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A survey of Listeria monocytogenes and its virulence factors in vegetable salads and fresh vegetables in Tehran, Iran

Maryam Shojaei Zinjanab[a], Masoumeh Douraghi[a,b], Mohammad Mehdi Soltan Dallal[a,c*]

[a] *Department of Food Microbiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.*
[b] *Division of Bacteriology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.*
[c] *Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Fruits and vegetables have high potential of contamination with pathogenic organisms such as Listeria monocytogenes. Illnesses and outbreaks due to consumption of contaminated fresh products have been increased in recent years. So the aim of this study was to investigate Listeria monocytogenes in vegetable salads and fresh vegetables. Identification carried out using culture method according to Food and Drug Administration (FDA) protocol. PCR assay also used to confirmation of isolates as well as recognition of Hly and actA virulence factors. One hundred vegetable salads and 100 fresh vegetables obtained from three regions under supervision of Tehran University of Medical Sciences, including Islamshahr, Shahr-e-Rey and south of Tehran city. Culture method identified 24% Listeria and 0.5% L. monocytogenes in 200 samples. Confirmation of isolates with PCR resulted in 12% Listeria and 0.5% L. monocytogenes. The only L. monocytogenes was obtained from a ready to eat (RTE) mixed leafy vegetable. PCR assay also recognized presence of Hly and actA virulence factors in isolated L. monocytogenes. The isolated L. monocytogenes was resistant to Ampicillin, Oxacillin and Chloramphenicol, susceptible to Gentamicin, Penicillin G, Erythromycin, Tetracycline, Vancomycin and Trimethoprim sulfamethoxazole and moderate resistant to Methicillin and Doxycycline. Furthermore, poor microbial quality of studied samples was revealed in terms of total microbial count, Enterococcus count and Escherichia coli contamination. Microbial quality assays was based on Iran national standards. About 76.5% of 200 samples exceeded allowed limits in at least one of three mentioned parameters.

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1. Introduction

Fruits and vegetables are one of the popular foodstuffs around the world and their consumption is on the rise. On the other hand, illnesses and outbreaks arising from them have increased in the recent years (1,2). Currently, outbreaks due to such products account for more diseases than outbreaks linked to beef, poultry and seafood (3). In particular, consumption of fresh products without much

One of the most important pathogens associated with vegetables is Listeria monocytogenes (4). It is a gram positive rod-shaped bacteria which can be isolated from animals, soil, sewage, plants, etc (5,6). Listeria monocytogenes can cause listeriosis which encompasses gastroenteritis to severe complications (7). As Listeria can survive and proliferate in harsh conditions such as refrigeration temperatures, high salt concentrations and low oxygen contents, it has become a major concern for food manufacturers (8,9). Furthermore, this pathogen has been one of the most

* Corresponding author. Tel.: +98-21-88992971
E-mail address: msoltandallal@gmail.com

important agents of product recalls (10). Although L.monocytogenes is the main pathogenic species, presence of any kind of Listeria can be indicative of poor sanitary conditions or potential presence of pathogen (11). So this study was performed to investigate Listeria monocytogenes in vegetable salads served in restaurants and delicatessens under surveillance of Tehran University of Medical Sciences, also in fresh vegetables offered in markets of Tehran city. Microbial quality of samples also examined in terms of total microbial count, Enterococcus count and Escherichia coli presence.

2. Materials and methods

2.1. Sampling

A total of 200 vegetable salad and fresh vegetable samples were collected from May to October 2014. 100 salads obtained from restaurants or delicatessens of three regions under supervision of Tehran University of Medical Sciences, including Islamshahr, Shahr-e-Rey and south of Tehran city, by Tehran deputy of health. 100 fresh vegetables of 10 kinds, consist of chopped spinach, green onion, ready to eat (RTE) mixed leafy vegetables, chopped Kuku (a Persian dish) vegetable, green pepper, mushroom, broccoli, wheat sprout, mung bean sprout and basil, also gathered. 10 basil samples prepared by Tehran deputy of health from Kebab retail shops and the rest purchased from markets of Tehran city, 10 samples of each one.

