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The occurrence and characteristics of *Listeria monocytogenes* in commercial and native chicken breeds

Azza S. El-Demerdash*, Ahmed A. Matter, Mona S. Ibrahim***, Adel A.A.M. El-Gmaal*, Salma Salah El-Deen Mohamed*, Rehab E. Mowafy****, Amera F. Ebrahem****

* Department of Microbiology, Agriculture Research Centre (ARC), Animal Health Research Institute (AHRI), Zagazig Branch, Egypt
** Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (AHRI), Gamasa Branch, ARC, Egypt
*** Department of Poultry Diseases, Agriculture Research Centre (ARC), Animal Health Research Institute (AHRI), Mansoura Branch, Egypt
**** Department of Pathology, Agriculture Research Centre (ARC), Animal Health Research Institute (AHRI), Zagazig Branch, Egypt

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ABSTRACT

The present study was designed to evaluate the potential presence and features of *Listeria monocytogenes* in different commercial and native poultry breeds. Overall, the isolation of *L. monocytogenes* strains from different chicken species was 42/550 (7.6%). *L. monocytogenes* showed high resistance to streptomycin (83.3%) and amoxicillin/clavulanic acid (78.5%) and was highly sensitive to vancomycin, gentamycin, and chloramphenicol. Multiple drug resistance (MDR) index values for 59.5% of the isolates were greater than 0.2. *L. monocytogenes* strains were screened for five virulence factors associated with genes, namely *inlA, act*A, *hly*A, *iap,* and *inlB*. The presence of the five virulence genes were 83.3%, 66.6%, 59.5%, 57.1%, and 45.2% for *act*A, *iap, inl*A, *hly*A, and *inl*B, respectively, in the identified *L. monocytogenes* isolates. Moreover, histopathological examination revealed marked changes in the brain and heart as the most affected tissues. Both the cerebral hemispheres and cerebellum were affected and exhibited marked encephalitis represented by diffuse congestion of cerebral blood vessels. These findings suggest that poultry may play a role in the zoonotic spread and transmission of multidrug-resistant and virulent *L. monocytogenes*, which can pose a health risk at human-poultry interface, especially in the absence of stringent hygienic standards and preventive measures.

Corresponding author: Azza Salah El-Demerdash, Zagazig Branch, Animal Health Research Institute, Agricultural Research Center (ARC).
E-mail: dr.azza@ahri.gov.eg
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INTRODUCTION

Listeria monocytogenes (L. monocytogenes), a Gram-positive facultative anaerobic bacillus can cause severe diseases in human and poultry. In chickens, listeriosis has been associated with two major forms of disease: septicemic and encephalitic. The World Health Organization has identified *L. monocytogenes* as one of four foodborne pathogens causing invasive infections in both humans and animals with a death rate of 20–30%. It can lead to severe listeriosis, which can cause meningoencephalitis, sepsis, fetal infection, or miscarriage in pregnant women (**Radoshevich and Cossart, 2018**). Consumption of processed chicken, poultry, and poultry products may significantly contribute to the spread and transmission of virulent and multiple drug resistance (MDR) *L. monocytogenes* to humans **(White et al. 2002**). *L. monocytogenes* has been found at all stages of poultry production and processing (**Rothrock et al. 2017**). The intestinal colonization of chickens and the presence of *L. monocytogenes* in their feces is a possible source of the organism, leading to contamination of the environment and litter in the chicken production facility (**Dhama et al. 2013**). Ingestion, inhalation, or wound contamination are the three main modes of transmission. Symptoms of infection can include sudden death, ataxia, lateral recumbency with leg paddling, twisting and backward neck retraction, and paralysis (**Abdel Aziz and Mohamed, 2020**).

The overuse of medications in clinical settings and their extensive use as growth promoters for farm animals, as well as increased global trade and travel, which favor the spread of antimicrobial resistance between nations and continents, are all accelerating factors of antimicrobial resistance in *L. monocytogenes* (**Moreno et al. 2014**). Among high-risk groups, the multi drug resistant (MDR) *L. monocytogenes* found in ready-to-eat foods is being regarded as a public health indicator, thus increasing understanding of the laws governing food safety and of medications used in both people and animals is strongly advised (**Garedew et al. 2015**).

