Research Article

Evaluation of *prf A* Gene in *Listeria monocytogenes* Isolated from Dairy

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Abstract

Background: A cross-sectional study was conducted to investigate *Listeria monocytogenes* contamination in dairy products from Ardabil, Iran. This foodborne pathogen is known to cause listeriosis, particularly in vulnerable populations such as immunocompromised individuals and pregnant women.

Objectives: This novel study focuses on an extensive analysis of *L. monocytogenes*.

Methods: DNA extraction of the bacteria was performed, followed by amplification and evaluation of the *prf A* gene in *L. monocytogenes* to assess its potential for virulence, pathogenicity, antibiotic resistance, and biofilm formation. Additionally, this study targeted the CFU/mL count and evaluated the antibiotic resistance of *L. monocytogenes* against multiple antibiotics, including ampicillin, kanamycin, penicillin G, co-trimoxazole, tetracycline, ciprofloxacin, cephotaxime, erythromycin, imipenem, and chloramphenicol.

Results: The presence of the *prf A* gene was detected in nearly all samples containing *L. monocytogenes*, and it was determined to be significantly responsible for the pathogenicity of the bacteria in listeriosis.

Conclusions: Understanding the frequency of virulent strains containing the *prf A* gene could support the development of improved prognostic models and interventions to prevent future outbreaks.

Keywords: *Listeria monocytogenesis*, Zoonotic, Infection

1. Background

More than 200 foodborne illnesses are transmitted to humans, among which one of the most severe diseases is listeriosis, caused by consuming food contaminated with *Listeria monocytogenes* (1, 2).

Listeria is one of the four major foodborne pathogens identified by the WTO (1). Milk, being one of the most consumed and perishable animal foods, is highly vulnerable to contamination by *Listeria*, particularly *L. monocytogenes* (3, 4). Among high-risk groups such as neonates, pregnant women, immunocompromised individuals, and older people, *listeriosis* has a hospitalization rate of 91%, with a fatality rate of 50% among neonates and a mortality rate of 20 - 30% (5, 6).

Of the six *Listeria species* studied, *L. monocytogenes* is the most prevalent and antibiotic-resistant food pathogen. The pathogenicity of *L. monocytogenes* is influenced by various virulence factors, including actin

(actA), internalins (*in1A*, *in1B*, in1C, and *in1J*), phosphatidylinositol phospholipase C (PI-PLC, plcA), listeriolysin O (*hlyA*), virulence regulator (*prf A*), and invasion-associated protein (iap) (7, 8). These bacteria can survive in both extracellular and intracellular environments, transitioning from a saprophytic to a pathogenic stage via a complex network of several regulators such as *prf A*, *σB*, and *CodY* (9, 10).

There are several *Listeria* pathogenicity islands, i.e., *LIPI-1* to LIPI-4, but the genes that determine the potential virulence of isolates (especially *prf A* and *hly* genes) are present on *LIPI-1* (5, 6, 11).

Listeria monocytogenes possesses numerous regulatory genes and transport proteins that enable it to colonize diverse ecosystems (7, 8).

2. Methods

A descriptive cross-sectional study was conducted to examine the prevalence of *L. monocytogenes* in dairy

products and to evaluate the *prf A* gene for its virulence and pathogenicity.

2.1. Sample Collection

This study involved collecting 100 dairy product samples from Ardabil city, Iran. The samples included n = 60 milk samples, n = 35 cheese samples (n = 15 soft cheese samples and n = 20 plain cheese samples), and n = 5 samples from infected animals.

2.2. Sample Preparation, Processing and Strain Isolation

The samples were prepared for processing. Milk samples were filtered to remove impurities, and 20 mL was taken for further analysis. Cheese samples were carefully crushed, and 20 g was taken for testing. Similarly, samples from infected animals were filtered, and 20 mL was taken for processing. The samples were processed in the laboratory according to ISO 11290-1:2017 (https://www.iso.org/standard/60313.html, accessed on 1 January 2018) with slight modifications (9). The double enrichment technique was employed for the isolation of *Listeria* species. Twenty grams of each sample were introduced into brain heart infusion (BHI) broth (12), *Listeria* enrichment broth (3), and Mueller Hinton agar. The samples were then incubated at 4°C for 7 days with shaking at 100 - 120 rpm in a shaker incubator and adjusted to 0.5 MacFarland before handling (12, 13).

The inoculum was streaked onto agar plates and incubated at 37°C. After incubation, colonies were selected for secondary enrichment at 30°C for 24 hours. Finally, these colonies were streaked onto fresh agar plates and incubated at 37°C (14).

