Integrated Bacteriological and Molecular Surveillance of *Listeria monocytogenes* in Food and Environmental Samples from Nineveh, Iraq

Ali Adnan younis [1] Hala A. Salih [2] Taha mohammed [3]

[1,2,3] Food Science Department, College of Agriculture and Forestry, University of Mosul, Nineveh, Iraq.

[1]Email: ali.25agp74@student.uomosul.edu.iq

[2]E-mail: halahadi@uomosul.edu.iq

[3]E-mail: tahataqi@uomosul.edu.iq

Abstract.

The study aimed to investigate the occurrence of *listeria monocytogenes* in selected food samples collected from Nineveh, Iraq, 100 food samples were collected, including raw milk, Arab cheese, canned food sausages, local meats, and leafy green vegetables. Suspected samples were divided into 6 PCR and 13 VITEK samples using an integrated diagnostic approach, Detection was performed though a comprehensive sequential workflow combining conventional culture methods, chromogenic media, automated identification using the (VITEK 2 compact system) and molecular confirmation by PCR targeting the *16S rRNA* and *hlyA* genes.

No confirmed isolates of Listeria monocytogenes were detected of *Listeria monocytogenes* were detected among the examined samples during the study period, the consistency of negative findings across all diagnostic platforms, together with successful amplification of the positive control strain, supports the analytical reliability of the methods used and indicate that negative results were unlikely to be technical limitations.

These findings suggest a low detectable occurrence of *Listeria monocytogenes* under the conditions of the present study, nevertheless, continued molecular surveillance remains essential to monitor potential contamination risks and to support food safety assessment in the local production environment.

Keywords: Listeria monocytogenes, PCR, VITEK, Microbial test

Introduction

Foodborne diseases remain a major global public health concern, posing substantial and economic burdens in both developed and developing countries, food safety is particularly important in urban environments, where variation in climate, handing practices, storage conditions and distribution systems may influence the likelihood of microbial pollution, Among foodborne bacteria pathogens, *Listeria monocytogenes* is considered one of the most clinically significant agents due to its ability to cause LLO, a severe disease associated with hospitalization, septicemia, meningitis, abortion and relatively high mortality rates in susceptible population, particularly pregnant women, neonates, older adults

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and immunocompromised individuals [1].

Listeria monocytogenes remains a major target of microbiological surveillance because of high pathogenicity and exceptional ecological adaptability, the bacterium is capable of surviving and multiplying under condition that limit the up growth of many other foodborne organisms , including refrigeration temperatures and elevated salt concentration, it can persist in a wide variety of food matrices, particularly dairy products, raw animal derived foods and ready to eat products, in addition, it ability to form biofilms on food contact surfaces significantly enhances its resistance to cleaning and sanitation procedures thereby contact surfaces significantly enhances it resistance to cleaning and sanitation procedures, thereby facilitation it long term persistence in food processing and handing environments [2].

The detection of *Listeria monocytogenes* in food samples remains analytically challenging, particularly when bacterial loads are low or when the organism is exposed to environmental stress during food storage and handling, for the reason, accurate diagnosis often requires the integration of multiple laboratory approaches rather than reliance on a single detection technique, conventional bacteriological culture methods remain essential for the recovery of viable organisms, however biochemical assays provide preliminary phenotypic characterization, chromogenic selective media further improve colony differentiation through the characteristic color development , allowing more efficient screening of suspected isolates [3].

Additionally, automated identification systems such as the (VITEK 2 Compact) can facilitate rapid biochemical identification and improve laboratory workflow, although complementary confirmatory methods remain necessary for the accurate identification of *Listeria monocytogenes* particularly in atypical isolates [4].

The molecular level, polymerase chain reaction PCR has become an important confirmatory tool because of its high analytical sensitivity and specificity, detection of the hlyA virulence gene is widely used for the specific identification of *Listeria monocytogenes* and provides strong of its pathogenic potential [5].

