Some virulence genes and biofilm formation capabilities of *Listeria monocytogenes* isolates from different sources

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ABSTRACT

In this study, it was aimed to determine the biofilm-forming abilities of both clinical and food-borne isolates of *Listeria monocytogenes*, to investigate the presence of nine different virulence genes, and to consider the current threat status of this agent. A total of 28 isolates, 21 from food and seven from clinical origin, were used in the study. Two different methods, namely "tube adherence" and "microplate" were used to determine the biofilm formation abilities of isolates. For the determination of nine different virulence genes of *Listeria monocytogenes* (*inlA*, *inlC*, *inlJ*, *hylA*, *luxS*, *flaA*, *prfA*, *inlB*, *actA*), the method of polymerase chain reaction (PCR) was used. As a result, all isolates were found to be able to form a biofilm to varying degrees by both tube and microplate methods. These two methods were similar in terms of their results. All nine different virulence gene regions were detected at various rates in the isolates. Although the genes directly related to biofilm formation for the isolates weren't detected, to form biofilm was observed. The virulence genes detected in clinical origin isolates were proportionally higher than in food-borne isolates (except for *flaA* and *prfA* gene regions). It was concluded that bacteria of *Listeria monocytogenes* continue to form biofilm and carry virulence genes regardless they are from food or clinical origin. Also, food-borne contaminations continue to be a severe threat to human health. So, to prevent listeriosis, cases of both humans and animals should be taken required precautions and all cases should be considered carefully.

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a zoonotic pathogen that causes infections in animals and humans (36). Moreover, this agent, reported to cause infections in humans even with its non-invasive forms, is one of the most important bacterial foodborne pathogen (41). Due to the high number of infections caused *by L. monocytogenes* resulting in death in some countries, this disease has been an important issue closely related to the professional groups dealing with public health in recent years (10).

According to some sequencing information and multilocus enzyme analysis, the genus of *Listeria* is composed of 20 species that are *L. monocytogenes*, *L. innocua*, *L. marthii*, *L. welshimeri*, *L. grayi*, *L. seeligeri*, *L. ivanovii*, *L. costaricensis*, *L. weihenstephanensis*, *L. rocourtiae*, *L. riparia*, *L. fleischmannii*, *L. newyorkensis*, *L. floridensis*, *L. aquatica*, *L. thailandensis*, *L. goaensis*, *L. cornellensis*, *L. grandensis*, and *L. booriae* (13). In addition, Quareda et al. (40) reported *Listeria valentina* sp. nov. as a new species. *L. monocytogenes* and *L. ivanovii* are virulent (29). Some *L. monocytogenes* strains have also been reported to be avirulent (28). Subtyping processes of *L. monocytogenes* are important for identifying sources and following strains of the agent involved in listeriosis outbreaks and determining the population genetics, taxonomy, and epidemiology of this pathogen (17). For subtyping, many methods could be roughly grouped as Phage typing, Multilocus enzyme electrophoresis, Amplified fragment length polymorphism, Randomly amplified polymorphic DNA,

Repetitive Element Sequence (REP), and Enterobacterial repetitive intergenic consensus (ERIC) - PCR and Ribotyping. Serotyping is a phenotypic subtyping method. Serotyping is performed using the slide agglutination method that characterizes the agent into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (14). The isolates from food and environmental samples belonged to a small number of serotypes 1/2a, 1/2b, and 4b (44).

The virulence potential of *L. monocytogenes* has shown great genetic diversity and variability over time. This has separated foodborne isolates into different groups, lineages, sequence types, and clonal complexes. All strains, therefore, have different abilities to cause human infection. Virulence factors constitute the pathogenicity of *L. monocytogenes*. Subgroups have different virulence phenotypes. The genetic diversity of *L. monocytogenes* is crucial for understanding the ecology and epidemiology of this bacterium (39).

Virulence determinants of *L. monocytogenes* are clustered along the chromosome in *Listeria* pathogenicity island (LIPI), 1, 3, and 4. These contain virulence factor genes of internalin proteins (*InlA*, *InlB*, *InlC*, *InlD*, *InlE*, *InlF*, *InlG*, *InlH*, and *InlJ*) that serve on adhesion and invasion of the host cell, listeriolysin O (Hyl) and phosphatidylinositol phospholipase C (plcA and plcB) require for lysis of vacuole membranes, listeriolysin S, only induced under oxidative stress conditions and contributes to virulence of the pathogen, actin polymerizing protein (ActA) facilitate movement of *L. monocytogenes* both inter and intra-cellularly, metalloprotease (mpl) require for maturation of plcB and assists hly, plcB, and plcA. The invasion-associated protein (iap) facilitates septum separation during the final stage of cell division and adherence of *L. monocytogenes* to the host cell. The positive regulatory factor A (*prfA*) controls these genes' expression (33).

Considering these characteristics of *Listeria*, the virulence properties of isolates from clinical cases, foods, or various materials have been a matter of curiosity for researchers and have been investigated by many researchers (29, 34, 42, 43).

It was reported that biofilm formation in *Listeria* spp. might be affected by some factors (differences in strains, physical and chemical properties of the external environment, growth period of bacteria, temperature, environmental conditions, and other microorganisms) (33). The ability of *L. monocytogenes* to form biofilm is also recognized as an essential virulence determinant affecting the pathogenicity of the strain, and researchers reported that biofilm formation can be regulated by many genes (7). In a recent study, researchers also strongly suggested that *mgtB*, *clsA*, *uvrB*, and *mltD* genes are involved in biofilm formation (35).