2.2. Listeria detection by biochemical tests

Listeria detection protocol released by Food and Drug Administration (FDA) was chosen as the reference method. For this purpose, 25 gr of each sample was added to 225 ml of Listeria enrichment broth (Difco) and incubated at 30°C for 48 h. A loopful of the enriched broth was streaked onto Palcam agar (Merck), incubated at 35°C for 24-48 h and then examined for listeria colonies based on colony morphology, gram staining, catalase and motility tests. Colonies confirmed as listeria were evaluated for sugar fermentation using rhamnose, mannitol, xylose and esculin and also hemolytic activity using blood agar medium.

2.3. Polymerase chain reaction (PCR)

This method was used for confirmation of biochemical tests of Listeria and L.monocytogenes isolates. It was also to detect Hemolysin (hly) and ActA virulence factors in isolated L.monocytogenes.

2.4. DNA extraction

DNA extracyion was carried out based on an article with some change (12). A microtube containing 1 ml sterile distilled water and L. monocytogenes isolate cultured on Tryptic soy yeast extract agar was heated in boiling water bath for 10 min and centrifuged at 4000 rpm for 5 min. The supernatant with equal volume of chilled ethanol was centrifuged at 12000-13000 rpm for 10 min. The supernatant was discarded and elution with ethanol and centrifuging repeated once again. The supernatant was discarded again and 50µl sterile distilled water added after drying the pellet at about 45°c.

2.5. Primers

16srRNA and Hemolysin (hly) genes were used to confirm Listeria genus and monocytogenes species, respectively. The latter gene is considered as a virulence factor in addition to use for species identification. ActA was the other virulence factor which was detected only in isolates confirmed as L. monocytogenes. Applied primers are shown in Table 1 (13-15).

2.6. PCR conditions

PCR assay was carried out in 20 µl reaction mixture consisting of 15.2 µl distilled water, 2 µl 10X PCR buffer, 0.4 MgCl2, 0.6 µl dNTPs (Cinagen,Iran) , 0.2 µl Taq

Table 1. Gene targets, annealing temperature, PCR amplicon sizes 111and primer sequences in the PCR assay

Gene	Primer	annealing	size (bp)	Sequence
16srRNA	Lis-F	47.5	863	TAAGAGTAACTGCTTGTCCCT
	Lis-R	56.5		GAGTTGCAGCCTACAATCCGA
Hly	Hly-F	50.8	702	CCTAAGACGCCAATCGAA
	Hly-R	53.8		AAGCGCTTGCAACTGCTC
ActA	actA-F	56.2	618	ACCGCCTCCAACAGAAGATG
	actA-R	55.0		GGATTACTGGTAGGCTCGGC

DNA polymerase (Cinagen,Iran), 0.3 µl of each primer (Takapouzist,Iran) and 1 µl DNA extract. Pre-denaturated mixture at 94°c for 2 min, was subjected to 35 cycles of 94°c for 30 sec, 51 or 56°c (51°c for 16s rRNA, 56°c for hly and ActA) for 30 sec, 72°c for 30, followed by a cycle at 72°c for 10 min. PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and observed using gel documentation system (gel doc).

2.7. Antimicrobial susceptibility test

Disc diffusion method was used based on Clinical and Laboratory Standards Institute (CLSI) guideline(16) using Ampicillin, Gentamicin, Trimethoprim sulfamethoxazole, Methicillin, Erythromycin, Tetracycline, Doxycycline, Oxacillin, Penicillin G, Chloramphenicol and Vancomycin discs (Mast). For this purpose a dilution equal to 0.5 McFarland standard was prepared from L. monocytogenes isolates. Dilution was cultured on mueller hinton agar plates and antibiotic discs were located on the plates. Incubation was performed at 37 for 16-18 h and finally halo around discs were examined.

