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Highlighting on *Listeria monocytogenes* in sheep with special reference to its antimicrobial resistance

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Abstract

Listeriosis, induced by the bacterium *Listeria monocytogenes*, represents a notable zoonotic infection with considerable impact on both animal and human health.

This study aimed to investigate the occurrence of *L. monocytogenes* in sheep farms in Sharkia governorate, identify the distribution of virulence-associated genes in isolates, evaluate antibiotic resistance patterns, perform molecular detection of virulence genes, and conduct phylogenetic analysis of the inlA and inlB genes. A total of 290 samples were collected from sheep suspected of listeriosis between November 2022 and May 2024, representing three clinical forms: nervous (n=50), abortive (n=90), and septicemic (n=150). Isolation and identification of *Listeria spp.* were carried out using standard microbiological techniques and confirmed with the VITEK-2 system. Out of the 290 samples, 15 (5.2%) were identified as *L. monocytogenes*, with 8 (2.8%), 4 (1.4%), and 3 (1.0%) isolates from nervous, abortive, and septicemic forms, respectively.

Phenotypic characterization revealed that all isolates were positive for Anton's test, CAMP test, and hemolysis test. Antimicrobial susceptibility testing showed varying levels of resistance, with 7 out of 15 isolates (46.6%) exhibiting multidrug resistance (MDR) with MAR indices ranging from 0.25 to 0.8. Resistance was most common to penicillin (60%), cefotaxime (53.3%), and cephalexin (46.7%). Molecular characterization via PCR detected the presence of virulence genes including 16S rRNA, prfA, inlA, inlB, and hlyA. The *16S rRNA* and *inlA* genes were identified in all isolates (100%), whereas the *prfA* gene was successfully amplified

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in 14 isolates (93.3%). Similarly, the *inlB* and *hlyA* genes were each detected in 14 isolates (93.3%). Sequencing and phylogenetic analysis of inlA and inlB genes revealed that all isolates belonged to serotype 1/2a – lineage II, indicating genetic diversity among isolates from different disease forms.

Introduction

Listeria monocytogenes is a small, Gram-positive, rod-shaped bacterium that is non-spore-forming and facultatively anaerobic. It grows well at 30-37°C temperatures, but it can grow also at colder temperatures (refrigerator), and in the laboratory can be separated from other bacteria by taking advantage of this fact, this technique known as cold enrichment **(Wellinghausen *et al*., 2011).**
Listeriosis is an important bacterial infectious disease most commonly caused by Listeria monocytogenes that can affect all ruminants, other animal species and humans and most commonly cause encephalitis, but it can also cause septicemia and abortion. Animals from 1 to 4 years of age are more susceptible to listeriosis than older animals. The abortive form of listeriosis is typically asymptomatic and can only be confirmed through laboratory diagnostic methods. Encephalitis develops very quickly and the death occurs within 24 to 48 hours from symptoms that include; circling in one direction, high fever, lack of appetite, red tissues around the eyes, usually with blindness, and depression **(Metzger, 2018).**
One of the most important causes of foodborne infections is *Listeria monocytogenes* whish is capable of infecting a wide range of hosts, including humans, domestic animals, companion pets, avian species, rodents, cattle, fish, and rabbits.
The principle route of transmission of listeriosis is the contaminated food, **Hassan *et al*. (2024)**.

L. monocytogenes is usually susceptible to antibiotics active against Gram-positive bacteria but, levels of resistance are varied and influenced by antimicrobial use in animals, as well as geographical differences Consequently, continuous monitoring of the antibiotic susceptibility and resistance profiles of *Listeria monocytogenes* is essential for effective disease management and the development of appropriate therapeutic strategies.

Molecular methods such as conventional PCR and real-time PCR have been developed as safe, useful, sensitive, and accurate methods for the detection of L. monocytogenes in clinical specimens **(Amagliani *et al*., 2006).** Differentiation between virulent and avirulent monocytogenes strains could be carried out by detection of more than one virulence gene which responsible for its pathogenicity that includes; listeriolysin O (encoded by *hlyA*), internalins (encoded by *inlA, inlB,* and *inlJ*), **(Liu *et al*., 2007).**

Aim of The present study:

- Determination of the incidence of *Listeria monocytogenes* in sheep farms and assess its involvement in disease manifestations within these settings.
- Characterization of the distribution of virulence-associated genes among *L. monocytogenes* isolates.
- Evaluation of the Antibiogram patterns of L. monocytogenes isolates in order to recommend successful therapy.
- Sequencing and phylogenetic analysis of *L. monocytogenes inlA* and *inlB* genes.

Material and Methods

Sampling:

During the period from November 2022 to May 2024 a total number of 290 samples were collected aseptically from different sheep disease conditions suspected of being infected with listeriosis in Sharkia governorate. Samples were representative for the 3 different disease forms in sheep as following; 50 samples from animals have nervous manifestation, 90 samples from aborted animals collected from Brucella free sheep farm suffering from abortion at third trimester of gestation period and 150 samples from animals have septicemic signs. All samples were transported in ice boxes to the laboratory with minimal delay.