In this study, it was aimed to evaluate the biofilm formation ability of *L. monocytogenes* isolates obtained from both food and clinical materials by different methods, to investigate the presence of nine gene regions that may be responsible for virulence properties, and thus to raise awareness against the danger of listeriosis.

Materials and Methods

***Isolates and Reference Strains*:** The isolates of *L. monocytogenes* (n=28), 21 of them from food samples and seven from clinical material origin, were isolated between the years 2015 and 2018 and then added to the strain collection of the Microbiology Laboratory of the Faculty of Veterinary Medicine of Harran University. Before using, they were inoculated on Trypticase soy agar (TSA) (Merck) medium for reliving and then confirmed on both VITEK (bioMérieux, France) and MALDITOF-MS (Bruker Corporation, Billerica, MA, USA) devices.

L. monocytogenes ATCC 7644, ATCC 19115, and ATCC 13932 strains situated in the department's strain collection were used as reference strains in all tests.

***Biofilm Detection Using The Tube Method*:** The "Tube Adherence Method" described by Christensen et al. (11) was used. Trypticase soy broth (TSB) (Merck) medium prepared according to the "product preparation instructions" was poured into 10 ml glass tubes for inoculation of bacterial cultures and evaluation of biofilm formation. The glass tubes were autoclaved at 121 °C for 15 min. and stored in cold conditions at 4 °C until use. In this test, one loop full of bacterial culture was inoculated into 10 mL of TSB. The tubes were incubated under aerobic conditions at 37 °C for 24 hours. After incubation, the tubes were gently emptied, and the empty tubes were washed with PBS (pH 7.3) and dried. Then, 0.1% crystal violet dye (Merck) was added to the tubes equal to the volume of the evacuated liquid medium, and the tubes were stained. The excess stain was washed with deionized water, and the tubes were dried inverted. All tubes were scored according to the biofilm results of the reference strains prepared as a control. Biofilm formation was considered positive when a visible film was observed on the wall or bottom of the tube. Biofilm results were graded as no biofilm (-), weak (+), moderate (++), and strong (+++). All tests were repeated twice to verify the results.

***Biofilm Detection Using The Microplate Method*:** The method described by Stepanovic et al. (45) was used. Three or four identical colonies were taken from the strains incubated in TSA and suspended in 5 ml of TSB medium. The culture was incubated in aerobic conditions at 37°C for 24 hours without shaking. After incubation, the culture was thoroughly mixed, and 20 µl of this culture was added to 230 µl of TSB, previously divided into

microplate (Greiner Bio-One) wells. Sterile TSB was used as a negative control. All samples were added in 3 replicates in the wells. After incubation, the contents of the wells were emptied, and each well was washed three times with 300 µl of sterile distilled water. Bacterial particles on the plates were fixed with 250 µl methanol for 15 min. The microplates were then emptied and allowed to dry again overnight in the inverted position at room temperature. The biofilm layer was stained by adding 250 µl crystal violet to each microplate well for 5 min. at room temperature, and the wells were rewashed under running water. The process was continued until the stain was cleared. After drying the microplates again at room temperature, the dye bound to the cells was dissolved with 33% glacial acetic acid (Merck) in each well. After the dye was dissolved with glacial acetic acid, each well's optical density (O.D.) was measured at 570 nm on a microplate reader (VersaMax, Molecular Devices), and the cut-off O.D. (O.D.c) was determined as three standard deviations from the mean O.D. values of the negative controls. The results were evaluated as (O.D.) ≤ (O.D.c) = no biofilm, O.D.c < O.D. ≤ (2 x O.D.c) = weak biofilm, (2 x O.D.c) < O.D. ≤ (4 x O.D.c) = moderate biofilm and (4 x O.D.c) < O.D. = strong biofilm formation accordingly. The final results were calculated based on the average O.D. of the wells analyzed in 3 replicates.

***DNA Isolation for Molecular Analyses*:** Genomic DNA was provided from all isolates by boiling method (22). A quarter loop of *Listeria* colonies grown on the TSA surface was taken. These colonies were suspended in cryovials filled with 400 µl PCR grade, DNase-RNase free water. The suspensions were mixed thoroughly using a vortex device (Heidolph-Reax) and boiled for 10 min. The boiled suspensions were centrifuged (Thermo Scientific-microCL 21R) at 13,000 rpm for 10 min. From these cryovials, 200 µl of supernatant was taken and transferred into 1.5 µl DNase/RNase-free microcentrifuge tubes (Isolab) and stored at -20ºC until use.

***PCR Amplification*:** PCR amplification was performed in a Thermo Scientific-Arctic thermal cycler. Taq DNA Polymerase Enzyme (5IU/µl, Thermo Scientific), 25 mM $MgCl_2$ (Thermo Scientific), 10X Taq PCR Buffer (Thermo Scientific), 10 mM dNTP mix (Fermentas) and PCR grade water (Ambion) were used for amplification. The primers (Table 1) were obtained from Sentebiolab, Türkiye. Each primer master stock was diluted for a microliter to contain 100 pmol of nucleotide chain. According to the datasheet of the primers provided by the manufacturer, different amounts of PCR-grade water were added to each primer bottle for reconstitution. Thus, they were adjusted in the amount of 100 pmol/µl. In short, to adjust 100 pmol/µl primer, the amount of nucleotide chain of each primer in nmol was multiplied by 10, and later, the PCR-grade water was added as much as the resulting amount in µl into the primer bottle. For PCR tests, from each primer master stock, both forward and reverse were taken 20 µl and added to water in 160 µl. As a result, mixes of primers were prepared from the master stocks with 10 pmol of reverse and forward nucleotide chains in each microliter. These mixes were used in PCRs.