2.8. Microbial quality assays

Methods used for total microbial count, Enterococcus count and detection of E coli were based on Iran national standards number 5272, 2198 and 2946 (17-19). According to Iran national standard number 10082 (20) and also Iran's Food and Drug administration guidelines, allowed limits were considered 1×10-6 CFU/g for total microbial count, 1×10-2 CFU/g for Enterococcus count and lack of E coli in 1 gram of samples.

3. Results

3.1. Phenotypical and biochemical analysis of Listeria

Overall, 48 (24%) Listeria isolates were detected by phenotypic tests in 200 examined samples from which, 15 isolates (7.5%) were belonged to salads and 33 isolates (16.5%) to vegetable samples. From 15 isolates of 100 salad samples, 5 isolates were for Islamshahr, 5 isolates for Shahr-e-Rey and 5 for south of Tehran, so the prevalence of Listeria genus was equal in three regions. Among 100 vegetable samples, 8 mixed leafy vegetables (8%), 5 spinaches (5%), 5 broccolis (5%), 4 basils (4%), 4 mushrooms (4%), 4 peppers (4%), 2 Kuku vegetables (2%) and 1 green onion were contaminated with this bacteria. Two contaminated basils were prepared from Shahr-e-Rey, 1 from Islamshahr and 1 from south of Tehran. Listeria wasn't isolated from sprouts. Differential tests (sugar fermentation) identified two species of Listeria. One of the 48 Listeria isolates identified as monocytogenes with the exception that its hemolytic activity was negative (so CAMP test didn't performed). The only L. monocytogenes was obtained from an RTE mixed leafy vegetable. All other isolates (47 ones) had sugar fermentation exactly similar to Listeria grayi (mannitol positive, rhamnose negative, xylose negative and esculin positive) (21) but unlike grayi, all of them had hemolytic activity of beta type. As grayi is the only mannitol positive Listeria species, these isolates couldn't be identified using biochemical tests. Frequency distribution of Listeria and L. monocytogenes contaminated samples using culture method is demonstrated in figure 1. Table 2 also

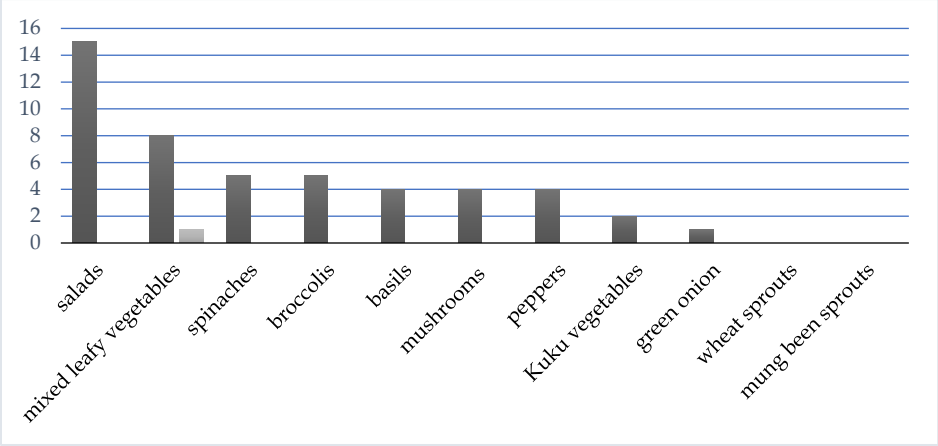

Figure 1. Frequency distribution (%) of contamination by Listeria and L. monocytogenes in salad and vegetable samples using culture method

Table 2. Sugar fermentation and hemolysis of L. monocytogenes, L. grayi and isolates

	Monocytogenes	Grayi	Listeria like isolates
Mannitol	-	+	+
Xylose	-	-	-
Rhamnose	-/+	variable	-
Esculin	+	+	+
Hemolysis	β	negative	β

indicates differences of sugar fermentation and hemolysis between monocytogenes, grayi and our isolates (21).

3.2. Polymerase chain reaction (PCR) analysis of Listeria

PCR of 16srRNA gene was performed on 48 biochemically detected Listeria isolates to confirm the genus. Overall 24 isolates were confirmed in this method which 16 of them were related to vegetables and 8 of them to salads.

