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Detection of *Listeria monocytogenes* from fresh vegetables in Amol, northern Iran by PCR method

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Abstract

Bacterial contamination of food is an important threat to food safety, which negatively affects health, economy, and society's well-being. *Listeria monocytogenes* is one of the pathogenic bacteria whose contamination in food is continuously increasing. Fresh vegetables are one of the food groups that can serve as a reservoir for *L. monocytogenes* bacteria. The increased consumption of fresh vegetables in recent years has led to increased cases of gastrointestinal infections caused by *L. monocytogenes*. The purpose of this study was the molecular isolation and sequencing *of L. monocytogenes* bacteria in fresh vegetables offered in the markets of Amol and Babol cities, Mazandaran province, Iran. Coriander and oregano were obtained, and the samples were analyzed using polymerase chain reaction (PCR) to identify *L. monocytogenes* bacteria. Among the samples, five samples were positive for the presence of *Listeria* genus and two samples were positive for the presence of *L. monocytogenes*, which indicated the contamination of vegetables from Mazandaran with *L. monocytogenes*. Although the prevalence of *L. monocytogenes* in this study was small, the presence of any type of *Listeria* can indicate improper sanitary conditions in different stages of production until the supply of vegetables or the possible contamination of vegetables by other pathogens.

Introduction

Food contamination and spoilage are of great importance in the food industry and are a constant concern for the World Food Safety Organization because they negatively impact human health and create adverse consequences for the economic and welfare dimensions of society. Various factors cause the contamination and spoilage of food, including pathogenic bacteria, which are among the most significant issues in the field of public health. Today, despite improvements in food safety, the incidence of deaths and illnesses caused by the consumption of contaminated food is increasing daily and poses a constant threat to public health, as well as being an important obstacle to social and economic development worldwide (Elbehiry *et al.,* 2023).

One of the food groups that can act as a reservoir for pathogenic bacteria is fresh vegetables. Vegetables, as a valuable food group, are rich in minerals, vitamins, and fibers, which, due to their benefits and people's interest in consuming organic food to maintain a healthier lifestyle, now make up a significant portion of

the human diet. The increase in the consumption of fresh vegetables in recent years has led to a rise in cases of gastrointestinal infections and human epidemics. In fact, vegetables can be infected with various pathogenic bacteria at any stage from production to consumption, and can cause disease when consumed by humans (Gopalakrishnan *et al.,* 2019).

Fresh vegetables often do not undergo any processes to eliminate pathogens and carry a diverse range of human enteric pathogens. Intestinal bacteria, most of which cause gastroenteritis in humans, are very important for diseases caused by consuming raw vegetables (Al-Kharousi *et al.,* 2016). *Listeria monocytogenes* is one of the most dangerous intestinal bacteria that contaminate vegetables and is recognized by the World Health Organization (WHO) as one of the four main food pathogens. It is found in 0.6 to 1.9% of patients with gastrointestinal symptoms (Shamloo *et al.,* 2019).

L. monocytogenes is a common gram-positive, facultatively anaerobic bacillus. This bacterium can aggressively infect humans and animals, causing severe listeriosis that can lead to meningoencephalitis, septicemia, and fetal infection or abortion in pregnant women, with a mortality rate of 20-30% in these cases and among high-risk groups. These high-risk groups include pregnant women, infants, immunocompromised patients, and the elderly (Bongiovanni *et al.,* 2024).

Bacteria enter the human body after consuming contaminated food through the digestive system. This bacterium has adhesion proteins that bind it to the cells of epithelial tissues, allowing it to multiply and spread throughout the body (Osek *et al.,* 2022).

This bacterium is naturally present in agricultural environments such as irrigation source water, soil, fertilizers used in fields, and decaying plant material, making its presence in vegetables a constant risk and an important source of contamination. Additionally, this bacterium has the ability to grow at different temperatures, including freezing temperatures, and even at pH levels below 4.4, high salt content, low humidity, and in the absence of oxygen, which is relevant in clinical samples and foods. It is common (Osek and Wieczorek**,** 2022).

To date, the actual situation regarding the presence of *L. monocytogenes* in fresh vegetables from Mazandaran province has not been determined, and little information is available on this bacterium's presence. This study aimed to evaluate the level of qualitative and quantitative contamination of *L. monocytogenes* in fresh vegetables in the markets of Mazandaran province using the sensitive and rapid technique of polymerase chain reaction (PCR).

The detection of *L. monocytogenes* in food and a comprehensive study on it is a solution to prevent and address problems related to public health and safety, as the information provided in these studies can be a useful resource for making decisions in the field of food safety and for the food industry. It allows for the adoption of more effective control measures to manage *L. monocytogenes* in the food production and preparation process (Shamloo *et al.,* 2019).