2.3. Identification and Characterization of Listeria Species Pluralis

The isolated bacterial colonies were screened for identification and characterization using various biochemical tests, including Gram staining, catalase test, oxidase test, motility test at 25°C and 37°C, methyl red/Voges-Proskauer (MR/VP) test, nitrate reduction test, citrate test, hydrolysis of esculin, fermentation of sugars (glucose, mannitol, xylose, maltose, α-methyl-D-mannoside, and rhamnose), β-hemolytic activity, and Christie–Atkins–Munch-Peterson (CAMP) test. All tests were conducted according to the guidelines provided by FDA BAM and ISO 11290 methods (15, 16).

2.4. DNA Extraction and PCR Amplification of prf A Gene

After the identification of *L. monocytogenes* through biochemical tests, further molecular characterization was conducted via DNA extraction followed by *prf A* gene

amplification for evaluation purposes. The strain was molecularly identified by 16S rRNA gene sequencing. The PGA DNA extraction kit (Pouya Gene Azma Co, Iran) was used to extract genomic DNA from the *Listeria* isolates according to the manufacturer's instructions. The extracted DNA was then preserved at -20°C for PCR (17).

For the evaluation of the *prf A* gene in *L. monocytogenes*, conventional PCR was performed, which is an in vitro enzymatic amplification of the targeted DNA sequence based on oligonucleotide primer-directed DNA synthesis by DNA polymerase. A 217 base pair fragment of the *prf A* gene was amplified using the routine PCR method (18). The primers LIS-R: TGA GCA ACG TAT CCT CCA GAG T and LIS-F: TCA TCG ACG GCA ACC TCG G were designed for this purpose (19, 20).

The process began with pre-incubation of DNA samples at 95°C for 5 minutes, followed by 40 cycles of denaturation, where double-stranded DNA (dsDNA) was converted into single-stranded DNA (ssDNA) at 95°C for 30 seconds. The next step involved hybridization or annealing of the two primers to the complementary region of the DNA template at 54 °C for 30 seconds, followed by primer extension at 72°C for 30 seconds. The final step was elongation or synthesis of DNA from sites dictated by the primers at 72 °C for 10 minutes. Each newly formed amplified fragment served as a template for subsequent cycles, resulting in an exponential increase in the number of target DNA copies. In just 2 - 3 hours and 30 - 40 cycles, up to 10^6 copies of the target DNA were amplified.

All amplified samples were run on 1% agarose gel for gel electrophoresis and visualized as bands on the gel using a DNA stain (21).

2.5. Enumeration of Listeria monocytogenes

After the molecular and biochemical characterization, the CFU/mL of *L. monocytogenes* was analyzed. All the positive milk samples were subjected to serial dilution to determine the colony-forming units per milliliter in each sample. A 10 μL culture was suspended in 10 mL of BHI broth, incubated at 37°C for 24 hours, and then subjected to serial dilution. Then, 1 mL of the sample from an appropriate dilution was taken and subjected to the pour plate and spread plate methods. The CFU/mL was measured by counting the colonies at specific dilutions (21, 22).

Listeria monocytogenes isolates from dairy products and infected animals were tested for antibiotic susceptibility. The disc diffusion method was used to evaluate the effectiveness of various antibiotics against these bacteria, following the guidelines of the Clinical

and Laboratory Standards Institute (13). A well-isolated colony of *L. monocytogenes* was transferred to 10 mL of BHI broth and incubated at 37°C for 24 hours, then diluted 1:10 in 9 mL of 0.1% peptone water to achieve a 0.5 MacFarland standard. The diluted culture was spread on Mueller-Hinton agar plates, and antibiotic discs were placed on the plates (3, 14).

The antibiotics used for testing included ampicillin, kanamycin, penicillin G, co-trimoxazole, tetracycline, ciprofloxacin, cephotaxime, erythromycin, imipenem, and chloramphenicol. The agar plates were then incubated at 37°C. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), various dilutions of antibiotics were prepared, and the isolates were cultivated to identify the concentration at which no visible growth was observed (1).

2.6. Congo Red Phenotype Detection

For detecting the pathogenicity and virulence of *L. monocytogenes*, it was also subjected to the Congo red agar method. All the isolates were tested for the production of exopolysaccharides by streaking them onto Congo red agar plates and incubating them at 37°C for 24 hours under aerobic conditions, followed by incubation at room temperature for another 24 hours. The biofilm-forming ability was assessed based on media discoloration and colony morphology. The results were considered positive when the isolates displayed colonies in a black to brown, wrinkled, or matte form (23).

3. Results

The results of the current study are as follows:

The collected samples were analyzed for the presence of *Listeria* spp. A cross-sectional descriptive analysis was employed to determine the prevalence of *Listeria* spp. across different sample types. Descriptive cross-sectional statistics provide an overview of the data, offering a clear picture of the attributes of the analyzed population at a specific point in time. Here, the total number of samples analyzed and the corresponding positive results, along with percentages, are presented, as frequencies and percentages are the most common ways to describe categorical data.