Several virulence factors, which are responsible for microbial invasion and adhesion as well as entry and replication within host cells, have been found to influence the pathogenicity of L. monocytogenes (**Matereke and Okoh, 2020**). In particular, the *inlA* and *inlB* genes promote bacterial uptake by host cells., and the secreted pore-forming toxin listeriolysin O (LLO), disrupts the phagosome to allow bacterial proliferation in the cytosol. It has also been demonstrated that *L. monocytogenes* internalization into epithelial cells is facilitated by LLO perforating the plasma membrane (**Phelps et al. 2018**). In real-time PCR-based studies, the hemolysin gene (*hlyA*) has been identified as a useful genetic marker for *L. monocytogenes* (**Salim and Othman, 2017**). The actin gamma gene (*actA*), a critical factor in *L. monocytogenes* persistence in the host and transmission, is a primary virulence determinant (**Travier et al. 2013**). Finally, the extracellular invasion-associated protein (IAP), encoded by the *iap* gene, plays a crucial role in the bacterium's virulence and pathogenicity (**Soni et al. 2014**).

Given its potential to cause a variety of health issues in humans, it is important to understand the prevalence of this bacterium in different breeds. Therefore, this study was designed to study the pathogenicity pattern and antibiotic resistance profiles of *L. monocytogenes* isolated from various chicken commercial and native breeds in Egypt.

MATERIALS AND METHODS

Samples collection

Five hundred and fifty randomly collected cloacal swab samples and internal organ were collected from commercial and native chicken breeds (diseased and apparently healthy at ages ranging from 8 to 12 weeks. Three hundred samples were isolated from broilers, 150 from layers, and 100 from Baladi chicken farms in Sharkia and Dakahlia Governorates in Egypt during the period from January to June 2023. The samples were collected in sterile plastic bags, kept in ice boxes, and with a minimum delay were transferred to the laboratory to study the presence of *Listeria* species.

Pathological examinations

The investigations were performed on samples taken from the brains and hearts of infected birds that had been either slaughtered or found freshly dead. Following a fixation step in 10% buffered neutral formalin, the specimens were cut into paraffin slices 2-3 microns thick and stained with hematoxylin and eosin. These sections were then inspected under a microscope **(Suvarna et al. 2013**).

Bacteriological examination

Primary Enrichment: Twenty-five grams of the sample were inoculated into 225 ml of tryptone soya broth (TBS) and incubated aerobically at 30 ± 1C for 24 ± 2 hours. Secondary Enrichment: 0.1 ml of the incubated broth was inoculated into 10 ml of Fraser broth and incubated at 37 °C for 24±2 hours. A loopful from the incubated Fraser broth was streaked onto the following media: ALOA agar; PALCAM agar and Oxford agar plates then the plates were incubated at 37 °C for 48 hours and examined after 24 ± 3 hours. The listeria-like colonies were picked and streaked onto Tryptic Soy agar (TSA) and then, incubated at 37°C for 24 hours. The isolates were morphologically identified by Gram stain and biochemical tests according to **ISO 11290-1, (2017).**

Antibiogram Profile

The isolated *L. monocytogenes* strains were subjected to a sensitivity test, using the Kirby-Bauer disk diffusion method. The 11 antibiotic discs belonging to 8 different antibiotic classes included: erythromycin(E), gentamycin (CN), danofloxacin (DA), ampicillin (AMP), sulfamethoxazole/trimethoprim (SXT), chloramphenicol (C), streptomycin (S), doxycycline (DO), amoxycillin/Clavulanic Acid (AMC), vancomycin (VA), and norfloxacin (NOR). The result was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines of 2022. *L. monocytogenes* ATCC 7644 was used as a control. The MARI values were calculated according to **Krumperman (1983**) using the mathematical formula: MARI= a/b (where a is the sum of the test antibiotics the isolates displayed resistance to and b is the total sum of antimicrobial agents used). Molecular confirmation and genotypic characterization of *L. monocytogenes* isolates.