Materials and Methods

Sampling

In this research, 148 samples of fresh vegetables, including 37 samples each of lettuce, watercress, chives, and oregano, were collected from different regions, specifically the cities of Amol and Babol. The samples were transported to the laboratory while maintaining the cold chain alongside ice. They were stored in a freezer at -40^{o}C until the intended tests were performed.

Preparation of samples and extraction of genetic material

To homogenize and prepare the samples, 10 g of each sample were homogenized in 5 ml of Trizol (Intron, Korea) for 20 minutes using a Stomacher (Instruments, France). Three hundred microliters of the homogenized product were used to extract the material, following bacterial genetics protocols (Baert *et al.,* 2008). The extraction of genetic material was carried out using protocol G from the commercial nucleic acid extraction kit by Intron (Korea), according to the manufacturer's instructions. The purity and concentration of DNA were determined by spectrophotometry (Intron, France) at wavelengths of 260 nm and 280 nm.

PCR test

To detect the genus *Listeria* in the samples, the forward primer LisUni1 and the reverse primer LisUni2 were used. Samples that tested positive for the genus *Listeria*, using the forward primer LM1 and the reverse primer LM2, were then used to detect the

species *L. monocytogenes* in PCR. Detailed information related to the primers, reaction components, and description of the temperature program was specified in Tables 1, 2, and 3, respectively.

Examining PCR products using the electrophoresis method

The products generated after PCR test for the presence of the fragments synthesized by the primers were placed on a 1.5% agarose gel. They were then detected and observed using a Gel documentation machine (Nogen, Iran) with Safe Stain staining (Pars Toos, Iran).

Sequencing and comparison with the gene bank

For sequencing, forward primer LisUni1 and reverse primer LisUni2 were used for two samples, and forward primer LM1 and reverse primer LM2 were used for one sample. The PCR product was produced with a volume of 50 µl and was synthesized from PCR products with an approximate size of 370 bp and 702 bp, respectively, and together with 15 µl of specific forward primers for one-way reading of the sequence, it was sent to Pishgam Biotechnology Company.

After receiving the sequence of PCR products, the obtained sequences were evaluated with the help of MEGA7 software. The sequences obtained in the forward reading using the link related to the portal of NCBI (National Center for Biotechnology Information of the United States) with the address http://www.ncbi.nlm.nih.gov/ Sequences with resources registered in the gene bank and were compared with the help of BLAST software. Additionally, the three obtained sequences were registered in the GenBank and the request to receive the accession number for the isolates was sent to the NCBI international portal.

Table 1. Sequence of primers.

Name	Sequence (3′to 5′)	Length (bp)	Reference
LisUni1	GCTGAAGAGATTGCGAAAGAAG	370	Chen *et al.,* 2017
LisUni2	CAAAGAAACCTTGGATTTGCGG		
LM1	CCTAAGACGCCAATCGAA	702	Chen *et al.,* 2017
LM2	AAGCCTTGCAACTGCTC		

Table 2. Volume and reaction components.

Reaction components	Volume (µl)
Nuclease-free water	8.5
PCR Mastermix	12.5
Forward Primer	1
Reverse Primer	1
DNA	2
Total	25

Table 3. Description of the temperature (Temp) program.

Steps	LisUni1 & LisUni2		LM1 & LM2		Cycle
	Temp	Time	Temp	Time	
Initial Denaturation	94	5 min	94	5 min	1
Denaturation	94	1 min	94	1 min	30
Annealing	56	1 min	54	1 min	
Extension	72	25 sec	72	45 sec	
Final Extension	72	10 min	72	10 min	1
Incubation	10	5 min	10	5 min	1

Table 4. Accession number of three sequences registered in GenBank.

Admission number GenBank	The type of species after Blast
PQ421123	*Listeria monocytogenes*
PQ421124	*Listeria monocytogenes*
PQ421125	*Listeria monocytogenes*

Results

Among the collected samples, 3.78% (five samples) and 1.35% (two samples) were infected with *Listeria* genus and *L. monocytogenes* species, respectively. Fig. 1 showed the representative bands obtained from the fragments synthesized by the primers in the PCR test to confirm the presence of the *Listeria* genus and *L. monocytogenes* species.

The results of determining the sequence of PCR products and the results of comparing the sequences with the gene bank

The nucleotide sequence related to the present study, after being registered in the gene bank, received the following acceptance numbers according to Table 4. The sequence alignment of the three products extracted from the PCR reading product of *L. monocytogenes* bacteria with the sequences recorded was shown in Fig. 2.