Accordingly, the present study aimed to evaluate the microbiological safety of selected local food products in Mosul, Iraq with particular emphasis on the detection and identification of listeria monocytogenes, by integrating conventional biochemical assays, chromogenic selective culture media , automated identification using the VITEK 2 Compact system, and PCR targeting the *16S rRNA* and *hlyA* genes, this study sought to assess the possible occurrence of this pathogen and to support the strengthening of local food safety monitoring and surveillance efforts.

Materials and Methods

Isolation and identification of *Listeria monocytogenes*

Total of (100 samples) were collected from various locations across Mosul , Iraq these included 40 samples of raw milk,10 of local Arab cheese, 10 of sheep yogurt,5 of local meat,5 of pastrami, 5 of sausage, 5 of luncheon meat, and 12 of paper samples, as part of a cross sectional survey, the sampling framework was designed to cover

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representative food categories commonly available to consumers , the samples were obtained under sterile conditions, transported to the laboratory in cooled containers, and analyzed within 24 h to ensure microbiological.

The search for *Listeria monocytogenes* was performed using conventional bacteriological as described by [6]. Briefly, 25 gm of each sample was homogenized in peptone water using a BagMixer, incubated at 37 °C For (24 h) and subjected to serial dilution and streaking, hemolytic activity was assessed on blood agar, followed by G staining and catalase, oxidase testing, additional enrichment was carried out using TSA and TSA according according to [7]. and BHI enrichment as described by [8]. Selective enrichment using Fraser and Half Fraser broth was performed following [9]. and cold enrichment at 4 °C was applied to improve bacterial recovery according to [10] Oxford agar blood agar and TSA were used during the isolation procedures.

Chromogenic identification

Selective enrichment was additionally performed using Fraser broth followed by subculture on Hicrome Agar as a chromogenic medium, after incubation at 36-37 °C for 24 h**,** colonies were examined for characteristic color development according to the manufacturer 's guidelines, colonies showing typical chromogenic reactions were selected for further confirmation according to [11].

Automated identification using VITEK 2 Compact

Pure isolates obtained from repeated subculturing were subjected to confirmatory identification using the VITEK 2 system(bioMérieux, France), fresh colonies 18-19 h were suspended in sterile saline and adjusted to 0.5 McFarland turbidity using a densitometer, the standardized suspension was loaded into G-positive identification cards and processed according to the manufacturer 's instructions, the system automatically monitored biochemical reactions and generated identification results based on it internal database, only high confidence identifications were accepted for final confirmation according to [4].

Molecular Detection by PCR

Molecular detection of Listeria monocytogenes was performed by conventional polymerase chain reaction (PCR) targeting the universal 16S rRNA gene (approximately 1500 bp) and the hlyA virulence gene (456 bp) using previously published primer sets (Table 1). Genomic DNA was extracted from the bacterial isolates using the AddPrep Bacterial Genomic DNA Extraction Kit (AddBio, Korea) according to the manufacturer's instructions. DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260/280 nm before amplification. PCR was carried out in a final reaction volume of 25 µL containing 12.5 µL of 2× PCR Master Mix (AddBio, Korea), 1 µL of each forward and reverse primer (10 pmol/µL), 2 µL of template DNA, and nuclease-free water to complete the final volume. Amplification was performed using a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: an initial denaturation at 95°C for 10 min, followed by (35)cycles of denaturation at 95°C for 45 s, annealing at 55°C for the 16S rRNA gene and 57°C for the hlyA gene for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on 1.5% agarose gel prepared in 1× TBE buffer and stained with GelRed. Electrophoresis was carried out at

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80 V for 60 min using a 100-bp DNA ladder as the molecular size marker. PCR products were visualized and photographed using a gel documentation system [12].

