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Molecular characterization of virulence and antibiotic resistance genes and genotyping of *Listeria monocytogenes* isolated from cheese, beef, chicken, and milk

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Abstract

The current study was conducted to detect *Listeria monocytogenes*, associated antibiotic resistance and virulence genes possessed by these strains, and the genotype of identified isolates from milk, cheese, beef, and chicken. Accordingly, 203 samples (53 milk samples, 52 cheese samples, 48 beef samples, and 50 chicken samples) were collected from local markets in Al-Qadisiyah Province, Iraq. These samples were used for conventional and selective cultivation and biochemical studies. Eight isolates were molecularly detected using the PCR and sequencing based on the 16S rRNA gene. All the physically detected isolates were recruited for antibiotic resistance tests. Furthermore, all isolates were exposed to the detection of virulence genes, which included actin assembly inducing protein (*actA*), listeriolysin (*hlyA*), invasive associated protein (*iap*), internalin A (*inlA*), and phospholipase C (*plcA*). Random amplified polymorphism DND-polymerase chain reaction (RAPD-PCR) was utilized to genotype these isolates. The cultivation revealed the identity of 63 isolates (16 milk, 14 cheese, 16 beef, and 17 chicken). The molecular detection confirms the identity of the eight tested isolates. Various antibiotic and pattern resistance profiles were detected for the isolates, which included the highest resistance rates, which reached up to 100% and 94% in penicillin and ampicillin, respectively. The virulence genes that reached up to 94% were identified in all isolates. The sequencing findings demonstrated strong alignment with world isolates from the GeneBank. The data here reveal the vital presence of *Listeria monocytogenes* in food products, which pose high health risks to consumers.

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Introduction

The relationship between food intake and human diseases has been known since ancient times. Foodborne pathogens or contaminating agents include viruses, bacteria, and parasites. An incident of a foodborne illness is defined as two or more cases of similar diseases linked to eating a common food substance (1). Foodborne illness results from a pathogen ingested with food that finds a human niche and then, often, proliferates there or a toxigenic pathogen ingested with food that finds (and then produces a toxin in) a food niche (2). Foodborne illness is divided into either foodborne infection or foodborne intoxication. Foodborne infections have longer times between ingestion and presentation because of an incubation period. More than 200 different food-borne illnesses have been identified. The worst cases are in the very old, young, immunocompromised, and otherwise robust individuals exposed to many of a particular organism (3). The pathogenic bacterium that causes listeriosis is called *L. monocytogenes*. Listeriosis is a sporadic disease in humans and animals, with high rates of hospitalization and death, and

it is considered one of the most severe foodborne diseases (4). Of the 21 known species of *Listeria*, a small rod-shaped found virtually everywhere, only *L. ivanovii* and *L. monocytogenes* are pathogenic in mammals (5). Avoiding foodborne zoonoses is an excellent procedure for human health and creates demand for certified food. *Listeria* species emerged as a critical foodborne pathogen in Western countries around the mid-20th century, and both human and animal listeriosis have brought high costs to society and the food industry. Listeriosis has increased in Europe since 2008 (6). Although members of the *Listeria* genus evolved from a bacterium fed by decaying organic matter, their ability to find tolerance for abiotic stressors can be a key to their evolutionary success. *Listeria* stress tolerance allows it to make its way in food from contaminated food to the mammalian gastrointestinal tract. Upon entering the host, *L. monocytogenes* employs many sophisticated mechanisms to invade different eukaryotic cells, survive inside them, avoid host immune detection, and disseminate throughout the body's other organs (7). *L. monocytogenes* is a food-borne pathogen that primarily afflicts immunocompromised individuals and can provoke septicemia, meningitis, and fetal infection or abortion in infected pregnant women (8). *L. monocytogenes* can cause diseases in humans and farm animals, and it is one of the most important causes of food poisoning; therefore, it is urgently needed to develop targeted therapies to control the mortality. Consequently, the molecular and cellular pathogenesis of *Listeria* has become an attractive field of investigation (9,10).

The current study was performed to identify *Listeria monocytogenes* and its virulence antibiotic resistance genes and to genotype isolated strains from milk, cheese, beef, and chicken.

Materials and methods

Ethical approve

The study protocol was approved for the ethical procedures by the Committee of Research Ethics, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq, under the issue No. 4413 at 23/11/2023.

Samples and cultivation

Two hundred three samples (53 milk samples, 52 cheese samples, 48 beef samples, and 50 chicken samples) were collected from local markets in Al-Qadisiyah Province, Iraq. These samples were employed in conventional and selective cultivation and biochemical methods.