Table 1. Gene regions and primer sequences.

Target region	Primary sequence (5'-3')	Product size (bp)	Reference
luxS	F-GGA AAT GCC AGC GCT ACA CTC TTT R-ATT GCA TGC AGG AAC TTC TGT CGC	208	(47)
flaA	F-GCG CAA GAA CGT TTA GCA TCT GGT R-TTG AGT AGC AGC ACC TGT AGC AGT	363	(47)
prfA	F-GAT ACA GAA ACA TCG GTT GGC R-GTG TAA TCT TGA TGC CAT CAG G	274	(12)
actA	F-GCT GAT TTA AGA GAT AGA GGA ACA R-TTT ATG TGG TTA TTT GCT GTC	827	(49)
inlB	F-TGG GAG AGT AAC CCA ACC AC R-GTT GAC CTT CGA TGG TTG CT	884	(29)
inlA	F-ACG AGT AAC GGG ACA AAT GC R-CCC GAC AGT GGT GCT AGA TT	800	(29)
inlC	F-AAT TCC CAC AGG ACA CAA CC R-CGG GAA TGC AAT TTT TCA CTA	517	(29)
hylA	F-GAA TGT AAA CTT CGG CG R-GCC GTC GAT GAT TTG AAC TTC ATC	388	(34)
inlJ	F-TGT AAC CCC GCT ACA CAG TT R-AGC GGC TTG GCA GTC TAA TA	238	(29)

***Investigation of The FlaA and LuxS Gene Regions*:** The molecular method from Warke et al. (47) was used by slightly changing the amounts of the reacting chemicals. Thus, PCR mixtures were prepared by adding enzyme 0.25 µl, $MgCl_2$ 4 µl, PCR buffer 5 µl, dNTP mix 2 µl, primer mix 2 µl, water 34.75 µl and finally DNA templates 2 µl to make a final volume to 50 µl. These mixtures were placed in the thermal cycler, and the first denaturation step was set at 94 °C for 2 min. The PCR recipe was then applied for 35 cycles, denaturation step at 94 °C for 30 sec., annealing at 58 °C for 30 sec., extension at 72 °C for 1 min., and final extension at 72 °C for 7 min. The amplicons obtained were electrophoresed into 1% agarose gel at 90 V for 50 min.. The band formations were investigated after visualizing with the UV illuminator (Vilber-Lourmat).

***Investigation of The PrfA Gene Region*:** The molecular method from D'agostino et al. (12) was used by slightly changing the amounts of the reacting chemicals. Thus, PCR mixtures were prepared by adding enzyme 0.25 µl, $MgCl_2$ 5 µl, PCR buffer 5 µl, dNTP mix 1 µl, primer mix 2 µl, water 35.75 µl and finally 1 µl of the DNA templates to make a final volume to 50 µl. These mixtures were placed in the thermal cycler, and the first denaturation step was set at 94 °C for 2 min. The PCR recipe was then applied for 40 cycles, denaturation at 94 °C for 30 sec., annealing at 55 °C for 30 sec., extension at 74 °C for 1 min., and final extension at 74 °C for 5 min. The amplicons obtained were electrophoresed into a 2% agarose gel at 90 V for 60 min. Band formations were investigated after visualizing with the UV illuminator.

***Investigation of The InlA, InlC, InlJ, and HlyA Gene Regions*:** The molecular method by Liu et al. (29) was used to investigate gene regions by slightly changing the amounts of reacting chemicals and adding *hlyA* primers. For this modified multiplex PCR, four gene regions can be examined at the same time with a single PCR; enzyme 0.2 µl, $MgCl_2$ 3 µl, PCR buffer 2.5 µl, and dNTP mix 0.5 µl were used. Primers, reverse and forward, were used separately at the amount of 0.2 µl from *inlA*, 0.15 µl from *inlC*, 0.1 µl from *inlJ*, and 0.2 µl from *hlyA* primers after they reconstituted to 100 pmol/µl. PCR mixtures were prepared by adding 15.5 µl of PCR-grade water and 2 µl of the final DNA templates to adjust the final volume to 25 µl. In the thermal cycler, the PCR recipe consisted of an initial denaturation at 94 °C for 2 min., followed by 30 cycles, denaturation at 94 °C for 20 sec., annealing at 55 °C for 20 sec., extension at 72 °C for 50 sec., and final extension at 72 °C for 2 min. The amplicons obtained were electrophoresed into 2% agarose gel at 90 V for 60 min. The band formations on the agarose gel were investigated after visualizing them with the UV illuminator.