Isolates confirmed as Listeria, were subjected to hly gene PCR. This gene was present only in the isolate recognized biochemically as monocytogenes and as previously mentioned, had been detected in an RTE mixed leafy vegetable. Therefore, this isolate confirmed as monocytogenes. However, prevalence of Listeria and L. monocytogenes in 200 examined samples achieved 12% and 0.5%, respectively. In other words, L. monocytogenes was detected in 1% of fresh vegetables and none of salads. PCR of actA gene also carried out for L. monocytogenes isolate and its presence was approved. Among 8 Listeria isolates obtained from salads, 4 isolates were for Shahr-e-Rey, 4 for south of Tehran and no Listeria was isolated from Islamshahr salads. The contaminated basil had been sampled from Shahr-e-Rey. Frequency distribution of Listeria and L. monocytogenes contaminated samples using PCR assay is shown in figure 2. Table 3 indicates frequency distribution of Listeria and L. monocytogenes and 3 mentioned genes in examined samples.

Table 3. Frequency distribution of Listeria and L. monocytogenes genes evaluated in PCR assay

	Vegetables	salads
16srRNA	16	8
Hly	1	0
actA	1	-*
Listeria	16	8
L. monocytogenes	1	0

* actA is only detected for hly positive isolates (L. monocytogenes)

3.3. Antimicrobial susceptibility

The antimicrobial susceptibility of one isolated L. monocytogenes is demonstrated in table 4.

3.4. Microbiological quality

This study revealed poor microbiological quality of samples, in terms of total microbial count, Enterococcus count and E coli presence. Overall, 76.5% of 200 samples exceeded allowed limits in at least one of three mentioned parameters. Contamination rate of vegetables and salads were 82% and 71%, respectively.

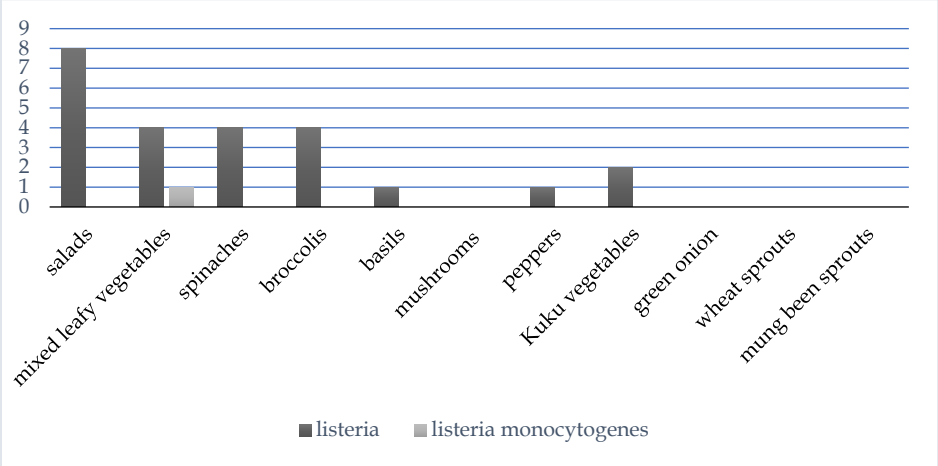

Figure 2. Frequency distribution (%) of contamination by Listeria and L. monocytogenes in salad and vegetable samples using PCR assay

Table 4. Antimicrobial susceptibility of isolated L. monocytogenes

Resistance	Resistant	Mod resistant	Susceptible
Antibiotic	Ampicillin Oxacillin Chloramphenicol	Methicillin Doxycyclie	Gentamicin Penicillin G Erythromycin Tetracycline Vancomycin Trimethoprim sulfamethoxazoe