Table (1). Types and Number of Samples Collected from Different Forms of Diseased Sheep:

Form of Disease	Types of samples	No. of samples
Nervous	Brain stem and Spinal cord	50
Abortion	Placenta and aborted foeti	90
Septicemia	Blood, internal organs, and intestinal content	150
Total no. of samples		290

Isolation and Identification of *Listeria* spp.:
The isolation and identification of *Listeria* species were performed following the protocol established by the U.S. Food and Drug Administration (FDA) **(Hitchins, 1992).** Briefly, collected samples were initially pre-enriched in buffered peptone water (Himedia Lab, Mumbai, India) and incubated at 37 °C for 48 hours. Subsequently, 5 mL of the pre-enriched culture was transferred into 10 mL of *Listeria* enrichment broth (Himedia Lab, Mumbai, India) and incubated at 30°C for an additional 48 hours.
Following enrichment, a loopful of the broth culture was streaked onto Oxford agar (Himedia Lab, Mumbai, India) and incubated at 37 °C for 24–48 hours. Three to four presumptive *Listeria* colonies were subcultured overnight at 35 °C for 24–48 hours on tryptic soy agar supplemented with yeast extract (TSAye). Colonies displaying typical morphology were subjected to Gram staining and further identification using the VITEK-2 system (bioMérieux, Marcy l'Étoile, France).
Bacterial suspensions were standardized to a McFarland turbidity of 0.50 in 0.45% saline solution and inoculated into the appropriate VITEK-2 identification cards for Gram-positive bacteria. The time between suspension preparation and card inoculation did not exceed one hour. The VITEK-2 system automatically recorded readings every 15 minutes, and data analysis was conducted using VITEK-2 software version VT2-R03.1 according to the manufacturer's instructions (bioMérieux, 2015). Additionally, all samples were cultured on blood agar plates to assess the presence of other potential infectious agents.

Phenotypic identification of some virulence factors of *L. monocytogenes*:
Anton's Rabbit Eye Test:
Anton's test was conducted by instilling 0.1 mL of a *Listeria monocytogenes* suspension containing 10^9 colony-forming units (CFU) into the conjunctival sac of one eye of a rabbit, while the contralateral eye served as a negative control. A positive Anton's test was indicated by the development of purulent conjunctivitis within 24–48 hours, often followed by keratitis, as described by **(Markey *et al*, 2013).**

CAMP Test:
The CAMP test was performed following a previously established method **(Markey *et al*, 2013)**, utilizing a standard β-hemolytic Staphylococcus aureus strain (ATCC 25923) inoculated in a straight line along the center of blood agar plates (Oxoid, UK). The Listeria monocytogenes isolates were then streaked perpendicularly to the S. aureus line without making contact. The inoculated plates were incubated at 37 °C for 18–24 hours. Positive CAMP test results were indicated by enhanced hemolysis at the intersection zone, typically appearing as an arrowhead, circular, or rectangular zone of clearing, signifying synergistic hemolytic activity.

Hemolysis test:
Selected colonies from tryptic soya broth yeast extract (TSBYE) agar (Oxoid, UK) plates were streaked onto 5% sheep blood agar (Oxoid, UK) plates and incubated at 37°C for 24-48 h to detect hemolysis **(Markey *et al* ,2013).**

Antibiogram and identification of MDR *L. monocytogenes* isolates: Antibiogram disc diffusion assay:
It was performed according to the instructions of Standards Institute **(CLSI, 2025).**

Table (2). Interpretation of antimicrobial sensitivity test

Antibiotic Groups	Antibiotic	Resistant	Intermediate	Sensitive
Fluoroquinolones	Ciprofloxacin (CIP 5µg)	≤12	13-17	≥18
	Norfloxacin (NOR 5µg)	≤12	13-17	≥18
Chloramphenicol	Chloramphenicol (C 30µg)	≤12	13-17	≥18
Sulphonamides	Trimethoprim/Sulfamethoxazole (SXT 25µg)	≤10	15-Nov	≥16
Aminoglycosides	Gentamicin (CN 10µg)	≤12	13-14	≥15
Tetracycline	Oxytetracycline (TE 30µg)	≤15	16-25	≥26
Cephalosporins	Cephalexin (CL 30µg) (1st generation)	≤14	-	15-17
	Cefotaxime (CTX 30µg) (3rd generation)	≤14	15-21	≥22
Macrolides	Tylosin (TL 15µg)	≤21	22-30	≥30
Penicillins				
Natural	Penicillin (P 10µg)	≤11	21-Dec	≥22
Aminopenicillins	Ampicillin/sulbactam (SAM 20µg)	≤11	14-Dec	≥15
Penicillin-like antibiotics + β-lactamase inhibitors	Amoxicillin/clavulanic acid (AMC 30µg)	≤13	14-17	≥18

The Multi-Drug Resistance (MDR) characters of the isolates were identified by observing the resistance pattern of the isolates to the antibiotics. The Multiple Antibiotic Resistance (MAR) Index of an isolate is calculated as a/b and done as following (a) represents the number of antibiotics that the isolate was resistant to and (b) the number of antibiotics that the isolate has been exposed to **Jayaraman *et al*., (2012).** Bacteria having MAR Index > 0.2 are considered to have a very high
MAR index value and originate from an environment where several antibiotics are used **(Tambekar *et al*., (2006)).**

Molecular characterization of *L. monocytogenes* and related virulence genes:
Extraction of DNA: According to QIAamp DNA mini kit instructions
Preparation of PCR Master Mix:
According to Emerald Amp GT PCR mastermix (Takara)
Code No. RR310A kit .
The PCR Master Mix was prepared in a total volume of 25 µl per reaction. Each reaction mixture contained 12.5 µl of Emerald Amp GT PCR mastermix (2x premix), 5.5 µl of PCR grade water, 1 µl of forward primer (20 pmol), 1 µl of reverse primer (20 pmol), and 5 µl of template DNA.

The cycling conditions for conventional PCR (cPCR) varied depending on the target gene. For all primers, the initial (primary) denaturation was performed at 94°C for 5 minutes. For the **16S rRNA** gene, the cycling included a secondary denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, for 35 cycles, followed by a final extension at 72°C for 12 minutes. For the **prfA** gene, the conditions included a secondary denaturation at 94°C for 30 seconds, annealing at 50°C for 50 seconds, extension at 72°C for 1 minute, for 35 cycles, and a final extension at 72°C for 10 minutes. The **inlA** gene was amplified with a secondary denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 45 seconds, over 35 cycles, and a final extension at 72°C for 10 minutes. For the **inlB** gene, the secondary denaturation was at 94°C for 30 seconds, annealing at 55°C for 40 seconds, and

extension at 72°C for 40 seconds, repeated for 35 cycles, with a final extension at 72°C for 10 minutes. Lastly, the ***hlyA*** gene underwent secondary denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, for 35 cycles, and a final extension at 72°C for 7 minutes.