***Investigation of The ActA Gene Region*:** The molecular method used by Arslan and Baytur (5) was preferred to investigate the *actA* gene region. Thus, PCR mixtures were prepared to a final volume of 50 µl by taking the amount of 0.25 µl from the enzyme, 2 µl from the $MgCl_2$, 5 µl from the PCR buffer, 1 µl from the dNTP mix, 1.5 µl from the primer mix, 39.25 µl from the water and finally 1 µl from the DNA templates. The PCR recipe in the thermal cycler was based on the molecular method used by Cai et al. (9). According to this molecular method, the PCR recipe consisted of initial denaturation at 94 °C for 2.5 min., followed by 40 cycles, 3 min. denaturation at 94 °C, 1 min. annealing at 53 °C, 2 min. extension at 72 °C, and 5 min. final extension at 72 °C. The amplicons were electrophoresed into 1.5% agarose gel at 90 V for 60 min., and band formation was analyzed with the UV illuminator.

***Investigation of The InlB Gene Region*:** The molecular method used by Arslan and Baytur (5) was used to investigate the *inlB* gene region. Thus, PCR mixtures were prepared by adding enzyme 0.25 µl, $MgCl_2$ 2 µl, PCR buffer 5 µl, dNTP mix 1 µl, primer mix 1.5 µl, water 39.25 µl and finally 1 µl of DNA templates to make a final volume of 50 µl. The PCR recipe in the thermal cycler was determined according to the molecular method used by Pangallo et al. (37). The PCR recipe consisted of initial denaturation at 94 °C for 2 min., followed by 35 cycles, denaturation at 94 °C for 45 s., annealing at 60 °C for 45 s., extension at 72 °C for 1.5 min., and final extension at 72 °C for 8 min. The amplicons were electrophoresed into a 1.5% agarose gel at 90 V for 60 min., and band formation was analyzed with the UV illuminator.

Statistical Analyses: The statistical relationship between the presence of virulence genes and the biofilm formation ability of these isolates was analyzed with Fisher's Exact Test by creating 2x2 contingency tables separately for each gene region on GraphPad Prism 10.

Results

***Results of Tube Method*:** According to the results of investigating the biofilm formation abilities of the isolates by tube method, all food-origin isolates were positive. Of the positives, out of six were weak, four were moderate, and 11 were strong. All clinic-origin isolates were positive, while five of them were moderate, and two were strong (Table 2).

***Results of Microplate Method*:** All isolates have biofilm formation abilities using the microplate method. Five isolates of clinical materials were evaluated as moderate, and two of them were evaluated as strong. Isolates of foods were evaluated as weak in four isolates, moderate in five isolates, and strong in 12 isolates (Table 2).

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Table 2. Table of all results.

Origin	Isolate No	Sources of isolates	Investigated gene regions of the isolates									R1	R2
			luxS	*flaA*	*prfA*	*inlA*	*inlC*	*inlJ*	*hylA*	*inlB*	*actA*		
Food	2	Butter	+	+	+	+	+	+	+	-	-	(+)	W
	3	Cheddar Cheese	+	+	+	+	+	+	+	+	+	(++)	M
	4	Cocoa Cake	+	+	+	+	+	+	+	-	-	(+)	W
	5	Plain Ice Cream	-	+	+	-	-	-	-	-	-	(+++)	S
	6	Cream	-	+	+	+	-	-	+	-	+	(+++)	S
	7	Milk Skin	-	+	+	+	-	-	+	-	+	(+++)	S
	8	Melting Cheese	-	+	+	-	-	-	-	-	-	(+)	M
	9	Sausage	+	+	+	-	-	-	-	-	-	(++)	M
	10	Roasting	+	+	+	+	+	+	+	+	+	(+)	M
	11	Meatball	+	+	+	-	-	+	+	-	-	(+)	W
	12	*Lahmacun	+	+	-	+	+	+	+	+	-	(+)	W
	13	Hamburger	+	+	+	+	+	+	+	+	+	(+++)	S
	14	**Börek	-	+	+	+	+	+	+	+	+	(+++)	S
	17	Pizza	+	+	+	+	+	+	+	+	+	(+++)	S
	18	Wet Cake	+	+	+	+	+	+	+	+	+	(+++)	S
	20	Sausage	+	+	+	+	+	+	+	-	+	(++)	S
	21	Ice Cream	+	+	+	+	+	+	+	-	+	(+++)	S
	22	Beans	-	+	+	+	+	+	+	+	+	(+++)	S
	23	Raw Meat	+	+	+	+	+	+	+	+	+	(++)	M
	30	Doner Kebab	+	+	+	-	-	+	+	-	-	(+++)	S
	31	Wet Cake	+	+	+	-	-	-	-	-	-	(+++)	S
Clinic	24	Bovine Brain	+	+	+	+	+	+	+	-	+	(+++)	S
	25	Aborted Fetus	+	+	-	+	+	+	+	+	+	(++)	M
	26	Aborted Fetus	+	+	+	+	+	+	+	+	+	(++)	M
	27	Aborted Fetus	+	+	+	+	+	+	+	+	+	(++)	M
	28	Aborted Fetus	+	+	+	+	+	+	+	+	+	(++)	M
	29	Aborted Fetus	+	+	+	+	+	+	+	+	+	(+++)	S
	32	Sheep Brain	+	-	+	-	-	+	+	-	-	(++)	M
PC		ATCC 19115	+	+	+	+	+	+	+	+	+	(++)	M
PC		ATCC 13932	+	+	+	+	+	+	+	+	+	(+++)	S
PC		ATCC 7644	+	+	+	+	+	+	+	-	+	(+++)	S
NC		PCR Water	-	-	-	-	-	-	-	-	-	-	-

(-)= no formation of biofilm, (+)= weak formation of biofilm, (++)= modarete formation of biofilm, (+++)= strong formation of biofilm, *= a kind of pizza with minced meat, **= a kind of fritter with salt and cheese, PC=Positive Control, NC=Negative Control, R1= Results of Tube Method, R2=Results of Microplate Method, W= Weak, M= Moderate, S= Strong.