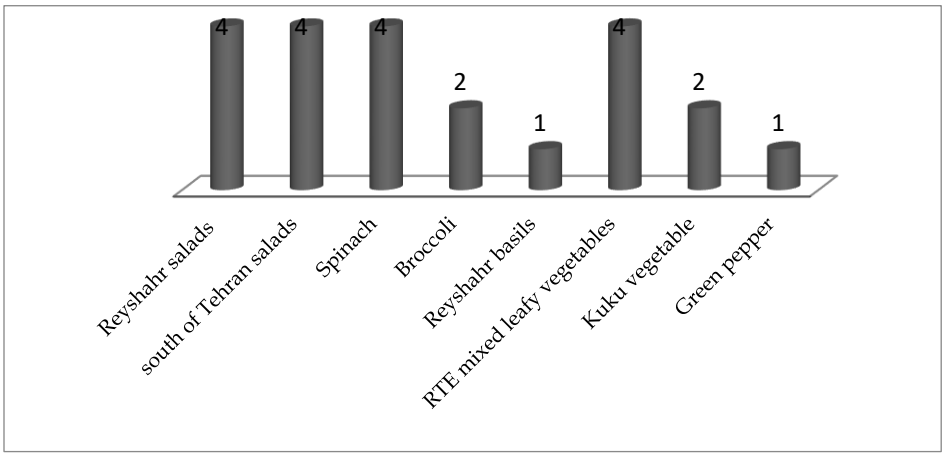

Figure 3. Frequency distribution of listeria contaminated samples with low microbiological quality

3.4. Microbiological quality

This study revealed poor microbiological quality of samples, in terms of total microbial count, Enterococcus count and E coli presence. Overall, 76.5% of 200 samples exceeded allowed limits in at least one of three mentioned parameters. Contamination rate of vegetables and salads were 82% and 71%, respectively.

3.5. Microbiological quality of Listeria positive samples

The Mixed leafy vegetable sample with L. monocytogenes contamination, had total microbial count and Enterococcus count higher than allowed, but it wasn't contaminated with E coli. Among 23 samples contaminated with other Listeria species, 69.56% and 91.30% exceeded acceptable limits of total microbial count and Enterococcus count, respectively. E coli contamination rate was 13.04% in these 23 samples which correlated to one mixed leafy vegetable, one Kuku vegetable and one salad of south of Tehran. Indeed, these samples were contaminated to three mentioned microbial parameters, as well as Listeria. Broccolis with better quality level compared to other vegetables, had listeria contamination yet. There were two broccoli samples in compliance with microbiological acceptable limits but contaminated with Listeria. Figure 3 demonstrates frequency distribution of 22 Listeria contaminated samples with low microbiological quality.

4. Discussion

Prevalence of Listeria species was obtained 48 (24%) by biochemical analyses. The highest level of contamination in fresh vegetables belonged to mixed leafy vegetables followed by spinaches and broccolis. The only L. monocytogenes (0.5%) was obtained from an RTE mixed leafy vegetable. The isolated L. monocytogenes had no hemolytic activity. The sugar fermentation of other isolates (47 ones) was according to Listeria grayi but all of them had beta hemolytic activity unlike grayi. As grayi is the only mannitol positive Listeria species, these isolates couldn't be identified using biochemical tests. Previously, unusual Listeria phenotypes have been observed in some studies. For example, L. monocytogenes strain ATCC 15313 is nonhemolytic, CAMP negative and nonmotile. Hemolytic L. Innocua and nonhemolytic L. seeligeri

have also been identified (10). Therefore, it is likely that these isolates are Listeria species with unusual phenotypes. On the other hand, some novel Listeria species have been introduced which do not conform to six well known Listeria species, such as our isolates. These recent species are marthii, rocourtiae, weihenstephanensis, fleischmannii, floridensis, aquatic, cornellensis, riparia and grandensis (22-26). Thus, another possibility is that the isolates are novel species that should be studied.