DNA extraction and *16S rRNA* gene identification and sequencing

The DNA of *L. monocytogenes* was extracted by employing the Wizard Genomic DNA Purification Kit (Promega, USA). All isolates were subjected to the kit protocol. The primers 27F: 5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R: 5′-GGTTACCTTGTTACGACTT-3′ were used (10-12). In total reaction, 50 µl, 10-100 ng DNA, 1.5 mM MgCl2, 2 µl dNTPs, each primer at 0.4 µM, and 2.5 U DNA polymerase were included in the PCR reaction. The conditions were initial denaturation (denaturation, annealing, and extension) and final extension at one cycle at 120 s-94 °C, 35 cycles at (60 s-94 °C, 90 s-55 °C, and 60 s-72 °C), and one cycle at 180 s-72°C. The PCR product was at 1500 bp. The electrophoresis runs a 1% agarose gel at 80-90 Volt for 60 mins. The products were visualized using GeneSys G: BOX EF2 (Syngene, USA). These eight PCR-purified products were sent out for sequencing (Bioneer, Korea). The phylogenetic tree was computed using NCBI websites and MEGA X software.

RAPD-PCR

All 63 isolates were subjected to RAPD-PCR in a total reaction of 50 µl, 0.5 µl RP1: 5'GGTGTGCTGT'3, 25 µl 10x master mix (EconoTaq®, Lucigen), 1.0 µl DNA, and H_2O to complete the volume. The conditions were initial denaturation (denaturation, annealing, and extension) and final extension at one cycle at 300 s-94 °C, 45 cycles at 60 s-94 °C, 60 s-35 °C, and 120 s-72 °C, and one cycle at 420 s-72°C, respectively. Agarose gel at 1% was performed for electrophoresis analysis. The products were screened using a UV-transilluminator imager followed by ImageJ.

Antibiotic resistance profile and multi-resistance index (MAR)

All isolates were exposed to antibiotic resistance tests using 10 antibiotics, which included 10 µg ampicillin (AM), 30 µg chloramphenicol (C), 10 µg gentamicin (GN), 15 µg erythromycin (E), 5 µg flucloxacillin (FL), 30 µg kanamycin (K), 10 µg penicillin (P), 10 µg streptomycin (S), 30 µg sulfisoxazole (ST), and 30 µg tetracycline (TE) (Oxoid, UK). The disk diffusion method (13) was followed using Muller-Hinton agar media. After 24 hrs, the zones of inhibition were read. Matyar *et al.* (14) employed a method for detecting the multi-resistance index (MAR). MAR = Number of resistant antibiotics / Number of antibiotics tested. MAR >0.2: indicates an isolation source with high usage of antibiotics. MAR≤ 0.2: indicates less usage of antibiotics (15).

Results

The cultivation findings revealed the identity of 63 isolates (16 milk, 14 cheese, 16 beef, and 17 chicken) (Table 1). The molecular detection confirms the identity of the eight tested isolates. The results were further confirmed using sequencing (Figures 1 and 2). Various antibiotic and pattern resistance profiles were detected for the isolates, including the highest resistance rates, which reached up to 100% and 94% in penicillin and ampicillin, respectively (Table 2,

Figures 3 and 4). The highest resistance genes were detected of ermB with 74%. The lowest was of the aad6 gene, which had 23.92%. Beef isolates were of bla_{SHV} gene with 87.5% (the highest). The least was of the aad6 gene, with 25%. In the case of the cheese isolates, the highest was of the ermB gene, with 78.57 %. The least was of aad6 and aac (3)-I genes, both at 21.42%. Chicken isolates showed the highest bla_{SHV} gene at 94.11%. The lowest was the aad6 gene at 11.76%. Milk isolates showed the ermB gene at 81.25% (the highest). The lowest was the aad6 gene at 37.5%. The patterns that showed frequent appearance were the A1 pattern (aac (3)-I, aad6, blaSHV, catA, ermB, sul, tetA) (Table 3 and Figures 5-8). All eight isolates had virulence genes identified, reaching up to 94%. All eight isolates had all tested genes (V4); however, at least two genes were present in each isolate. On the other hand, WB02, WB13, WC01, WC08, WK05, WK15, WM04, and WM10 had five genes. The V3 pattern of genes was the most frequent, while V1 and V5 were the least frequent in the isolates (Table 4, Figures 9-11). The RAPD-PCR generated different patterns, including 6-12 bands. The RAPD-PCR separated these isolates into 11 clusters (Figure 12).