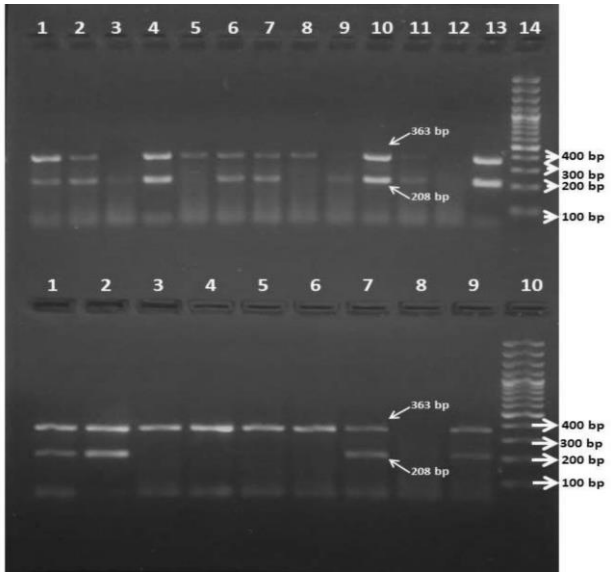
***Identification Results of The LuxS and FlaA Gene Regions*:** While the *luxS* gene was detected in all clinic-origin isolates (n=7), 15 out of 21 were detected in food-origin isolates (Figure 1 and Table 2).

The *flaA* gene was detected in all food-origin isolates and identified in six clinic-origin isolates (Figure 1 and Table 2).

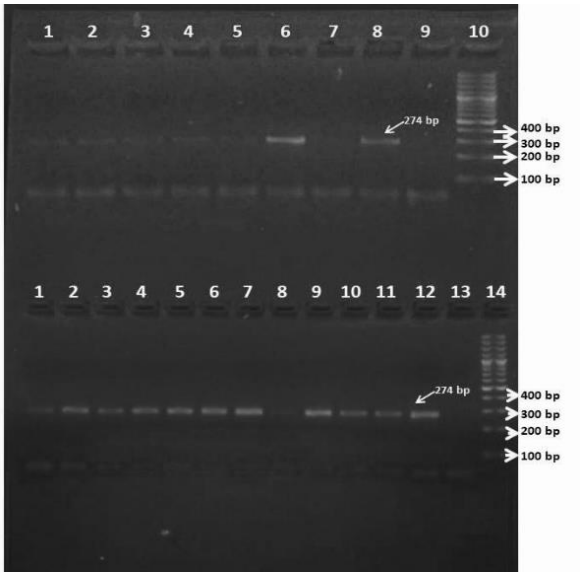
***Identification Results of The PrfA Gene Region*:** The *prfA* gene region was detected in 20 food-origin isolates (n=21) and six in the clinic-origin isolates (n=7) (Figure 1 and Table 2).

***Identification Results of The InlA, InlC, InlJ, and HlyA Gene Regions*:** For the food-origin isolates (n=21), the *inlA* gene region was detected in 15, *inlC* in 13, *inlJ* in 15, and the *hlyA* gene region in 17 isolates. For the clinic-origin isolates (n=7), the *inlA* gene region was detected in six, *inlC* in six, *inlJ* in seven, and the *hlyA* gene region in seven isolates. (Figure 1 and Table 2).

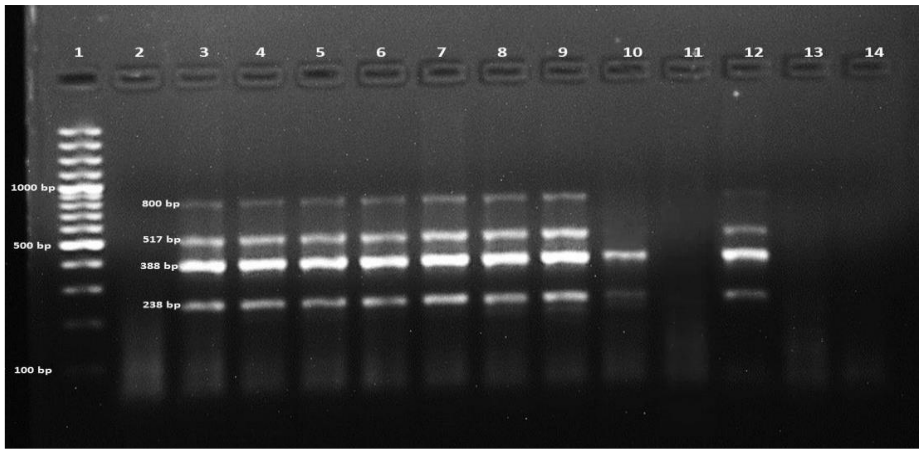
***Identification Results of The InlB Gene Region*:** The *inlB* gene region was detected in nine out of all food-origin isolates. In terms of the presence of this gene region, five of the clinic-origin isolates were positive (Figure 1 and Table 2).