The target virulence genes, corresponding oligonucleotide primer sequences, and the sizes of the amplified PCR products used for *Listeria monocytogenes* detection are as follows: The **16S *rRNA*** gene was amplified using the primer sequence 5'-GGACCGGGGCTAATACCGAATGA-TAATTCATGTAGGCGAGTTGCAGCCTA–3', yielding a 1200 bp product, as described by **Kumar *et al*. (2015).** The ***prfA*** gene was targeted using the sequence 5'-TCTCCGAGCAACCTCGGAACCTG-GATTGACAAAATGGAAACA–3', producing a 1052 bp fragment, based on the work of **Dickinson *et al*. (1995).** For the ***inlA*** gene, the primer sequence was 5'-ACGAGTAACGG-GACAAATGCCCCGACAGTGGTGCTAGA TT–3', resulting in an 800 bp product, according to **Liu *et al*. (2007).** The ***inlB*** gene was amplified using the primer 5'-CTGGAAAAGTTTGTATTTGG-GAAATTTCATAATCGCCCATCATCACT–3', generating a 343 bp fragment. Finally, the **hlyA** gene was amplified with the primer 5'-GCATCTGCATTCAATAAA-GATGTCAC-TGC-ATC-TCC-GTG-GT–3', producing a 174 bp fragment, as reported by **Deneer and Boychuk (1991).**

Genotypic characterization of *L. monocytogenes* by sequencing of internalin A *(inlA)* and internalin B *(inlB)* genes isolated from the 3 different disease forms:

Method of Sequencing:

-The residual RT-PCR products obtained after electrophoresis were stored at −80 °C for preservation.

-An aliquot of 20 µL from each primer was transferred into thin-walled PCR tubes for the sequencing reaction.

-The purified PCR products were subjected to bidirectional sequencing using the dideoxy chain-termination method with the same amplification primers described previously. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) at MACROGEN (Korea) using a 3730XL DNA sequencer.

-The entire experimental workflow was monitored and managed using a Laboratory Information Management System (LIMS).

-Sequencing results were delivered within 48 hours and analyzed using the BLAST tool available on GenBank (NCBI) at: http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome.

Method of Sequencing Analysis:

-The obtained sequences were imported into the alignment window of BIOEDIT software version 7.0.4.1, alongside highly similar reference sequences retrieved from GenBank.

-The GenBank database was screened using the Basic Local Alignment Search Tool (BLAST) to identify closely related sequences. Comparative sequence analysis was performed against publicly available entries in the NCBI Infectious Bronchitis Virus (IBV) resource database.

-Multiple sequence alignments were conducted using the Clustal W algorithm embedded within BIOEDIT version 7.0.4.1.

-Sequence editing, frame correction, and amino acid alignment were carried out using various functions available in BIOEDIT version 7.0.4.1.

-Finalized and curated sequences were exported from BIOEDIT in FASTA format.

-The FASTA files were then imported into MEGA version 4.0.2 for DNA alignment and saved in MEGA format (*.meg).

-The MEGA file served as the basis for phylogenetic analysis. Evolutionary relationships were inferred using the neighbor-joining method, and branch reliability was assessed by performing 1,000 bootstrap replicates **(Tamura *et al*., 2007).**

-Statistical analysis of the phylogenetic tree topology was confirmed by 1,000 bootstrap iterations.

-The final phylogenetic trees were exported as image files. In addition, phylogenetic tree construction was also conducted using the MegA-

lign module of DNASTAR Lasergene software version 12.1, applying the neighbor-joining method in MEGA6 for further validation.

Results

Incidence of *L. monocytogenes* isolated from different disease forms in sheep:

On the basis of cultural characteristics, biochemical reactions and VITEK-2 system; 15 (5.2%) *Listeria monocytogenes* isolates were isolated from different disease forms in sheep; represented as 8 positive samples (2.8%) from nervous form samples (Brain stem and Spinal cord), 4 positive samples (1.4%) from abortive form samples (Placenta and aborted foeti), followed by 3 positive samples (1.0 %) from septicemic form samples (Blood and intestinal content).

Table (3). Incidence of *L. monocytogenes* isolated from diseased sheep in different samples:

Disease Forms (Samples types)	No. of samples	No. of positive samples	Positive percentage %
Nervous form (Brain stem and Spinal cord)	50	8	2.8
Abortive form (Placenta and aborted foeti)	90	4	1.4
Septicemic form (Blood, internal organs and intestinal content)	150	3	1.0
Total	290	15	5.2

The percentages were calculated according to the total no. of collected samples (290)
All isolates exhibited positive results for Anton's, CAMP and hemolysis tests.