Table 1: Incidence rates of *Listeria monocytogenes*

Type	Total	Positive (%)
Cheese	52	14 (26.92)
Beef	48	16 (33.33)
Chicken	50	17 (34)
Milk	53	16 (30.18)
Total	203	63 (31.03)

Table 2: Rates of antibiotic resistance of *Listeria monocytogenes* isolates

Antibiotics (µg)	Beef n=16 (%)	Cheese n=14 (%)	Chicken n=17 (%)	Milk n=16 (%)	Total %
Ampicillin (10)	13(81.25)	13(92.85)	16(94.11)	15(93.75)	90.49
Chloramphenicol (30)	3(18.75)	5(35.71)	6(35.29)	5(31.25)	30.25
Gentamicin (10)	8(50)	9(64.28)	11(64.7)	11(68.75)	61.93
Erythromycin (15)	5(31.25)	11(78.57)	11(64.7)	10(62.5)	59.25
Flucloxacillin (5)	6(37.5)	9(64.28)	11(64.7)	11(68.75)	58.8
Kanamycin (30)	3(18.75)	5(35.71)	10(58.82)	11(68.75)	45.5
Penicillin (10)	16(100)	14(100)	17(100)	16(100)	100
Streptomycin (10)	5(31.25)	7(50)	9(52.94)	8(50)	46.04
Sulfisoxazole (30)	11(68.75)	11(78.57)	14(82.35)	11(68.75)	74.6
Tetracycline (30)	12(75)	5(35.71)	14(82.35)	12(75)	67.01

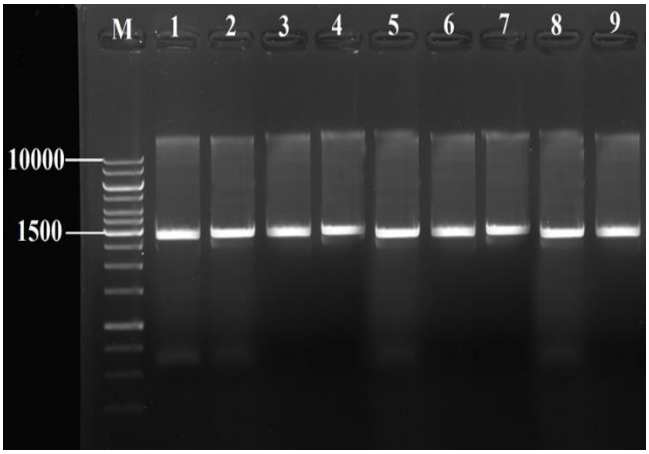

Figure 1: *Listeria monocytogenes* 16S rRNA-dependent analysis via agarose gel. M: ladder; Lane1: Positive control; Lane 2: WB02; Lane 3: WB13; Lane 4: WC01; Lane 5: WC08; Lane 6: WK05; Lane 7: WK15; Lane 8: WM04; Lane 9: WM10.

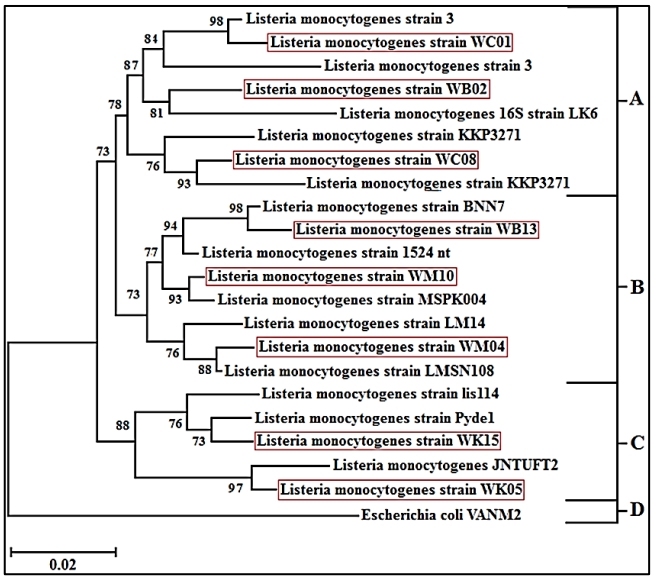

Figure 2: *Listeria monocytogenes* 16S rRNA-dependent analyses via phylogenetic tree.