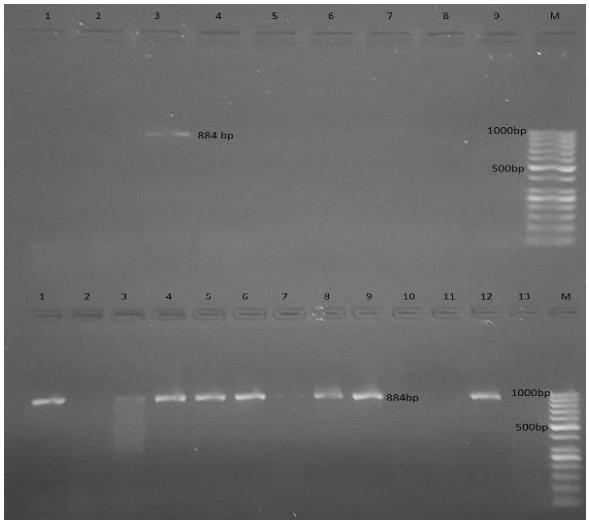
(**a)** PCR image for *luxS* and *flaA* genes



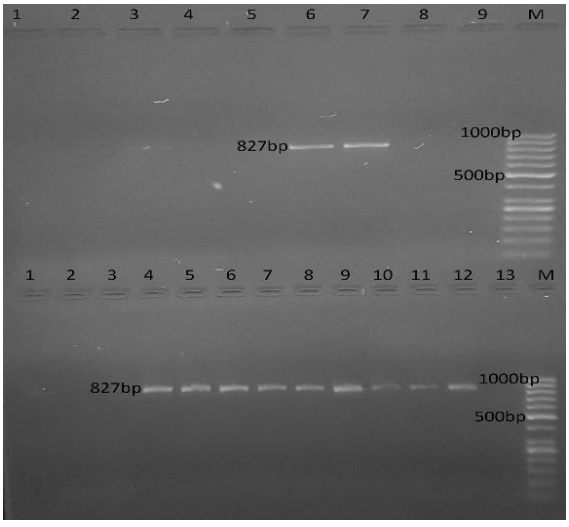
(b) PCR image for *prfA* gene



(c) PCR image for *inlA*, *inlC*, *hylA*, and *inlJ* genes



(d) PCR image for *inlB* gene



(e) PCR image for *actA* gene

Figure 1. PCR images for investigated gene regions.

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Table 3. Positivity rates of the isolates according to the presence of the gene regions.

	Positivity rates (%) and numbers of isolates								
Origin of isolates	***inl*A**	***inl*C**	***inl*J**	***hyl*A**	***luxS***	***flaA***	***prfA***	***inlB***	***actA***
Clinical (n=7)	85.7 n=6	85.7 n=6	100 n=7	100 n=7	100 n=7	85.7 n=6	85.7 n=6	71.4 n=5	85.7 n=6
Food (n=21)	71.4 n=15	61.9 n=13	71.4 n=15	80.9 n=17	71.4 n=15	100 n=21	95.2 n=20	42.8 n=9	57.1 n=12
Total (n=28)	75 n=21	67.8 n=19	78.5 n=22	85.7 n=24	78.5 n=22	96.4 n=27	92.8 n=26	50 n=14	64.2 n=18

***Identification Results of The ActA Gene Region*:** The *actA* gene region was detected in 12 food-origin isolates. Six clinic-origin isolates were positive for the presence of this gene region (Figure 1 and Table 2).

Results of Statistical Analyses: There was no statistically significant correlation between the presence of the gene regions and the biofilm formation ability (P >0.05)

Discussion and Conclusion

L. monocytogenes has different virulence genes and biofilm formation abilities. These properties have been the subject of many researchers' studies. Lee et al. (25) reported that the amount of biofilm might vary according to growth conditions, strain, serotype, and genotype. There wasn't a clear relationship between biofilm synthesis ability and biofilm formation under experimental conditions. The authors also reported that environmental factors influence certain stages of biofilm synthesis and that nutrient deficiency increases adhesion, while prolonged nutrient deficiency inhibits biofilm synthesis. In this study, the biofilm synthesis abilities of the isolates were examined at 37°C using both tube and microplate methods, and biofilm formation was detected in different amounts (Table 2). Some researchers evaluated the biofilm-forming capacities of food and clinical isolates of *L. monocytogenes* and observed that biofilms formed by strains from the food industry were thicker than those from sporadic cases (3). In this study, most of the food-origin isolates were evaluated as strong regarding biofilm synthesis ability, while the biofilm synthesis abilities of clinical isolates were generally found to be moderate and strong under the same conditions. In addition, it was observed that the results of the tube and microplate methods used in the evaluation of biofilm formation were very close to each other, and it was concluded that any of the methods could be used in line with the laboratory infrastructure (Table 2).

The *luxS* gene was detected in most food isolates (71.4%) and all clinical isolates (Table 3). Sela et al. (42) reported that the *luxS* gene plays a role in biofilm formation in some pathogenic bacteria. The findings obtained in this study, similar to the literature, can emphasize the relationship between the presence of the *luxS* gene and biofilm formation. The luxS gene region was not detected for the isolates, but biofilm formation was detected; the role of possible mutations in the gene region may be considered. Or, in the absence of this gene, different genetic mechanisms may be involved in biofilm formation. This hypothesis supports the ideas of some researchers that other gene regions may also be involved in biofilm formation (25, 26).