Table (4). Antimicrobial susceptibility testing of *L. monocytogenes* isolates:

Antibiotic (Concentration)	Sensitive no. (%)	Intermediate no. (%)	Resistant no. (%)
Ciprofloxacin (CIP 5µg)	5 (33.3%)	10 (66.7%)	0 (0%)
Norfloxacin (NOR 5µg)	4 (26.7%)	8 (53.3%)	3 (20%)
Chloramphenicol (C 30µg)	9 (60%)	5 (33.3%)	1 (6.7%)
Trimethoprim/ Sulphamethoxazole (SXT 25µg)	11 (73.3%)	4 (26.7%)	0 (0%)
Oxytetracycline (O 30µg)	4 (26.7%)	6 (40%)	5 (33.3%)
Tylosin (TL 15µg)	4 (26.7%)	9 (60%)	2 (13.3%)
Cefotaxime (CTX 30µg)	2 (13.3%)	5 (33.3%)	8 (53.3%)
Gentamicin (CN 10µg)	4 (26.7%)	7 (46.7%)	4 (26.7%)
Amoxicillin/Clavulanic acid (AMC 30µg)	10 (66.7%)	3 (20%)	2 (13.3%)
Cephalexin (CL 30µg)	1 (6.7%)	7 (46.7%)	7 (46.7%)
Penicillin (P 10µg)	0 (0%)	6 (40%)	9 (60%)
Ampicillin/Sulbactam (SAM 20µg)	2 (13.3%)	9 (60%)	4 (26.7%)

Table (5). Antibiotic Resistance Profile of *L. monocytogenes* isolates:

Isolate (ID no)	No. of Antibiotics the Isolate Was Resistant To	MAR Index	MDR
Nervous (1)	10 out of 12	0.8	+
Nervous (3)	9 out of 12	0.75	+
Nervous (4)	4 out of 12	0.3	+
Nervous (5)	7 out of 12	0.58	+
Nervous (6)	9 out of 12	0.75	+
Abortive (12)	6 out of 12	0.5	+
Septicemic (15)	3 out of 12	0.25	-

Results of genotypic characterization of *L. monocytogenes* isolates

Table (6). Results of PCR amplifications of different used genes of *L. monocytogenes*:

Serial	Disease form	16S rRNA	inlA	inlB	prfA	hlyA
1	Nervous	+	+	+	+	+
2	Nervous	+	+	+	-	+
3	Nervous	+	+	+	+	+
4	Nervous	+	+	+	+	+
5	Nervous	+	+	+	+	+
6	Nervous	+	+	+	+	+
7	Nervous	+	+	+	+	+
8	Nervous	+	+	+	+	+
9	Abortive	+	+	+	+	+
10	Abortive	+	+	+	+	+
11	Abortive	+	+	+	+	+
12	Abortive	+	+	+	+	+
13	Septicemic	+	+	+	+	+
14	Septicemic	+	+	-	+	-
15	Septicemic	+	+	+	+	+

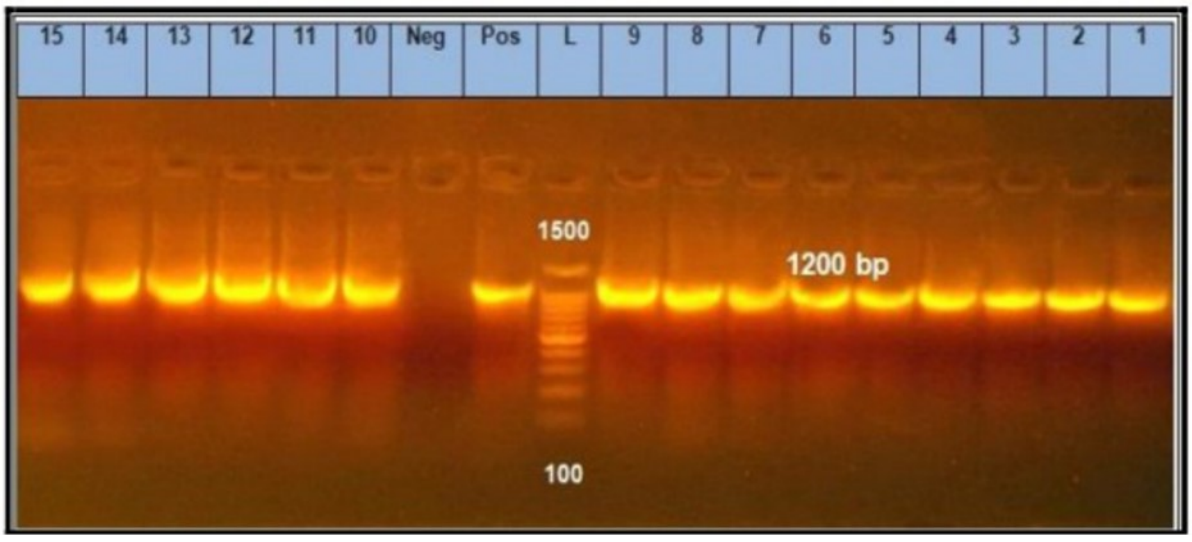

Fig. (1). PCR- electrophoretic pattern of 16S rRNA gene of *L. monocytogenes* at 1200 bp.
Lane L: 100 - 1500 bp Ladder.
Neg.: Negative control. Pos.: Positive control (at 1200 bp).
Lane (1:15): *L. monocytogenes* (16S rRNA) gene positive

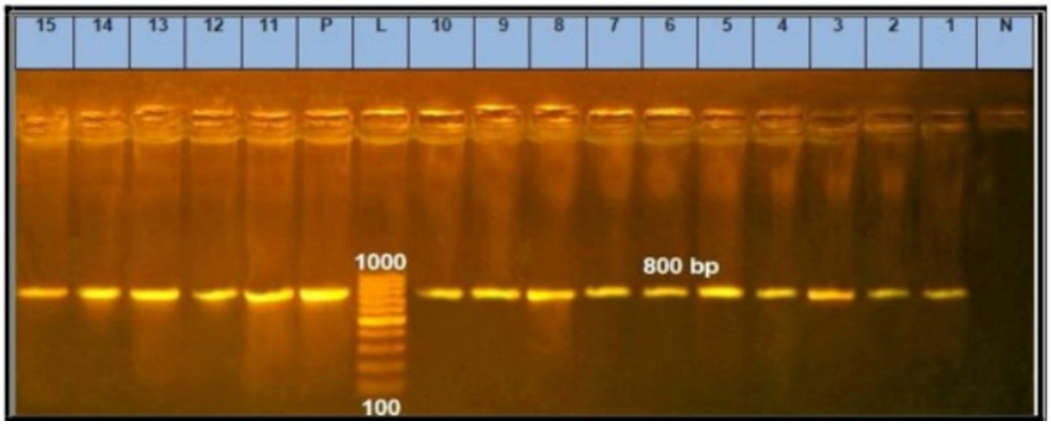

Fig. (2). PCR- electrophoretic pattern of *inlA* gene of *L. monocytogenes* at 800 bp. Lane L: 100 - 1000 bp Ladder.
Neg.: Negative control. Pos.: Positive control (at 800 bp). Lane (1:15): *inlA* gene positive *L. monocytogenes*