The bacteriologically identified *L. monocytogenes* isolates were confirmed by PCR using the 16S rRNA gene. Five sets of primers were used for genotypic detection of L. monocytogenes virulence genes: *inlA; inlB; hlyA; iap* and *actA*. This was applied on 41 isolated L. m following the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), Taq PCR Master Mix Kit and Agarose 1.5%. The specific sequence and amplification are shown in **Table 1**. Temperature and time conditions of the primers during PCR were as follows initial denaturation at 95 °C for 3 min; 35 cycles each consisting of denaturation at 94 °C for 30 s, annealing at 53 °C for 15 s, and extension at 72 ° C for 90 s; and final extension at 72 °C for 7 min.

Table 1. Oligonucleotide primers sequences used in genotypic characterization of *Listeria* isolates

Targets	Sequences (5 to 3)	Amplicon Size (bp)	Reference
16S rRNA	F: CTC CAT AAA GGT GAC CCT R: CAG CMG CCG CGG TAA TWC	938	**Usman et al., 2016**
hlyA	F: CCT AAG ACG CCA ATC GAA R: AAG CGCTTG CAA CTG CTC	702	**Usman et al., 2016**
actA	F: ACG TGA AGT AAG TCACGT GAT ATT G R: ACG TGA AGT AAG CTC ACG TGA TAT TG	268	**Usman et al., 2016**
inlA	F: AGA TCT AGA CCA AGT TAC AAC GCT TCA R: TAA TAT CAT TTG CTG TTT TAT CTG TC	255	**Usman et al., 2016**
inlB	F: CTGGAAAGTTTGTTTTGTAAAAGGGAAA R: TTCATAAATCGCCATCATCAC	343	**Liu et al., 2007**
iap	F: ACA AGC TGC ACC TGT TGC AG R: TGA CAG CGT TGT TAG TAG CA	131	**Usman et al., 2016**

RESULTS

The clinical signs

The diseased chickens showed depression, lethargy, loss of appetite, diarrhea, vomiting, ataxia, lateral recumbency with leg padding, twisting and backward retraction of the neck, and paralysis.

Postmortem examination

It revealed that the heart and brain were the most affected organs with petechial hemorrhages on the heart, meningitis (inflammation of the membranes surrounding the brain and spinal cord), and encephalitis (inflammation of the brain) observed yellow necrotic area on the myocardium and liver were detected. Additionally, septicemia (infection of the bloodstream), enlarged spleen, and enlarged liver were recorded in some cases.

Prevalence of *L. monocytogenes*

The overall prevalence of *L. monocytogenes* was 7.68% (42/550), Among the different poultry breeds, *L. monocytogenes* was most commonly detected in Baladi breeds (10%) followed by broilers (8.3%) and layers (4.6%) as shown in **Table 2**.

Antibiogram of *L. monocytogenes*

Antimicrobial resistance profiles of *L. monocytogenes* isolates were tested against eleven antibiotics belonging to eight different antibiotic classes using the Kirby-Bauer disc diffusion method as shown in **Table 3 and Figure 1**. *L. monocytogenes* isolates showed high susceptibility to vancomycin, whereas high resistance was detected against β-lactams (amoxicillin-clavulanic acid). Moreover, the incidence of MDR isolates was 25/42 (59.5%).

Detection of five virulence genes in *L. monocytogenes*

It was conducted on 42 confirmed isolates. Amplification of the 16S rRNA gene, was found in all of the isolates, confirming the *L. monocytogenes* species. The most prevalent gene was *inlA* (83.3%) followed by *actA* (66.66%), *iap* (59.5%), *inlB* (57.1%), and *hylA* (40.47%) indicating their pathogenic potential as illustrated in **Table 4 and Figure 2**.