In some serotypes of *L. monocytogenes*, the *prfA* gene is also shown to be required for biofilm formation (26). Poimenidou et al. (38) characterized the *prfA* gene expression of *L. monocytogenes* isolates and showed that *prfA* was among the most conserved genes. In this study, the *prfA* gene was detected in 92.8% of total isolates, while the food-origin rate was 95.2% and the clinical-origin rate was 85.7% (Table 3). The high parallelism between the proportion of *prfA* gene region detected and biofilm formation is consistent with previous studies.

In this study, no statistically significant correlation was found between the presence of the investigated gene regions and the biofilm formation ability of the isolates. In a similar study, researchers investigated the biofilm-forming ability of *L. monocytogenes* serotypes isolated from different (clinical and food) sources, and it was reported that no correlation was found between serotype and biofilm-forming ability. (16). Angelidis et al. (2) reported that the correlation between biofilm formation ability and serotype could not be evaluated due to the small number of *L. monocytogenes* isolates used in their study. Recently, Di Ciccio et al. (15) evaluated the biofilm-forming ability of 57 isolates of *L. monocytogenes* isolated from food and the existing environment in Italy and reported that all isolates were classified as weak or moderate biofilm producers, while the percentage of isolates from meat products considered as moderate or strong biofilm producers was higher than the percentage of isolates from dairy products. Lianou et al. (27) evaluated biofilm formation by foodborne pathogenic microorganisms as a highly complex phenomenon dependent on numerous internal and external factors. Nowaka et al. (35) reported that the regulatory cascades controlling biofilm formation may be specific to certain

environmental triggers and may be influenced by genetic variability between strains or serotypes.

The *flaA* gene is responsible for flagella synthesis and plays an essential role in the surface adhesion of *L. monocytogenes* (47). In this study, the *flaA* gene was detected in all foodborne isolates and most of the clinical isolates (85.7%) (Table 3). Also, some researchers have reported that some serotypes can be identified by detecting regions of the *flaA* gene (8).

Internal proteins of *L. monocytogenes* can enhance the invasion and virulence of the agent. Therefore, the detection of virulence markers is a rapid way for the preliminary differentiation of virulent *L. monocytogenes* strains from avirulent strains, and many PCR-based studies have been conducted for key virulence-related genes with rapid and reproducible results (17, 18).

Although some researchers have determined all of the *L. monocytogenes* isolates of clinical, food, or environmental origin as positive for the presence of *inlA*, *inlC*, *inlJ*, and *hylA* (5, 24, 44), some researchers reported that they detected three virulence genes, *inlA*, *inlC* and *inlJ* in all of the food-origin isolates (4). Liu et al. (29) also found all isolates (n=36) positive for *inlA*, but *inlC* and *inlJ* genes were detected in 29 and 28 isolates, respectively. In this study, the gene of *inlA* was determined at the rate of 75%, *inlC* 67.8%, *inlJ* 78.5%, and *hylA* 85.7% based on all isolates (Table 3). These results are consistent with the different results indicated in previous studies. *InlA*, *inlC*, *inlJ*, and *hylA* genes could not be detected in four food-origin isolates examined in this study. This result is consistent with the findings of some researchers (32, 36) that they could not find these four genes. The species of *L. monocytogenes* included many virulence factors, which may have contributed to this result.

Although studies have been conducted to determine whether the gene of *inlA* is species-specific for *L. monocytogenes* (20), the detection rate of the *inlA* gene in isolates were different proportions like 74.1% (36), 32.4% (32), and 99.6% (48) respectively. In this study, we could not determine species specificity regarding the primers used. Here, 85.7% of clinical samples and 71.4% of food-origin isolates were positive for the *inlA* gene (Table 3). It is seen that the rate of the *inlA* gene is similar to the rates determined by the researchers. Although the *inlA* gene region encoding the molecule that enables cell entry is considered to be species-specific for *L. monocytogenes*, some studies have shown that *inlA PMSC* mutations in isolates of *L. monocytogenes* may be responsible for low virulence (31, 46). It is thought that mutations in this region may prevent the detection of the *inlA* gene in all isolates examined.

A similar situation may exist for the gene of *hlyA*. Many researchers detected the *hlyA* gene region in *L. monocytogenes* isolates at a rate of 100% (24, 44). Although some researchers reported that the presence of the *hylA* gene to determine hemolysis ability, which is an essential factor determining virulence, can be an identifier to determine *L. monocytogenes* at the species level (6), Matle et al. (32) and Osman et al. (36) reported that positivity rates of the *hylA* gene as 45.6% and 92.5% respectively according to the results of their studies. In this study, the *hylA* gene was detected at 100% in the clinical samples; however, the positivity rate was determined at 80.9% in food-origin isolates (Table 3). It is seen that the rate of the *hylA* gene obtained in the study is similar to the rates determined by the researchers.