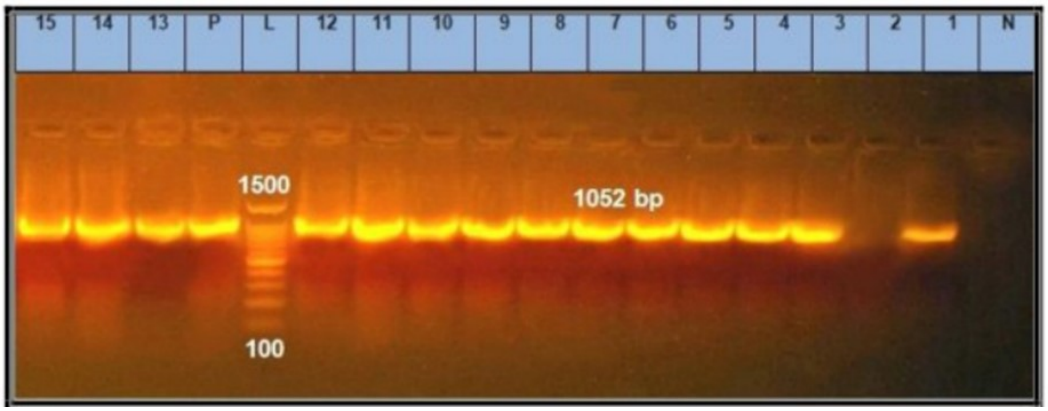

Fig. (3). PCR- electrophoretic pattern of *prfA* gene of *L. monocytogenes* at 1052 bp.
Lane L: 100 - 1500 bp Ladder.
Neg.: Negative control. Pos.: Positive control (at 1052 bp). Lane (1 and 3:15): *prfA* gene positive *L. monocytogenes*
Lane (2): *prfA* gene negative *L. monocytogenes*

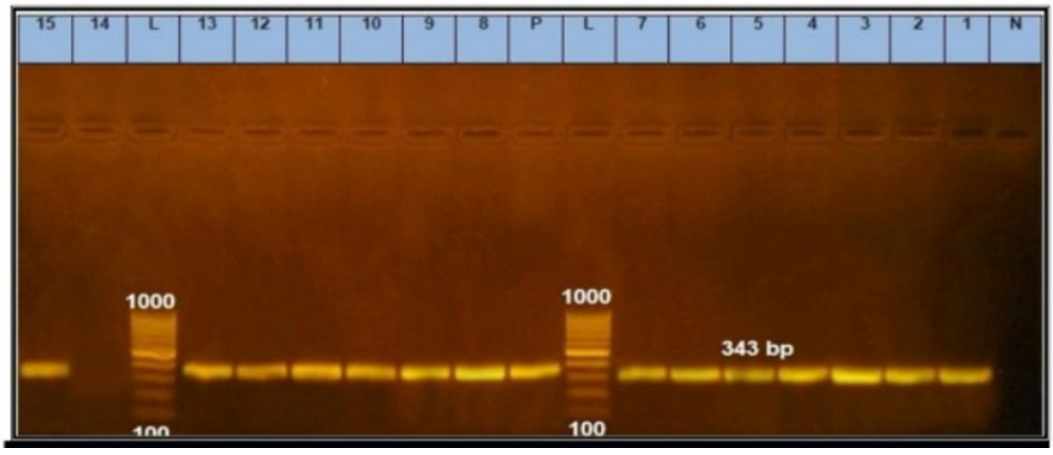

Fig. (4). PCR- electrophoretic pattern of *inlB* gene of *L. monocytogenes* at
343 bp.
Lane L: 100 – 1000 bp Ladder.
Neg.: Negative control. Pos.: Positive control (at 343 bp).
Lane (1:13 and 15): *inlB* gene positive *L. monocytogenes*

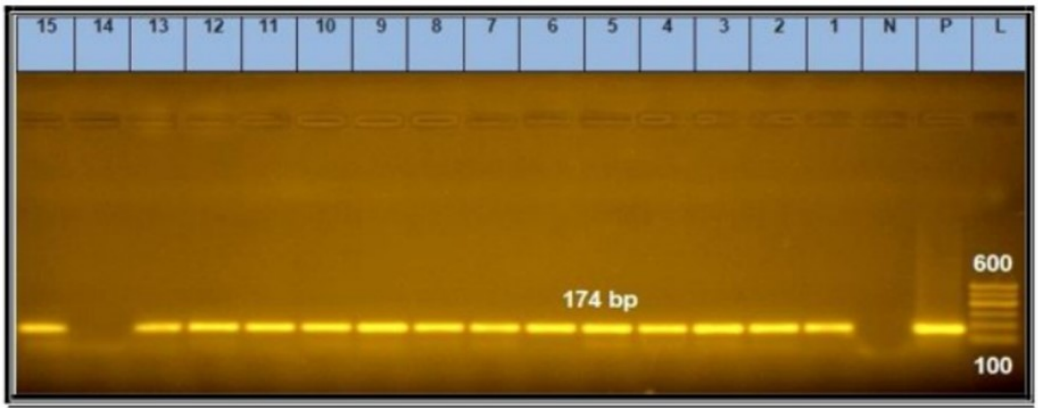

Fig. (5). PCR- electrophoretic pattern of *hlyA* gene of *L. monocytogenes* at 174 bp.
Lane L: 100 – 600 bp Ladder.
Neg.: Negative control. Pos.: Positive control (at 174 bp).
Lane (1:13 and 15): *hlyA* gene positive *L. monocytogenes.*
Lane (14): *hlyA* gene negative *L. monocytogenes.*