Table (1) primer sequences used for pcr amplification of the 16s rRNA and hlyA genen

الجين الهدف	البادئ	تسلسل البادئ (3′–5′)	حجم الناتج (bp)	درجة الحرارة التمائل (°C)
16S rRNA	Luni-F	TTAGTGGCGGACGGGTGA	~1500	55
	Luni-R	GGTATCTAATCCTGTTTGCTC		
hlyA	hlyA-F	GCAGTTGCAAGCGCTTGGAGTGAA	456	57
	hlyA-R	GCAACGTATCCTCCAGAGTGATCG		

Results and Discussion

All examined samples n 100 were subjected to a comprehensive microbiological workflow integrating conventional culture, chromogenic differentiation, automated biochemical identification and molecular, preliminary screening on Blood and Oxford agar yielded several colonies with partial morphological resemblance to *Listeria monocytogenes*; However, subsequent G staining and biochemical characterization did not assert the presence of *Listeria monocytogenes*, none of the tested exhibited the complete phenotypic profile typically associated with the pathogens.

the agarose gel electrophoresis result for amplification of the *16SrRNA* gene, as illustrated in Figures 1 and 2, revealed clear and consistent band across all samples at the expected molecular sizes, indicating successful the DNA extraction and efficient PCR performance, this confirms the suitability of the samples for molecular analysis and proposes that subsequent negative results cannot be attributed to technical failure, these findings are consistent with previous studies demonstrating that amplification of the highly conserved *16SrRNA* gen is widely used for reliable bacteria identification and phylogenetic analysis [12].

Table (2) Sample type and degree of growth on selective media

أنواع العينات	عدد العينات	كرموجيني		اكسفورد	
		سالبة	موجبة	سالبة	موجبة
حليب خام	40	35	5	30	10
جبن عرب محلي	10	8	2	5	5
لبن غنم	10	7	3	6	3
لبن بقري	8	8	0	6	2
لحم محلي	5	4	1	3	2
باسطرمة	5	3	2	3	2
موصج	5	3	2	4	1
لشون	5	2	3	4	1
المجموع الكلي	88	70	18	61	26

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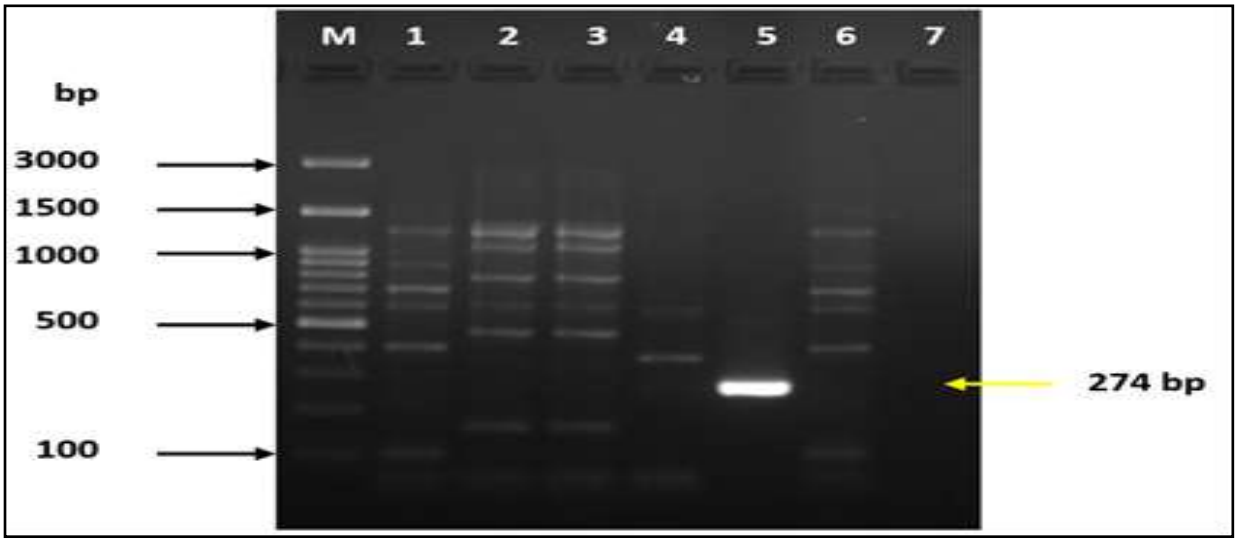