Table 3: Antibiotic resistance genes of *Listeria monocytogenes*

Strain	Beef	Cheese	Chicken	Milk	Total
Aminoglycosides resistance (*aac(3)-I*)	8(50%)	3(21.42%)	9(52.94%)	8(50%)	43.59
Streptomycin resistance (*aad6*)	4(25%)	3(21.42%)	2(11.76%)	6(37.5%)	23.92
β-lactamase resistance (bla_{SHV})	14(87.5%)	8(57.14%)	16(94.11%)	8(50%)	72.18
Chloramphenicol Resistance (*catA*)	12(75%)	8(57.14%)	10(58.82%)	12(75%)	66.49
Macrolides resistance (*ermB*)	12(75%)	11(78.57%)	11(64.7%)	13(81.25%)	74.88
Sulfonamides (*sul*)	9(56.25%)	5(35.71%)	8(47.05%)	8(50%)	47.25
Tetracycline Resistance (*tetA*)	9(56.25%)	10(71.42%)	7(41.17%)	10(62.5%)	57.83

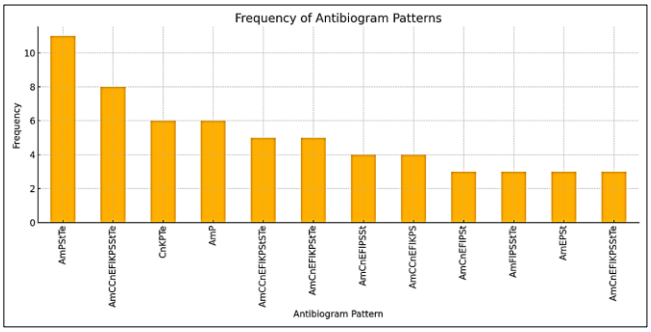

Figure 3: Antibiotic pattern distribution frequency among isolates of *Listeria monocytogenes*.

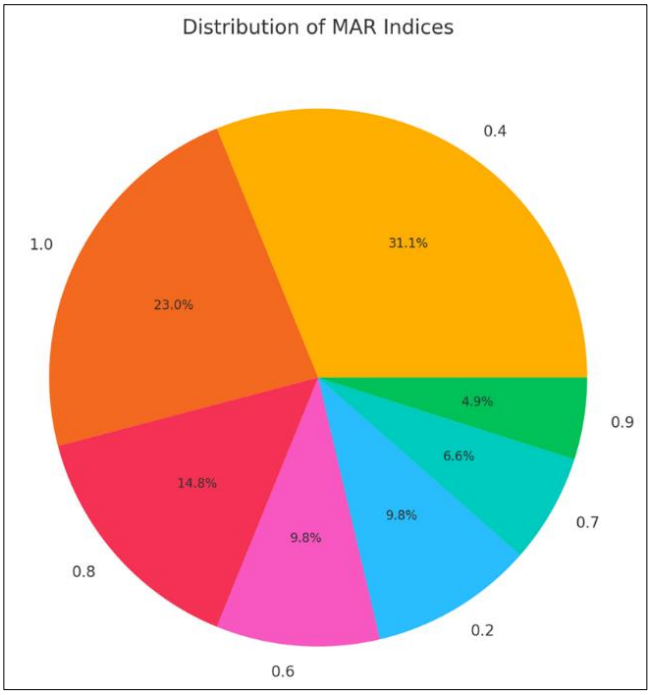

Figure 4: Pie chart of antibiotic pattern distribution among isolates of *Listeria monocytogenes*.

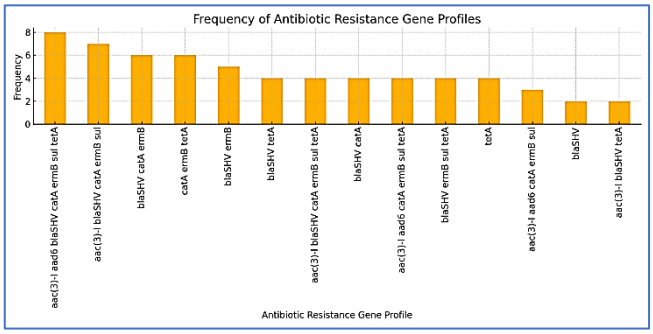

Figure 5: Antibiotic gene pattern distribution frequency among isolates of *Listeria monocytogenes*.