Results of sequencing of *L. monocytogenes* isolated from the different disease forms in sheep:

The sequencing result of the 2 genes indicated that there were some variations in the nucleotides sequence between the 3 *L. monocytogenes* isolates from the 3 different disease forms in sheep, however it was found that all of them belong to the serotype 1/2a – lineage II.

- Phylogenetic analysis of *L. monocytogenes* (inlA) gene:

In this study, MEGA 4.0.2 software was used. The evolutionary history was inferred using the neighbor-joining method and the reliability of each branch in the phylogenetic tree was assessed by conducting 1,000 bootstrap replicates, providing statistical support for the inferred evolutionary relationships. replicates. the phylogenetic tree was constructed for better understanding the genetic relatedness and evolution of the 3 isolated Egyptian *L. monocytogenes* MS-Alex isolates which is likely to belong to serotype 1/2a, which is classified under lineage II.

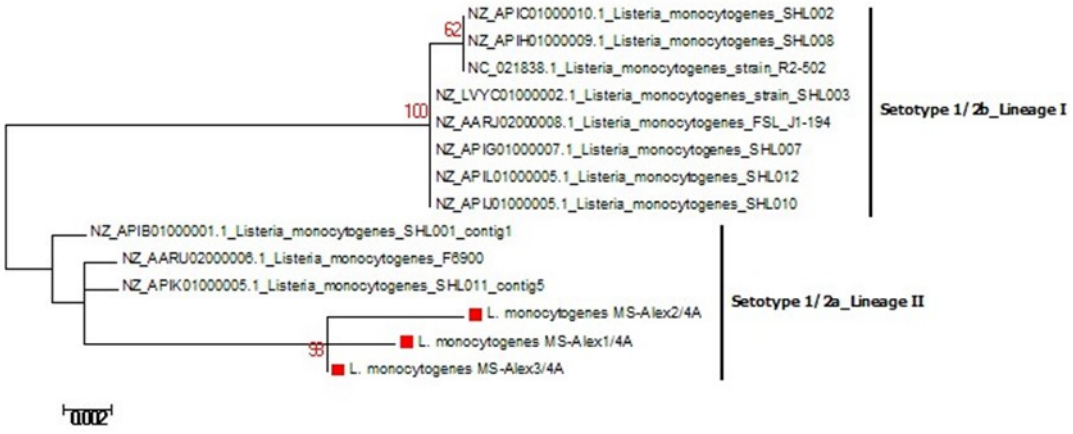

Fig. (6). Phylogenetic analysis of *inlA* gene based on nucleotide sequence showing the clustering of *L. monocytogenes* MS/Alex1/4A, *L. monocytogenes* MS/Alex2/4A and *L. monocytogenes* MS/Alex3/4A strains with other representative *L. monocytogenes* genetic groups. bootstrap values were represented by the numbers at the nodes. The scale bar represents the no. of substitutions for each site.

Table (7). Sequence identity matrix of *L. monocytogenes inlA* gene between the 3 isolates from different disease forms created by Clustal 2.1.:

Diversity	Identity Matrix
	L. monocytogenes MS-Alex1/4A (Abortive)
L. monocytogenes MS-Alex1/4A (Abortive)	100%
L. monocytogenes MS-Alex2/4A (Nervous)	2%
L. monocytogenes MS-Alex3/4A (Septicemic)	1%

-Phylogenetic analysis of *L. monocytogenes inlB* gene:

The phylogenetic tree of *inlB* gene was constructed for better understanding the genetic relatedness and evolution of the 3 isolated Egyptian *L. monocytogenes* MS-Alex isolates which found to be serotype 1/2a – lineage II.

Fig. (7). Phylogenetic analysis of *inlB* gene based on nucleotide sequence showing the clustering of *L. monocytogenes* MS/Alex1/4L, *L. monocytogenes* MS/Alex2/4L and *L. monocytogenes* MS/Alex3/4L strains with other representatives *L. monocytogenes* genetic groups. Bootstrap values were represented by numbers at the nodes. The scale bar represents the no. of substitutions for each site.

Table (8). Sequence identity matrix between the 3 isolates from different disease forms of *L. monocytogenes inlB* gene created by Clustal 2.1. :

Diversity	Identity Matrix
	L. monocytogenes MSAlex3/4L (Septicemic)
***L. monocytogenes* MSAlex3/4L (Septicemic)**	100%
***L. monocytogenes* MSAlex1/4L (Abortive)**	100%
***L. monocytogenes* MSAlex2/4L (Nervous)**	95%

Discussion

Listeriosis has great importance as it is associated with CNS infections, abortions, septicemic infection, gastroenteritis, and mastitis **(Maury *et al*., 2016).**