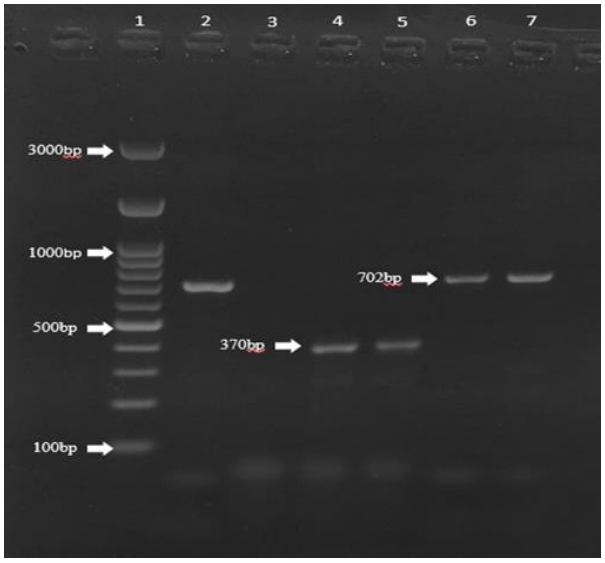

Fig. 1. Representative bands of vegetable samples infected with *Listeria* genus and *Listeria monocytogenes* species. Well 1, ladder100 bp; Well 2, *L. monocytogenes* positive control (ATCC19115); Well 3, negative control; Wells 4 and 5: positive samples of the genus *Listeria*; Wells 6 and 7: positive samples of *L. monocytogenes* species.

Discussion

Eating raw fresh vegetables exposes people to foodborne pathogens. Nowadays, with the allocation of a large amount of the human diet to fresh vegetables, the diseases and outbreaks of diseases caused by the consumption of vegetables have increased in recent years. Currently, the prevalence of food diseases due to such products is more than the prevalence of diseases related to other foods including beef, chicken, and seafood. Because they are often consumed fresh and no process is done on them to eliminate the pathogen (Santos *et al.,* 2023).

The pathogenic bacteria *L. monocytogenes* is one of the important pathogens transmitted through vegetables, which can be isolated from animals, soil, sewage, plants, etc. Vegetables can be infected with these bacteria at any stage from the farm to the table, and listeriosis has been caused by the consumption of vegetables by humans, which includes complications such as acute gastroenteritis and death (Osek and Wieczorek**,** 2022).

L. monocytogenes can survive in different conditions and multiply in harsh conditions such as refrigerator temperature, high salt concentration, and low oxygen content. Today, despite the increase in safety in the field of food, the death from bacterial poisoning caused by the consumption of vegetables infected with *L. monocytogenes* has been seen all over the world, which is a major concern for food producers, a constant threat to public health and an important obstacle to social development and economic worldwide (Shamloo *et al.,* 2019; Osek and Wieczorek**,** 2022).

Our study was the first investigation on the contamination levels of *L. monocytogenes* in fresh vegetables from different regions of Amol and Babol cities of Mazandaran province using PCR technique. To experiment, first, fresh vegetable samples were collected from the farms and vegetable shops of the province, they went through the stages of DNA preparation and enrichment, and then they were analyzed with the PCR molecular technique, which is a reliable method for identifying pathogenic pathogens.

The results of the prevalence of *L. monocytogenes* bacteria in this study showed that 3.78% of the samples were positive for *Listeria* genus and 1.35% were positive for *L. monocytogenes* species, which is consistent with the results of previous studies (Amajoud *et al.,* 2019) and showed *L. monocytogenes* is the contamination of vegetables in Mazandaran region. The presence of *L. monocytogenes* in food indicates recent exposure to fecal matter, either directly or indirectly, and indicates improper sanitary conditions at various stages of production to supply and the possibility of other intestinal pathogens that cause food-borne gastroenteritis and bacterial diarrhea.

Considering the threat that *L. monocytogenes* bacterium poses to human health through the consumption of vegetables, conducting studies to quantify the risks attributed to the consumption of fresh vegetables infected with *L. monocytogenes* and to increase epidemiological information and extensive study about them, to reduce and prevent of the diseases related to consumption of fresh food with the help of observational study designs along with molecular typing test methods in realizing the goal of identifying the bacteria that cause human infectious diseases that are related to the consumption of fresh food is of great importance. These studies can be a useful source for making decisions in the field of food safety and allow the food industry to adopt more effective control measures to control *L. monocytogenes* in the food production and preparation process (Koutsoumanis *et al.,* 2020).

Fig. 2. Alignment of the sequence extracted from the PCR product reading of *Listeria monocytogenes* with the sequences registered in the gene bank.

Therefore, considering the contamination of the examined samples, more monitoring, training of people involved in the production, public education, microbial evaluation of water sources and fertilizers, more extensive research in the field of vegetable contamination in our country, and improvement of rapid methods to identify such contaminants is recommended. In addition, the revision of national standards regarding the use of quality control of vegetables seems necessary for some reasons.

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Conflict of Interest

The authors do not have any potential conflict of interest to declare.

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