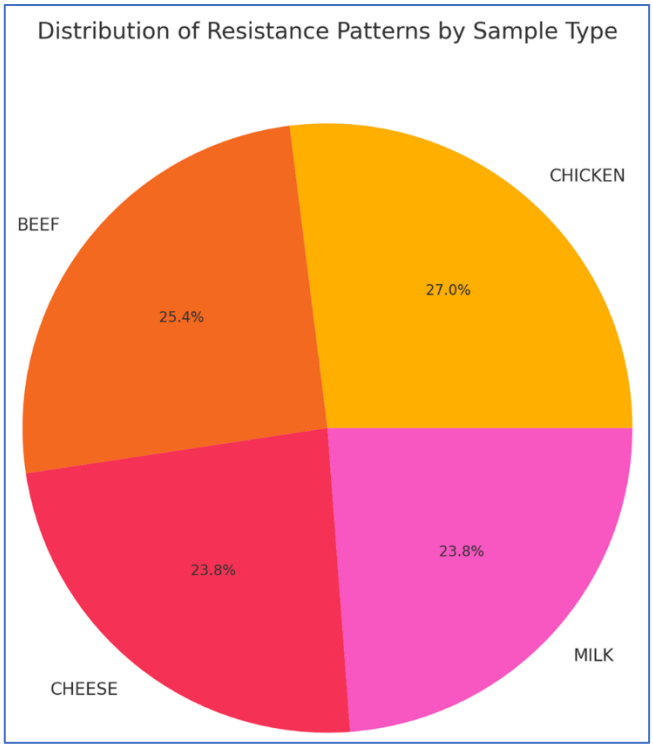

Figure 6: Pie chart of antibiotic gene pattern distribution among isolates of *Listeria monocytogenes*.

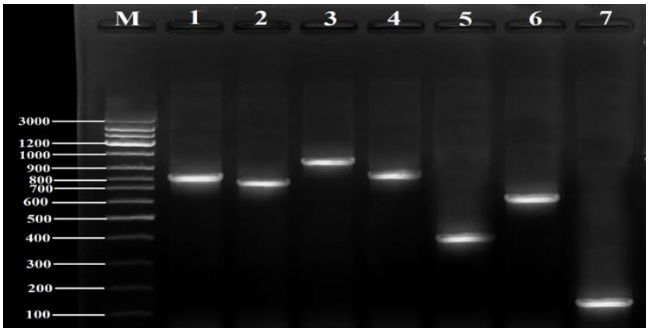

Figure 7: Antibiotic resistance genes in *Listeria monocytogenes* isolates. Image of 1.5 % agarose gel. M: Ladder. Lanes 1: *sul* gene in WB02. Lanes 2: *blaSHV* gene in WC01. Lanes 3: *aad6* gene in WB02. Lanes 4: *tetA* gene in WK05. Lanes 5: *catA* gene in WM04. Lanes 6: *ermB* gene in WM10. Lanes 7: *aac* (3)-I gene in WC08.

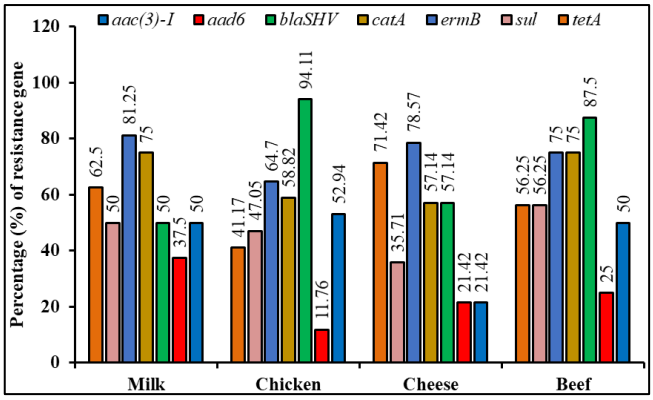

Figure 8: Percentage of antibiotic resistance genes in *Listeria monocytogenes* isolates.

Table 4: Percentage of virulence genes of *Listeria monocytogenes*

Gene	Milk n=16 (%)	Cheese n=14 (%)	Beef n=16 (%)	Chicken n=17 (%)	Total %
actA	9 (56.25)	7 (50.00)	7 (43.75)	10 (58.82)	52.3
hlyA	13 (81.25)	11 (78.57)	14 (87.05)	16 (94.11)	85.7
iap	13 (81.25)	10 (71.42)	12 (75.00)	9 (52.94)	69.8
inlA	3 (18.75)	5 (35.71)	7 (43.75)	10 (58.82)	39.7
plcA	6 (37.5)	11 (78.57)	9 (56.25)	11 (64.70)	58.7