PCR assay identified 24 (12%) Listeria species. The highest level of contamination in fresh vegetables in this method also belonged to spinaches, broccolis and mixed leafy vegetables. This method also confirmed the only monocytogenes isolate. This isolate had hly and actA virulence factors. Difference of detection rate between biochemical and molecular tests can be due to ability of selected primers in identifying or higher efficiency of molecular methods. However, Isolates weren't recognized by PCR can be gram positive, catalase positive and motile bacteries which are highly similar to Listeria or can be Listeria strains with genetic alterations that could not be detected by 16sr RNA PCR.

Despite possessing hly virulence factor, isolated L. monocytogenes didn't have hemolytic activity on blood agar, as noted above. Lack of hemolytic activity of it can be due to expression of hemolysin, poor culture condition or changes or deletion of hly or prfA genes (27). Inactivation of hly gene in mouse model studies has led to loss of hemolytic activity, obstruction of escape from phagosomes and complete non-pathogenicity (28).

Previously, other researchers have also studied prevalence of this pathogen in foodstuffs in Iran. For instance, 13.3% Listeria and 6.6% monocytogenes were identified in meat and meat products, 8.5% Listeria and 3.0% monocytogenes in ready to eat foods of restaurants and no L. monocytogenes in cheese samples (29-31). In a study in Spain on 191 ready to eat and processed vegetable foods, 4.19% and 10.47% L. monocytogenes were identified using culture method and multiplex PCR, respectively (32). These results are higher than ours despite the fact that Spain is a country with higher quality level. However, prevalence of L. monocytogenes in a previous study in Spain on fresh vegetables and sprouts was 0.7% which is consistent with 0.5% L. monocytogenes in our samples. Sprouts weren't contaminated with this pathogen, as our study (33). In another research carried out in Malaysia on fresh vegetables by MPN-PCR method, prevalence of Listeria was 23.6 and 34.2% and prevalence of L. monocytogenes was 16.7 and 31.5% in two hypermarkets (34). Investigation of 200 vegetable samples resulted in L. monocytogenes prevalence of 10% in India (35). The data of these two researches are also higher than our results which can be due to different climates. A survey performed in Brazil identified 0.6% L. monocytogenes in vegetable salads (in 1 sample) which is highly alike to our data (36). In another survey in Portugal, one spinach sample (0.66%) contained L. monocytogenes that is in similarity with our results (37). In a research in Chile on raw and cooked vegetables, prevalence of L. monocytogenes was 13.9, 5.6 and 11.1% in manually prepared salads of three markets and 0% in salads prepared industrially (38). So, prevalence of L. monocytogenes in manually prepared salads was so higher than our results. However, these statistics suggest that industry has played an important role in improving the quality of such foods in this country. Because of very low rates of L. monocytogenes in our samples and lack of that in manually prepared salads, definite statements can't be made about the effect of food industry on elimination of this pathogen in vegetables. It should be noted that other Listeria species ware present in manually prepared salads but not in industrially ones in our research.

However, despite the low prevalence of L. monocytogenes in our study, we can not consider the vegetable and salad products as high quality because presence of other species of Listeria also indicates poor sanitary conditions or potential presence of monocytogenes (11). The most important microbial contamination sources are soil, feces, irrigation water, water used to apply fungicides and insecticides, fertilizers, dust, insects, animals, handlings, containers and instruments (2). Prevention of contamination by microorganisms is the responsibility of all people involved in the pre harvest, harvest and post harvest activities (39). Therefore, considering high contamination rate of examined samples, more supervision, education of individuals involved in production, public education, microbial assessment of water sources and fertilizers, more extensive researches in the field of food contamination in our country and improvement of rapid methods for the identification of such contaminants are recommended. Furthermore, revision of national standards used to control of vegetables' quality seems necessary due to some existing defects, as lack of microbial parameters in some published ones (40-44). Restricted number of brands available in the market was the limitation of this research, so statistics may vary by development of new production units.

5. Conclusion

Vegetables and salads of Tehran, Iran, have potential to transfer pathogenic microorganisms such as Listeria monocytogenes to human body. On the other hand microbial quality of such products is very poor. So, it is important to have a appropriate washing and disinfection stage before consumption.

Conflict of interest

The authors have no conflict of interest.

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