The results obtained from the basic morphological characterization (microscopically by Gram staining and colonial appearance on different media) revealed that 25 isolates (8.6%) were suspected to be Listeria species. After biochemical characterization and differentiation (using conventional phenotypic characteristics) and VITEK-2 system only 15 isolates (5.2%) were identified as *L. monocytogenes*

isolates represented as 8 isolates (2.8%) from nervous form samples, 4 isolates(1.4%) from abortive form samples, followed by 3 isolates (1.0 %) from septicemic form samples. These results were relatively lower than those obtained by **Keelara *et al*., (2015)** who isolated 23 different
Listeria isolates from 880 samples collected from 220 ewes, out of them 15 isolates (6.8%) were *L. monocytogenes,* 2 were L. ivanovii and the other 6 isolates were other Listeria. Also, CNS form isolates were lower than those isolated by **Aldabbagh (2019)** who identified two *L. monocytogenes* isolates (4%) out of 50 sheep brain samples. But higher than those of **Shimaa *et al*., (2020)** who found that 16 (3.39%) out of 472 sheep samples were positive for *L. monocytogenes*. And higher than **Branka *et al*., (2006)** results who isolated 18 *L. monocytogenes* isolates out of 46 tissue samples (3.9%) from sheep having listeriosis signs. 12 isolates (2.6%) were from the brain stem and medulla oblongata samples, and 6 (1.3%) were from fetal tissue samples.
All isolates exhibited positive results for Anton's, CAMP and hemolysis tests.

Beta hemolytic activity is an important virulence factor that promotes RBCs lyses and cell death. These findings were largely in agreement with those reported in the OIE Terrestrial **Manual (2014)**, which described the hemolytic activity of Listeria monocytogenes on horse and sheep blood agar as typically presenting a narrow zone of hemolysis, often not extending significantly beyond the colony margins and becoming evident only upon removal of the colonies. The manual also noted the existence of rare non-hemolytic *L. monocytogenes* strains. Furthermore, the CAMP test was utilized to enhance the detection of hemolytic activity in L. monocytogenes. These observations are consistent with the findings of **Quinn *et al.* (2011),** who reported that *L. monocytogenes* yields a positive CAMP reaction in proximity to Staphylococcus aureus streaks and a negative reaction near Rhodococcus equi, whereas Listeria ivanovii demonstrates the opposite pattern.
Identification of antimicrobial susceptibility properties of *L. monocytogenes* is very essential in the reduction of antibiotic treatment costs and prevention of MDR bacteria increasing and persistence. Results of the antimicrobial susceptibility test of the 15 isolated *L. monocytogenes* bacteria to 12 different antibiotics and the MDR bacteria which have been found. Out of the tested 15 *L. monocytogenes* isolates, 7 isolates (46.6%) were MDR with medium to very high MAR index range from (0.25 to 0.8). Resistance to Penicillin, Cefotaxime and Cephalexin was widespread in most of the isolated *L. monocytogenes* (60%, 53.3% and 46.7%; respectively), followed by Oxytetracycline resistance (33.3%) then Gentamicin and Ampicillin/sulbactam resistance was (26.7% for both). Resistance to Norfloxacin, Tylosin and Amoxicillin/clavulanic acid was less common (20%, 13.3% and 13.3%; respectively). Finally, Chloramphenicol resistance was rare only (6.7%). However, all of the isolates were sensitive to Ciprofloxacin and Trimthoprim/ Sulphamethoxazole. These results mostly agreed with **Safarpoor *et al*., (2013)** who found that *L. monocytogenes* isolates showed the highest antibiotic resistance toward tetracycline (71.3%) and had great resistance to the penicillin, streptomycin, sulfamethoxazol, gentamycin, and erythromycin as; 43.6%, 34.8%, 33.9%, and 33.03%; respectively. Followed by resistance to chloramphenicol and ampicillin (29.9% and 29.5%), then resistance to enrofloxacin, lincomycin and cephalothin as; 20.7%, 16.7%, and 15.85%; respectively. The lowest resistance was to nitrofurantoin (5.72%), trimethoprim (11.01%) and ciprofloxacin (11.8%). The obtained MAR index in the present work was higher and the antibiotic resistance slightly differs from those of **Shimaa *et al*., (2020)** who found that 37.5% (6/16) of L. monocytogenes isolates were MDR with MAR index ranged from 0.1 to 0.6. A high resistance rates were found to amoxicillin, cefotaxime, erythromycin (50% each), then SXT and tetracycline as 25% and 12.5%; respectively. On the other hand, all isolates were found to be sensitive to ciprofloxacin, and norfloxacin. Also, these results disagreed with **Garedew *et al*., (2015) and Al Sharif *et al.* (2025)** who isolated four multi-drug resistant *L. monocytogenes* isolates. The higher rate of resistance was recorded for chloramphenicol, tetracy-

cline, nalidixic acid and penicillin in increasing order. And great susceptibility to amoxicillin, cephalothin, cloxacillin, sulfamethoxazole, gentamicin and vancomycin among all *L. monocytogenes* isolates.

Differences in resistance rates can be affected by country, regulations on antibiotic use, cultivation, and sample types **(Ruiz *et al*., 2011).** The existence of these multi-drug resistant bacteria is a serious economic problem and a serious public health threat **Sosnowski *et al*. (2023).**

L. monocytogenes produces a large number of virulence factors that give it various ways of pathogenicity **(Swetha *et al*., 2012)** as *prfA* gene, which has a role in the switch of *L. monocytogenes* from saprophytic to pathogenic form through virulence factor regulation; *hlyA* gene has a great role in intracellular parasitism and hemolytic activity and two invasion proteins; Internalin A and B (inl A and B) that present on the listerial cells surface, binds to a surface protein on the host epithelial cells surface. All of these genes facilitate the spreading and intracellular growth of *L. monocytogenes* within a mammalian host **(Ciolacu *et al*., 2015).** In the current study; PCR using five sets of primers was used for genotypic identification of *L. monocytogenes* isolates and detection of four virulence genes. The target genes included in the analysis were the *16S rRNA* gene, *internalin A (inlA)*, *internalin B (inlB)*, *Listeriolysin O/hemolysin (hlyA)*, and the *positive regulatory factor A (prfA)*.