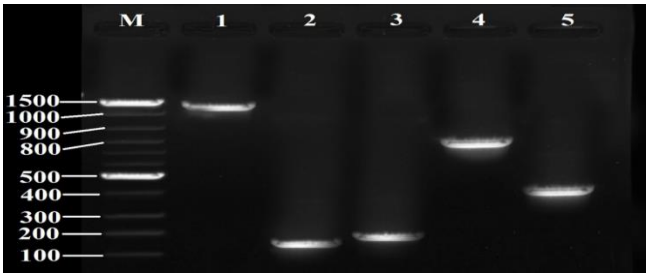

Figure 9: Image of 1.5 % agarose gel of virulence genes of *Listeria monocytogenes*. Lane M: ladder. Lanes 1: *plcA* gene in WB01. Lanes 2: *iap* gene in WC08. Lanes 3: *hlyA* gene in WK05. Lanes 4: *inlA* gene in WM10. Lanes 5: *plcA* gene in WM10.

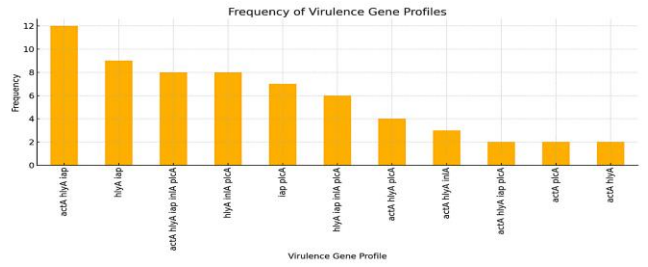

Figure 10: Virulence gene pattern distribution frequency among isolates of *Listeria monocytogenes*.

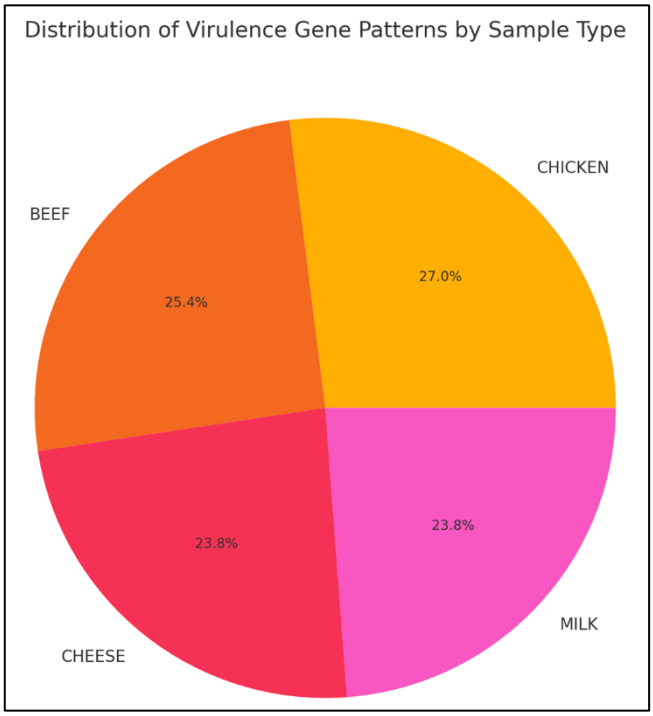

Figure 11: Pie chart of virulence gene pattern distribution among isolates of *Listeria monocytogenes*.