Two gene regions of the bacterium that can be considered virulence markers of the agent are *inlC* and *inlJ* regions. Because they are not present in avirulent *L. monocytogenes* strains. It has been reported that the *inlJ* (29) and the *inlC* (18) are specific gene regions that determine the virulence of the bacteria. Although some researchers (5, 24, 44) reported the detection rate of *inlC* and *inlJ* genes as 100%, some others reported them respectively as 70.4% - 66.7% (36) and 98% - 99.2% (48). Abdollahzade et al. (1) reported a positivity rate of 91.7% for both genes, while Sharma et al. (43) reported 100% for *inlC* and 0% for *inlJ* in five milk-origin isolates. In this study, positivity rates of the *inlC* and *inlJ* gene regions are consistent with the values reported by other researchers.

Although there was no statistically significant correlation between the presence of these four gene regions, *InlA*, *inlC*, *inlJ*, *hylA*, and the biofilm formation ability ($P > 0.05$) in this study, Maggio et al. (30) reported that the presence of internalin (*inl*), Stress Survival Islet (*SSI*) and erythromycin resistance (*ermC*) genes were associated with the ability to produce biofilms. This may have been due to differences in the strains or gene regions studied. Because the same researchers also reported that the biofilm production is strain-dependent.

Researchers have conducted many studies on the detection of *actA* and *inlB* virulence gene regions and emphasized that *actA* can be used for the diagnosis of *L. monocytogenes* at the species level (49), and *inlB* can be used for the same diagnosis at species and serogroup levels (19). Some researchers have detected this gene region in all strains they used in their studies (21, 24, 36, 44). While Pangallo et al. (37) found the *inlB* gene region in all 33 strains of *L. monocytogenes* using the primers they developed, some researchers detected this gene region in two out of five strains from milk (43), in 22 out of 27 food and clinical isolates (36) and, in 58 out of 59 food-derived isolates (21). Poimenidou et al. (38) reported that the *actA* gene region had the highest diversity among the gene regions they examined in their study. Haidar-Ahmad et al. (21) also reported that they detected the *actA* gene region in all isolates but obtained amplicons with

different sizes for three isolates and explained the reason for this with the possibility of genetic polymorphism. In this study, positivity rates for the *actA* and *inlB* gene regions were found to be 64.2% and 50%, respectively (Table 3). The positivities are not 100% as the results of Sharma et al. (43). This may be "because virulence genes follow different evolutionary pathways, which are influenced by the origin and serotype of a strain and may affect the virulence and/or epidemiological dominance of certain subgroups" (38). In addition, *actA* and *inlB* percentages showed significant differences between clinical isolates and food isolates, and this difference was mainly due to the low percentages in food isolates. In their study with a group of proteins, including *inlB* and *actA* proteins in human and foodborne strains, Jacquet et al. (23) evaluated the *actA* protein detected in isolates in 4 different serogroups. They emphasized that non-virulent or attenuated strains may be very common among foodborne strains and even *L. monocytogenes* strains with different virulence factors may play a role in various clinical pictures for clinical isolates.

In this study, the proportional differences in the presence of virulence genes between clinical and food isolates were higher in clinical isolates than in food isolates, depending on the gene region. This may be because isolates obtained from active infections that are direct disease cases come from naturally virulent strains. The researchers emphasized that the distinction between virulent and non-virulent strains is of great importance. However, they reported that attempts to use key virulence proteins and genes as targets were generally unsuccessful. They also reported that *L. monocytogenes* is a highly heterogeneous species, consisting of hypervirulent and hypovirulent clones in terms of pathogenicity (24). In addition, the presence of detected gene regions does not necessarily mean they are expressed. Depending on the detection method used, possible small mutations in the gene region may reduce the detectability of that gene region. Genomic analyses, phenotypic tests, animal experiments, clinical and epidemiological data may need to be evaluated to determine true pathogenicity. Takeuchi et al. (46) reported differences in virulence between different *L. monocytogenes* strains in the mouse bioassay, but no correlation was found among the origin of *L. monocytogenes* isolates (human, animal, food type, or environment). The researchers also reported that no correlation could be established between serotypes, phagovars, ribovars, or DNA macrorestriction patterns and the degree of *L. monocytogenes* virulence and that differences in the ability of *L. monocytogenes* strains to cause disease could be attributed to different virulence factors associated with each strain.

In conclusion, it was shown that *L. monocytogenes* isolates of both food and clinical origin carry gene regions that may contribute to biofilm synthesis, and these isolates can synthesize biofilm of various thicknesses. It was observed that the ability to synthesize biofilm, which is effective in the adhesion of microorganisms to living surfaces, especially to inanimate surfaces, and protection from environmental adverse effects, is an important virulence factor, and biofilm formation can occur in the absence of genes that may directly relate to biofilm synthesis. It was thought that this situation is essential for both animal and public health, especially due to foodborne contamination, and necessary measures should be taken for biofilm awareness.

The virulence-associated gene regions detected in this study were found in some proportions in *L. monocytogenes* strains, but no gene region was detected in all of the strains. Therefore, it was concluded that the primers used to detect these gene regions cannot be used directly for species-specific diagnosis. In addition, since foodborne strains were also found to carry virulence genes, it is recommended that human and animal health be protected by taking necessary precautions.

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Ethical Statement

The study was reviewed and approved by the Animal Experiments Local Ethic Committee of Harran University, Şanlıurfa, Türkiye (Decision number:01-14 / 2019).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

AMS, AA, KA, and OK conceived and planned the experiments. AMS, AA, KA, and AGY contributed to preparing the samples and performing the experiments. AMS and OK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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