA* gene is considered essential in pathogenicity as it controls and regulates the expression of some virulence genes and it is thought hypothetically that its absence lead the bacteria to lose its ability to cause infections (Osman et al., 2019). Our results revealed that 81.2% of *L. monocytogenes* isolates carried *prfA* gene. In consistent with the present study, the *prfA* gene was detected in 77.7% of *L. monocytogenes* isolates from different clinical and food samples in Egypt (Osman et al., 2019). On the other hand, the *prfA* gene was not found in any of the tested isolates in a study conducted in Egypt (Abdeen et al., 2021). However, in Northern region of Iran was found that *prfA* gene was in almost all of the investigated isolates (Jamali et al., 2015). This diversity in results need further studies for more comprehensive explanation.

Furthermore, the total prevalence of *InIA, InIB, InIC* and *InIJ* genes were 79.2%, 91.7%, 93.8% and 83.3%, respectively, in both human and food product samples. In contrast, the prevalence rate of the internalins genes in Egypt were somehow less than that of our finding rates that revealed the following results, *inIA* (74.1%), *inIB* (81.5), *inIC* (70.4%), and *InIJ* (66.7%) (Osman et al., 2019). In disagreement with our results, the *inIA* gene was not found in any of the tested isolates in a study performed in Egypt (Abdeen et al., 2021). However, in a study conducted in Iran that found the rates of *inIA, inIB, inIC, and inIJ* genes were almost in all of the investigated isolates (Jamali et al., 2015). The differences in the virulence genes rate might be due to the differences in the sample type or the presence of different strains in different regions.

Various studies showed that the presence of internalins (*InIA, InIB, InIC, and InIJ*) genes could raise the mortality rate as these genes have a significant potential to initiate human infections (Liu et al., 2007; Ireton et al., 2021). The incidence of multiple virulence factors has been reported in this study in which all tested isolates (100%) showed the presence of multi-virulence genes. Three virulence factors were noticed in 20.8%, four genes were detected in 33.3% and all five tested virulence genes were identified in 45.8% of *L. monocytogenes* isolates. In agreement with our results, Tahoun et al. (2017) reported that 60.8% *L. monocytogenes* isolates showed the presence of multiple virulence associated genes (Tahoun et al., 2017). The occurrence of multiple virulence genes in single *L. monocytogenes* isolate is also supported by several recent studies (Du et al., 2017; Kaur et al., 2018; El-Demerdash & Raslan, 2019). The presence of multiple virulent genes in the same isolate will increase its pathogenicity and its ability to cause life threating infections. Thus, further studies are needed to investigate the virulogentic

profile by recruiting a bigger sample size and following up the outcomes of these infections.

In conclusion, this study showed the prevalence of major virulence-associated genes in tested *L. monocyogenes* isolates. It also showed the occurrence of multiple virulence related genes in single isolates. Therefore, infection with such a microorganism may cause life-threatening infections. Additionally, application of multiplex PCR for detection virulence genes provides rapid and immediate confirmation of *L. monocytogenes* virulence-associated genes. The association between those genes and clinical outcomes has to be investigated further.

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