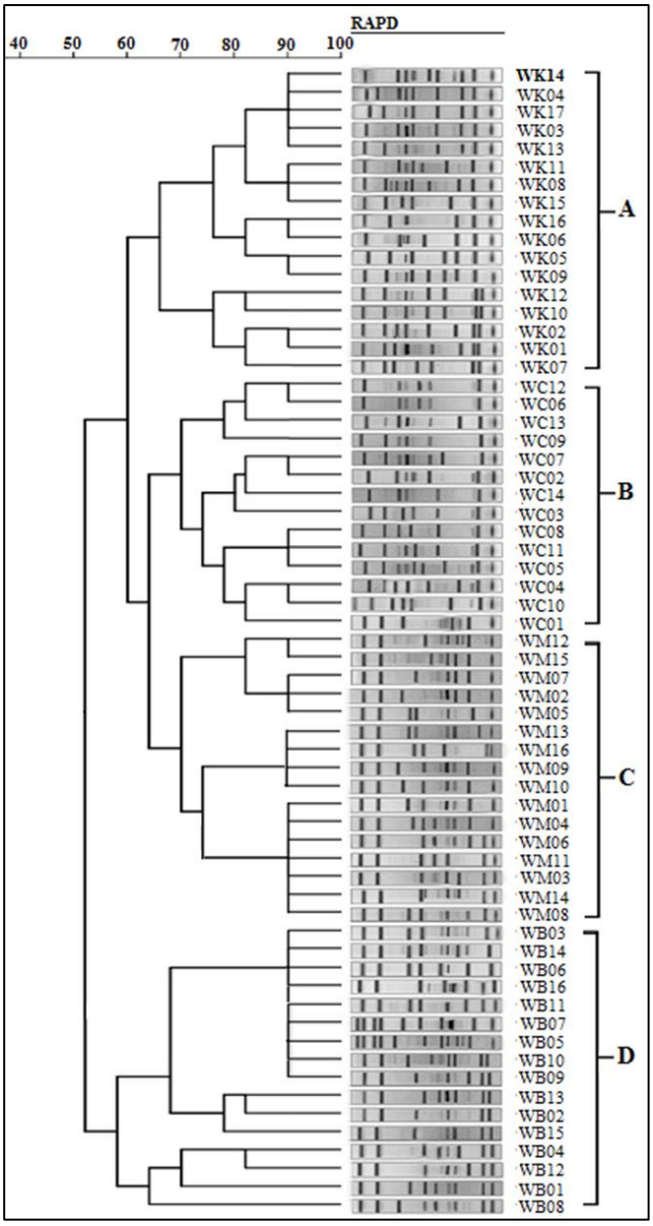

Figure 12: Dendrogram of typeable *Listeria monocytogenes* isolated from milk, cheese, beef, and chicken generated by RAPD-ImageJ-UPGMA analysis.

Discussion

Listeria spp. bacteria are found in soils, water, vegetation, and animals, and 27 species are currently described. At least five of these are pathogens. *Listeria monocytogenes* is particularly well-studied and is common in the environment and in food and animal feed. Listeriosis presents clinically as meningoencephalitis, abortions, sepsis, and gastroenteritis both in humans and ruminants (16). Many mechanisms that *L. monocytogenes* uses to cause disease to have been described, and important molecular factors have been identified. The main components are the six virulence genes (*prfA*, *plcA*, *plcA*, *mpl*, *actA*, and *plcB*) located in the *Listeria* pathogenicity island (LIPI) - 1 which are transcriptionally controlled by the transcriptional regulator *PrfA*. The infection cycle follows mechanisms utilizing phagosome lysis and actin-dependent intercellular bacteria motility for the bacterial release into the cytoplasm (17).

DNA sequencing techniques are presently performed in many areas of biology. In particular, this technique has numerous advantages in molecular biology research, genetics, anthropology, forensic sciences, biotechnology, and many other areas. 16S rRNA gene sequencing is commonly used for insights into the phylogeny and taxonomy of many bacteria, mainly for all the above-mentioned reasons (18). One of these is the possibility of identifying phylogeny and taxonomy using the 16S rRNA molecule itself, which is 1500 base pairs long. Another reason is that the molecule has not been modified through the evolutionary centuries, and third, 16S rRNA is present in all bacterial species. Secondly, finally, and probably most importantly, it can re-classify a bacterium into a rock-bottom novel bacterial species or even a genus. Furthermore, it can determine newly isolated species that are not yet successfully grown in culture (19). The VITEK 2 system does not have the same level of specificity as the 16S rRNA gene sequence, so the 16S rRNA gene sequence assembly-based tree is an optimal way to identify a species (20).

This study was one that determined the sequence of 16S rRNA for the genus *Listeria*. However, it is still not known whether it is unique for a certain species and also why the branches in the tree split at the nodes with a low bootstrap value, especially for the small number of strains in each taxonomic group. If chemotaxonomy and chemotherapeutic data are insufficient, it is important to fully compile the 16S rRNA sequence database for all the type strains of the unidentified taxa of the *Listeria* genus and to determine the sequence for as many strains as possible for each taxonomic group (21). Efforts continue to carry out additional searches for such sequences. Identification of a sequence that can detect pathogens is more important and beneficial for researching infections than isolating the bacterium, culturing it, and determining it using the standard biochemical tests of previously typed strains due to phenotypic divergence among strains of the genus (22). The phylogenetic position of all selected isolates was determined by sequence comparison. However, we could not determine the subspecies of the isolates due to the lack of a sequence database for the unidentified taxa. Efforts for building a database continuously improve the efficiency of 16S rRNA sequencing as a tool for bacterial classification. To get more reliable identification of closely related taxa, it is advisable to proceed with additional analyses and sequencing, for example, the 16S-23S intergenic spacer regions. These regions are known to be more variable for the bacterial

classification than 16S rRNA itself, and sequencing may give more exact results. These techniques of sequencing are highly advantageous for determining the necessity of performing the tedious and unreliable test (23).