Figure 1. Electrophoresis results for *16SrRNA* gen amplification, with clear bands in all samples

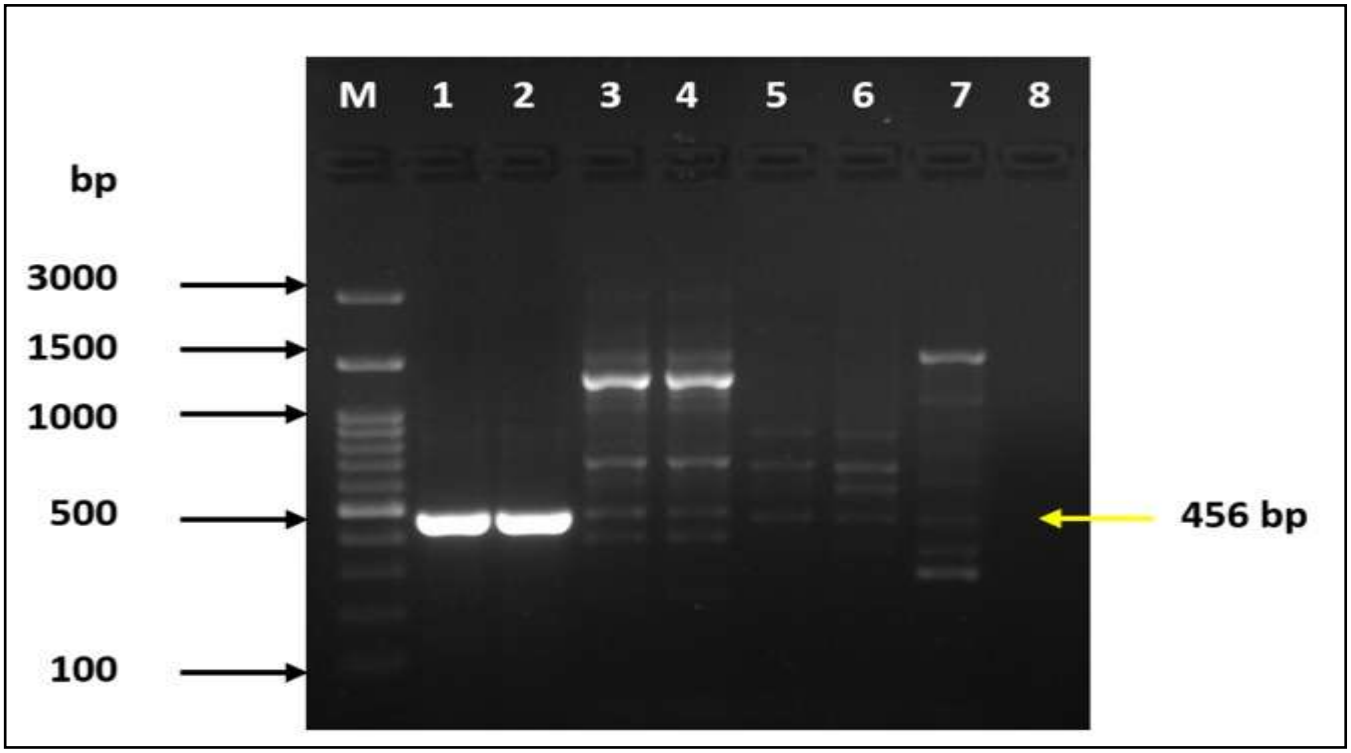


Figure 2. additional results of 16S RNA gen.

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In contrast, amplification of the virulence associated hylA gen, as shown in Figure 3, demonstrated the absence of bands in most isolates at the expected size(456 bp), while a distinct band was observed in the positive control, confirming the efficiency of the PCR reaction, the absence of amplification proposes that these isolates are unlikely to possess the virulence factor associated with *Listeria monocytogenes*, additionally, faint or non-specific bands observed in some lanes may be attributed to non-specific primer binding or primer dimer formation, which are commonly reported in conventional PCR assays and do not affect the overall interpretation of results, these findings are consistent with previous studies demonstrating that PCR targeting virulence associated gens such as hlyA provides a sensitive and reliable approach for the detection of *Listeria monocytogenes* in food samples [13].