Among 60 milk samples, 5 (8.3%) contained *Listeria* spp. About 9 (25.7%) of the cheese samples tested positive for *Listeria* spp., while 5 (100%) of the samples collected from infected isolates had *Listeria* spp., as shown in Table 1. Overall, of the 100 total samples, 19 tested positive, indicating a 19% prevalence of *Listeria*

spp. However, 55 milk samples and 26 cheese samples (plain cheese + soft cheese) tested negative for *Listeria* spp.

The collected isolates from all the samples were subjected to biochemical testing to identify and classify the different *Listeria* spp. The results confirm and verify the presence of *L. monocytogenes*, *L. innocua*, and *L. seeligeri*, as shown in Table 2.

After the biochemical characterization of *L. monocytogenes*, DNA was extracted and amplified by PCR. To confirm the identity and virulence of *L. monocytogenes*, the *prf A* gene was amplified. The frequency of the *prf A* gene observed in *L. monocytogenes* was approximately 94.73% (n = 18), indicating that *L. monocytogenes* strains possess the *prf A* gene responsible for their pathogenicity.

The results show that out of 19 positive samples, 14 milk samples contained *L. monocytogenes*, 4 cheese samples contained *L. innocua*, and 1 sample from an infected animal contained *L. seeligeri*. The corresponding percentages are presented in Table 3.

The isolated samples of *L. monocytogenes* were then enumerated, and the CFU details are provided in Table 4.

The enumeration of *L. monocytogenes* in milk samples indicates a wide range of microbial loads. As the sample numbers progress, the CFU/mL values also increase. The variation in results reflects the heterogeneity in the level of contamination of *L. monocytogenes* in the dairy samples. After determining the CFU/mL of *L. monocytogenes* in the dairy samples, the antimicrobial resistance profile of *L. monocytogenes* was assessed, as shown in Table 5.

The antimicrobial resistance, MIC, and MBC of *L. monocytogenes* were determined from both food-based and animal source samples. The list of antibiotics and their effects on *L. monocytogenes* are shown in Table 5. Ciprofloxacin exhibited the highest efficacy, while cephotaxime showed both sensitivity and resistance. The MIC and MBC of Ciprofloxacin were determined to be 4.15 and 8.5, respectively.

The use of Congo red agar further confirmed the presence of *L. monocytogenes* with the *prf A* virulent gene, as the change in color and biofilm formation indicated its presence. The results showed that 100% of isolates from infected animal sources tested positive with the Congo red test, while among food samples, 78.57% (n = 11) yielded positive results.

4. Discussion

The results indicate that milk has 8.3%, cheese has 25.7%, and infected animal isolates have 100%

Table 1. Processing of the Collected Samples

Types of Samples	No. of Samples Analyzed	Positive Results for *Listeria Species* Pluralis
Milk	60	5 (8.3)
Cheese (plain cheese+soft cheese)	35 (20 + 15)	9 (25.7)
Infected animal isolates	5	5 (100)
Total number	100	19

Table 2. Biochemical Testing

Serial Numbers and Test Names	*Listeria monocytogene*	*Listeria innocua*	*Listeria seeligeri*
1. Gram staining	Gram +	Gram +	Gram +
2. Tumbling/motility test at 25°C and 37°C	Motile at 25°C/non motile at 37°C	Motile at 25°C/non motile at 37°C	Motile at 25°C/non motile at 37°C
3. Nitrate reduction	-	-	-
4. MR/VP	+	+	+
5. Catalase test	+	+	+
6. Oxidase test	-	-	-
7. CAMP test	+	-	-
8. Hydrolysis of esculin	+	+	+
9. Citrate test	-	-	-
10. B-hemolysis	+	-	+
11. Mannitol fermentation	-	-	-
12. Dextrose fermentation	+	+	+
13. Rhamnose fermentation	+	-/+	-
14. Xylose fermentation	-	-	+
15. α-methyl-D-mamoside	+	+	-/+
16. Phosphatidylinositol phosphatase	+	-	-

Abbreviation: MR/VP, methyl red/Voges-Proskauer.

Table 3. Species Identified from the Contaminated Samples

Name of Isolates	Sample	No. (%)
Listeria monocytogene	Milk	14 (73.6)
Listeria innocua	Cheese	4 (21)
Listeria seeligeri	Animal isolates	1 (5.26)
Total	-	19

contamination from *Listeria species* ([1]). These findings show that 7.67% of *Listeria species* are found in milk and related products ([10]). Similarly, one study stated that about 8.5% of *Listeria species* were found in milk samples and 6.8% in cheese ([8]).