In contrast to our study, the prevalence of *L. monocytogenes* in foods is relatively lower in China 5.5%, Chile 7.5%, Uruguay 11.9%, Turkey 8.5%, and Poland 13.5%. Our investigation was able to identify specifically tetA and tetC genes, which confer resistance against tetracycline. Mafuna and coworkers (24) isolated various resistance genes (including fosX, lin, mprF and norB) from several sample strains. They indicated a continuous increase in the global prevalence of resistance genes in the food chain (25). The virulence genes extensively studied in these isolates are *hlyA* (listeriolysin O), which produces pores that allow pathogens and certain organelles containing virulence factors to enter the cell and *prfA*, which activates the virulence genes, particularly *prfA* and *inlA*. *prfA* is a global regulator of virulence gene expression in pathogenic strains of *L. monocytogenes*, and its activity is affected by environmental factors, such as high temperature and stress (26). The presence of Internalin A (*inlA*) was detected in all the isolated species to attach to intestinal cells (27). The genotyping method used in the present study showed high-quality results. This agrees with those of Kalekar *et al.* (28), who detected clusters in the genotypes of isolates from different samples. The relationship between food intake and human diseases has been known since ancient times. Foodborne pathogens or contaminating agents include viruses, bacteria, and parasites (29-50).

Conclusion

The data, here, reveal important presence of *Listeria monocytogenes* in food products, which apply high health risk on consumers.

Acknowledgement

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Conflict of interest

No conflict of interest is detected in the present study.

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التوصيف الجزيئي للجينات المسببة للضراوة ومقاومة المضادات الحيوية والنمط الجيني للستيريا مونوسايتوجينز المعزولة من الجبن، ولحوم البقر، والدجاج، والحليب

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الخلاصة

أجريت الدراسة الحالية للكشف عن جينات اللستيريا مونوسايتوجينز وجينات الفوعة المقاومة للمضادات الحيوية المرتبطة بها والتي تمتلكها هذه السلالات وكذلك النمط الجيني للعزلات التي تم تحديدها من الحليب والجبن ولحم البقر والدجاج. وبناء على ذلك تم جمع ٢٠٣ عينات (٥٣ عينة حليب، ٥٢ عينة جبن، ٤٨ عينة لحم بقر، ٥٠ عينة دجاج) من محافظة القادسية، العراق. واستخدمت هذه العينات للزراعة التقليدية والانتقائية والدراسات البيوكيميائية. تم الكشف جزيئياً عن ثماني عزلات باستخدام تفاعل البلمرة المتسلسل وتسلسل القواعد النتروجينية لجين ال *16S rRNA*. تم تجنيد جميع العزلات المكتشفة لاختبار مقاومة المضادات الحيوية. علاوة على ذلك، تم تعريض جميع العزلات للكشف عن جينات الفوعة، والتي شملت البروتين المحفز لتجميع الأكتين (*actA*)، listeriolysin (*hlyA*)، البروتين المرتبط الغازي (*iap*)، internalin A (*inlA*)، وphospholipase C (*plcA*). تم استخدام تفاعل البلمرة المتسلسل التكثيفي العشوائي في التنميط الجيني لهذه العزلات. كشفت نتائج الزراعة عن هوية ٦٣ عزلة (١٦ لبن، ١٤ جبن، ١٦ لحم بقر، ١٧ دجاج). وتم الكشف الجزيئي عن هوية العزلات الثمانية التي تم اختبارها. تم الكشف عن أنماط مقاومة مختلفة للمضادات الحيوية والنمطية للعزلات، والتي تضمنت أعلى معدلات المقاومة التي وصلت إلى ١٠٠% و٩٤% في البنسلين والأمبيسيلين، على التوالي. تم التعرف على جينات الضراوة في جميع العزلات والتي وصلت إلى ٩٤%. أظهرت نتائج التسلسل توافقًا قويًا مع العزلات العالمية من بنك الجينات. وتكشف البيانات هنا عن وجود بكتيريا اللستيريا مونوسايتوجينز الهامة في المنتجات الغذائية، والتي تشكل مخاطر صحية عالية على المستهلكين.