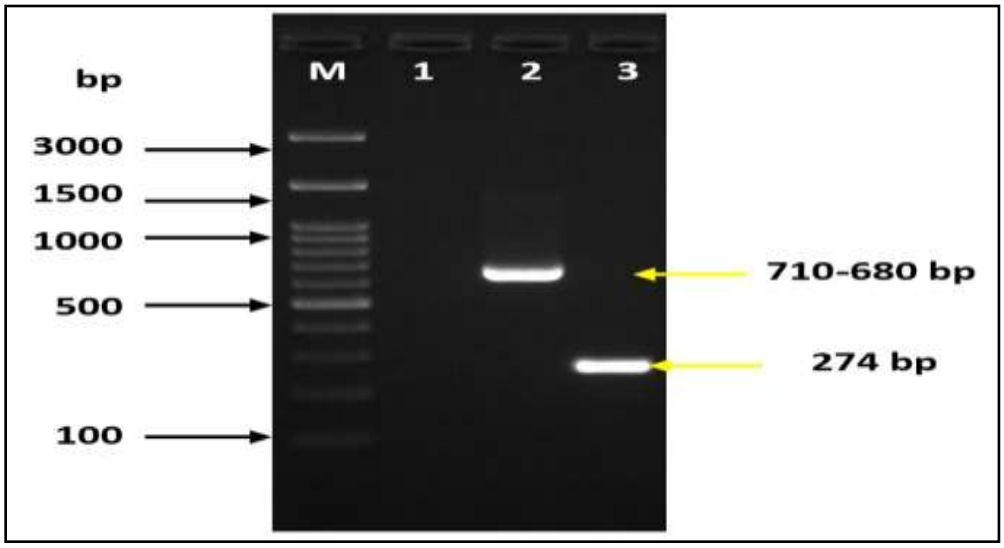


Figure 3. Electrophoresis results for amplification of the hlyA gen and the appearance of the clearly positive control sample.

From an automated diagnostic perspective, the results obtained using the VITEK 2 compact system revealed a clear variability in the identification of bacteria isolates, as presented in Table 1, several isolates were identified as bacterial species other than *Listeria monocytogenes*, including *Rothia kristinae, Granulicatella adiacens* and Enterococcus faecalis, in addition to some isolates were reported as unidentified organisms, while others failed to yield conclusive results due to operational limitations, such as insufficient incubation time, these findings proposes that certain bacteria present in the samples may share morphological and biochemical characteristics with *Listeria monocytogenes*, which could explain their initial classification as presumptive isolates during primary screening, this observation is consistent with previous studies indicating that automated identification systems may encounter limitation when differentiating between closely related bacteria species, particularly those exhibiting similar phenotypic profiles [4].

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Table (3) Bacteria diagnosis results using VITEK 2 compact

No.	Treatments	VITEK 2	Observations
1	**Sample 1**	Rothia kristinae	Physiologically similar bacteria
2	**Sample 2**	Granulicatella adiacens	Biochemically similar
3	**Sample 3**	Enterococcus faecalis	Gram positive
4	**Sample 4**	Unidentified organism	Not identified
5	**Sample 5**	No reading / insufficient incubation	Not read
6	**Pure comparative isolation**	Listeria monocytogenes (99%)	Confirmed

The one pure isolate showed a high identification confidence 99% as *Listeria monocytogenes* using the VITEK 2 compact system, as illustrated in Figure 4, this result was further confirmed at the molecular level using both the bacteria primer 16S rRNA and the virulence hen primer hlyA, where amplification products were observed at the expected molecular sizes in agarose gel electrophoresis, accordingly, this isolate can be considered a confirmed positive isolate, supported by the agreement between automated identification and molecular confirmation, rather than representing a discrepancy between diagnostic approaches, the molecular findings in this study are further supported by the use of primer sets that have previously been applied in local investigation of ovine LLO in Nineveh province, where *Listeria monocytogenes* was successfully detected in clinically infected animal samples, this supports the suitability of the selected molecular targets within the local epidemiological context [14].