PCR provides sensitive and rapid detection of *L. monocytogenes* in food products ([24]). The current study indicates a prevalence of the *prf A* gene (94.73%) in *L. monocytogenes* via PCR. A similar study using PCR detected virulence genes (*prf A*, *hlyA*, actA, and inlA) in *L. monocytogenes* isolated from contaminated milk samples ([24]). Another study also supports the prevalence of the *prf A* gene in *L. monocytogenes* isolates extracted from various sources such as the environment, milk, and infected animal samples analyzed via PCR ([14]). Another study in Italy supports these findings, showing that the *prf A* gene and *act A* gene were 100% dominant in *L. monocytogenes* ([15]). A previous study conducted in Nigeria aligns with the current study, stating that only three genes (*hly A*, *iap*, and *prf A*) are virulence-associated genes in *L. monocytogenes* ([15]).

Table 4. *Listeria monocytogenes* (CFU/mL) of in All 14 Positive Milk Samples

No. of Samples	CFU/mL
1.	320
2.	460
3.	570
4.	690
5.	780
6.	940
7.	1520
8.	1690
9.	1840
10.	1920
11.	2140
12.	2250
13.	2380
14.	2530

Table 5. Antimicrobial Resistance Profiles of *Listeria monocytogenes* Isolates

Variables	Dose (µg/disc)	Sensitivity of the Isolates (%)		
		R	I	S
Ampicillin	10 µg	1 (7.14)	5 (35.71)	8 (57.14)
Canamycin	10 µg	5 (35.71)	1 (7.14)	8 (57.14)
Penicillin G	10 U	4 (28.57)	5 (35.71)	5 (35.71)
Co-trimoxazole	25 µg	6 (42.85)	3 (21.42)	5 (57.14)
Tetracycline	30 µg	7 (50)	3 (21.42)	4 (28.57)
Ciprofloxacin	5 µg	2 (35.71)	1 (7.14)	11 (78.57)
Cephotaxim	30 µg	8 (57.14)	4 (28.57)	2 (18.18)
Erythromycin	15 µg	3 (27.27)	3 (18.18)	8 (57.14)
Imipeneme	10 µg	4 (27.27)	2 (14.28)	8 (57.14)
Chloramphenicol	30 µg	5 (27.27)	3 (27.27)	6 (42.85)

The current study found that among analyzed dairy product samples, milk had 73.6% (n = 14) *L. monocytogenes*, cheese had 21% (n = 4) *L. innocua*, and infected animal isolates had 5.26% (n = 1) *L. seeligeri*. These findings are supported by a previous study ([1]), which reported that 2.28% of *L. monocytogenes* was found in milk samples, 9.09% in bulk milk tanks, 2.85% in cheese, and 1.82% in infected cow milk ([1]). Another study exhibited the presence of *L. monocytogenes* in cheese and related processing facilities, though with a low prevalence, consistent with the current findings ([10]).

The presence of *L. seeligeri* in milk, feed, feces, water, and the environment and the presence of *L. innocua* in milk, feces, feed, the environment, and water have been documented previously ([25]). Another study supporting our findings reported that *L. monocytogenes* was found in milk (13%), udder swabs (19%), and cattle fecal samples (43%). Prior studies also mention that the prevalence rate of *L. monocytogenes* in raw cow milk can vary from 0% to more than 45% ([21]).

Another corresponding study shows that raw milk contains 25% *Listeria species*, among which approximately 2% of the isolates are *L. monocytogenes* and 5% are *L. innocua* ([22]). Another prior study reports about 5.90% occurrence of *L. monocytogenes* in raw milk ([18], [26]).

The prevalence of *L. monocytogenes* varies among different food sources due to several influencing parameters ([2]), including geographical region, season, type of sample, time, processing environment, farm size, transport conditions, isolation techniques, management practices, milking process, storage

methods, silage quality, sample size, and the level of sanitation in food processing and manufacturing facilities (2, 27, 28).

Approximately 60% of foodborne outbreaks are attributed to bacterial biofilm formation (26). One of the most common biofilm-forming, foodborne pathogens is *L. monocytogenes*, which is responsible for the spoilage of dairy and ready-to-eat products (20). The current study indicates biofilm formation verified by Congo red agar in *L. monocytogenes* isolated from food samples (78.5%) and from infected animal samples (100%). Previous studies also confirm the importance of the *prf A* gene in biofilm formation in *L. monocytogenes* (29, 30).

4.1. Conclusions

This study found that dairy products (milk, cheese) and samples from infected animals were contaminated with *Listeria species*. Molecular analysis confirmed the presence of *L. monocytogenes* and the virulence gene *prf A*. The study highlights the importance of *prf A* in the pathogenicity and biofilm formation of *L. monocytogenes*.

Footnotes

Authors' Contribution: Study concept and design, acquisition of data, analysis and interpretation of data and drafting of the manuscript was done by C. G.

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