It was applied on the 15 isolated *L. monocytogenes* from all disease forms. PCR results showed that 16SrRNA and *inlA* genes were detected in all 15 *L. monocytogenes* isolates (100.0%), *prfA* gene was amplified in 14 (93.3%) isolates and missed in one isolate. However; *inlB* and *hlyA* didn't detect in another one isolate and detected in the remaining 14 (93.3%) isolates. These results were mostly agreed with **Bouymajane *et al*., (2021)** who detected the presence of 16S *rRNA* gene in 15 isolates (100%), *hlyA*, *prfA* and *plcB* genes in 14 isolates (93.3%), but only 13 isolates (86.7%) carried *inlA* and *inlC* genes. Otherwise, the obtained results were lower than those obtained by **Abd El-Tawab *et al*., (2015)** who detected all the tested genes in all the 5 studied *L. monocytogenes* isolates (100%) when they identified isolates using PCR by targeting 16S *rRNA* gene and detected its virulence genes including *InlA, InlB, hlyA* and *PrfA*. However, my results were more than **Asmaa *et al*., (2017)** results who detected the presence of *prfA, hlyA, inlB* and *inlA* virulence-related genes in only 42 (60.8%) isolates out of 69 *L. monocytogenes* isolates, but the remaining 27 (39.2%) isolates did not harbor any virulence-related genes. The inability of some strains in the previous work to have the *prfA, hlyA, inlB* genes could be due to many mutations **(Cooray *et al*., 1994) and Çelik *et al*. (2024)**.

Sequencing of *L. monocytogenes* internalin A (*inlA*) and internalin B (*inlB*) genes isolated from the 3 different disease forms in sheep was detected by Applied Biosystems®, (Foster City, CA) at MACROGEN, Korea Using 3730XL DNA sequencer. Then determine identity percentage between *L. monocytogenes inlA* and *inlB* gene sequences and other related sequences from GeneBank database (NCBI) and finally, sequences identity matrix between different isolation forms (nervous, septicemic and abortive) of *L. monocytogenes inlA* and *inlB* genes was determined by the aid of Clustal2.1 software. The sequencing result of the 2 genes indicated that there were some variations in the nucleotides sequence between the 3 *L. monocytogenes* isolates from the 3 different disease forms in sheep, however, all of them belong to the serotype 1/2a – lineage II. These obtained results agreed with **(Mary *et al*., 2006)** who stated that *Listeria monocytogenes* isolates classified under lineage I exhibited clonal characteristics and were predominantly associated with encephalitic cases. In contrast, lineage II isolates demonstrated greater genetic diversity and were comparably distributed among the three clinical manifestations of listeriosis in ruminants: encephalitis, septicemia, and fetal infection.

Also, agreed with **(Jeffers *et al*., 2001)** who confirmed that *L. monocytogenes* strains of lineage II (serotype 1/2a and 1/2c isolates) are the most found among clinical cases of animal listeriosis and in food isolates. On the contrary, the present results disagreed with **Abd-Elghany *et al*. (2024)** who found that the *L.*

monocytogenes isolates isolated from the brain stem of sheep with rhombencephalitis during the study were belonging to lineage I.

In this study the *L. monocytogenes* isolates belonged to 1/2a _lineage II serotype, presence of this serotype in sheep samples indicated a risk factor to infect the human through cross-contamination as according to **(Jeffers *et al*., 2001)** The predominant *Listeria monocytogenes* serotypes associated with the majority of human clinical infections are serotypes 4b and 1/2b, which belong to lineage I, and serotype 1/2a, which is classified under lineage II.

Sequencing analysis of the virulence-associated *inlA* and *inlB* genes from the isolated *L. monocytogenes* strains revealed notable sequence identity when compared to reference sequences available in the GenBank database. Specifically, the *inlA* gene sequences from the three *L. monocytogenes* MS Alex isolates demonstrated 96% identity with other serotype 1/2a (lineage II) isolates and 94% identity with serotype 1/2b (lineage I) isolates.

Regarding the *inlB* gene, sequence identity ranged from 95–98% with serotype 1/2a (lineage II) and from 88–94% with serotype 1/2b (lineage I) strains in GenBank.

Analysis of the sequence identity matrix and sequence diversity matrix indicated that the isolate obtained from the nervous form of listeriosis exhibited the highest level of genetic divergence compared to the other isolates, with a maximum diversity score of 2% for the *inlA* gene and 5% for the *inlB* gene.

Conclusion

L. monocytogenes causes various clinical manifestations including; nervous signs, 3rd-trimester abortions and septicemia in neonates and young animals. PCR and sequencing analyses revealed minimal molecular variation among the *Listeria monocytogenes* isolates derived from different clinical forms, with all isolates belonging to serotype 1/2a, lineage II. This finding suggests that a single strain of *L. monocytogenes* can be associated with multiple clinical syndromes, implying that factors other than genetic differences such as host age, immune status, pregnancy, and overall health condition may play a critical role in determining disease manifestation.

Listeria monocytogenes isolates exhibited resistance to ampicillin, which is the primary antibiotic employed in the treatment of listeriosis in Egypt. This resistance raises significant public health concerns, as it suggests that cross-contamination or the consumption of undercooked sheep meat or unpasteurized milk may pose a serious risk, particularly to immunocompromised individuals, children, and the elderly. These findings underscore the urgent need to raise awareness about the dangers posed by antimicrobial-resistant pathogens. Special emphasis should be placed on minimizing the overuse and misuse of antibiotics in both human and veterinary medicine to prevent the emergence and dissemination of multidrug-resistant (MDR) *L. monocytogenes* strains.

Ethics approval

The study received approval from the Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) under approval number

ZU-IACUC/3/F/117/2025

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