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Figure 4. Documentation of the positive control isolate in VITEK 2 compact

The discrepancy between animal based studies and the findings of the present study may be attributed to differences in sample type and expected bacteria load, clinically infected tissues typically harbor higher bacterial concentrations, facilitating detection, whereas contamination in food samples is often low, intermittent, or absent under the examined conditions, this is consistent with previous reports indicating the challenges associated with detecting *Listeria monocytogenes* in food matrices with low bacterial loads[2], From an environmental perspective, moisture is considered one of the most important factors influencing the persistence and spread of *Listeria monocytogenes*, the bacterium tends to survive in humid environments and on contaminated surfaces, while reducing moisture levels may limit it persistence [15,16].

Accordingly, the relatively dry climatic conditions in the city of Mosul, Iraq, may represent one of the factors contributing to the reduced likelihood of bacterial survival along the local food chain, local dietary habits may also play an important role, as reliance on freshly prepared meals and reduced consumption of refrigerated ready to eat foods may decrease the risk of exposure to the pathogen, given that such foods are recognized as major sources of *Listeria monocytogenes* transmission [17].

Furthermore moreover, improved animal husbandry practices, along with enhanced hygiene, ventilation, and sanitation in production environments, may contribute to reducing the persistence of the bacteria in these settings (Chowdhury & Anand, 2023), with regard to leafy vegetables, the use of cleaner irrigation sources such as groundwater or fresh water, rather than stagnant water, may reduce the risk of contamination, Irrigation water quality plays a critical role in the dissemination of bacterial in agricultural environments [18].

The field observation from certain areas of Nineveh province have also indicated the occurrence of abortion cases of unknown etiology, particularly in rural regions located near water sources, these cases may be associated with the presence of

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pathogenic agents, including *Listeria monocytogenes*, though they are not always formally documented, this observation is consistent with the well - established epidemiological characteristics of the bacteria and its association with reproductive disorders and infectious diseases in both humans and animals [13].

environments more favorable for the survival of *Listeria monocytogenes* compared to relatively dry urban areas [19]. The based on above, the absence of *Listeria monocytogenes* in most of the examined food samples from the city of Mosul can be explained by the combined influence of several environmental and behavioral factors, including relatively dry climatic conditions, improved hygiene and animal production practices, and local dietary patterns, However, the presence of the bacteria in other areas, particularly rural regions, cannot be excluded, highlighting the importance of continued molecular surveillance and future epidemiological investigations.

Conclusion

The experiment utilized a mixed diagnostic method that involved the use of traditional culture, chromogenic media and automated identification of the VITEK 2 compact system, and molecular detection PCR targeting the (16S rRNA) and (hlyA) gen to investigate Listeria monocytogenes in the selected food and environmental samples in Nineveh, Findings revealed no confirmed

Beforehand studies have also shown that the persistence and dissemination of bacterium in the environment are influenced by multiple factors, including moisture, temperature, soil characteristics, and animal activity, these factors make rural

presence of *Listeria monocytogenes* in the examined samples, despite the application of multiple complementary detection methods, the consistency of negative results across all diagnostic platforms, together with successful amplification of the positive control, confirms the reliability and validity of the applied methodology, the absence of detectable isolates may be attributed to a combination of environmental and behavioral factors, including relatively dry climatic conditions, improved hygiene and animal production practices, local dietary patterns that favor freshly prepared foods over long term refrigerated products, However, these findings do not exclude the potentially presence of the pathogen in other settings, particularly in rural environments where ecological and management conditions may favor its persistence, therefore, continuous molecular surveillance and broader epidemiological studies are recommended to ensure food safety and to monitor potential contamination risk in the local production systems.

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