Histopathological findings

They revealed marked changes in the brain and heart as the most affected tissues. Both the cerebral hemisphere and cerebellum showed diffuse congestion of cerebral and cerebellar blood vessels, focal demyelination of some neurons with submeningeal encephalomalacia, encephalitis with focal inflammatory cells infiltrating the parenchyma, myocarditis with inflammatory cellular infiltration, perivascular

Table 2. Prevalence of *L. monocytogenes* among different commercial and native breeds

Breed	Flocks number	No. of examined birds	No. of positive *L. monocytogenes*	Percentage
Broiler	6	300	25	8.3%
Layer	3	150	7	4.6%
Baladi	2	100	10	10%
Total	9	550	42	7.6%

Table 3. Percentage of MDR among 42 *L. monocytogenes* isolates collected from commercial and native chickens

Resistance to one or more antibiotic classes	No. of isolates that showed resistance	Percentage of resistance
1	0	0
2	17	40.47
3	9	21.4
4	11	26.19
5	5	11.9

Table 4. Antimicrobial resistance profile and virulence determinant of each *L. monocytogenes* isolate collected from poultry

no	Breed	Type of samples	Resistance pattern	virulence determinant	MDRI
1	Broiler	organs	C-SXT	*act*A-*hly*A-*iap*-*inl*A-inlB	0.18
2	Broiler	organs	C- S- SXT	*act*A-*hly*A-*iap*-*inl*A-inlB	0.27
3	Broiler	organs	C-AMC-S-DA – SXT	*hly*A-*inl*A	0.45
4	Broiler	organs	AMC – S	*iap*-*inl*A-inlB	0.18
5	Broiler	organs	AMC-S-DA -E -DA -DO	*act*A-*inl*A-inlB	0.45
6	Broiler	organs	AMC - S - E -DA – DO	*act*A-*inl*A-inlB	0.45
7	Broiler	Cloacal swab	C-AMC-S	*act*A-*hly*A-*iap*-*inl*A-inlB	0.27
8	Broiler	Cloacal swab	C-AMC-S- NOR	*act*A-*hly*A-*iap*-*inl*A-inlB	0.36
9	Broiler	Cloacal swab	C -SXT	-	0.18
10	Broiler	organs	C -SXT – S	*act*A-*hly*A-*iap*	0.27
11	Broiler	organs	C -SXT	-	0.18
12	Broiler	organs	C-AMC-S-NOR	*act*A-*hly*A-*iap*-inlB	0.36
13	Broiler	organs	C-AMC-SXT-NOR	*act*A	0.45
14	Broiler	Cloacal swab	C-SXT	*act*A	0.18
15	Broiler	Cloacal swab	C-AMC-S	*hly*A-*iap*-*inl*A	0.27
16	Broiler	Cloacal swab	AMC- S	*act*A-*inl*A	0.18
17	Broiler	organs	C-AMC	*iap*-*inl*A	0.18
18	Broiler	organs	C-AMC-S-SXT	*hly*A-*inl*A	0.36
19	Broiler	organs	AMC – S	*iap*-*inl*A-inlB	0.18
20	Broiler	organs	AMC - S - E -DA – DO	*act*A-*inl*A-inlB	0.45
21	Broiler	Cloacal swab	AMC- S -NOR	*act*A-*inl*A	0.27
22	Broiler	organs	C -AMC	*iap*-*inl*A	0.18
23	Broiler	organs	C - AMC - S - DO – NOR	*hly*A-*nl*A	0.45
24	Broiler	organs	AMC – S	*iap*-*inl*A-inlB	0.18
25	Broiler	organs	CN- AMC - S -E -DA - DO	*act*A-*inl*A-inlB	0.54
26	Layer	Cloacal swab	C-AMC-S	*act*A-*hly*A-*iap*-*inl*A-inlB	0.27
27	Layer	Cloacal swab	C-AMC-S	*act*A-*hly*A-*iap*-*inl*A-inlB	0.36
28	Layer	Cloacal swab	C-AMC-S-DA	*act*A-*hly*A-*iap*-inlB	0.36
29	Layer	Cloacal swab	AMC-S-DA -E -DA -DO – NOR	*act*A-*iap*-*inl*A-inlB	0.54
30	Layer	Cloacal swab	AMC - S - E -DA – DO	*act*A-*iap*-*inl*A-inlB	0.45
31	Layer	organs	AMC - S - E -DA – DO	*act*A-*inl*A-inlB	0.45
32	Layer	Cloacal swab	AMC - S -E -DA - DO -NOR	*act*A-*iap*-*inl*A-inlB	0.54
33	Baladi	organs	S- DA	*act*A-*hly*A-*iap*-*inl*A-inlB	0.18
34	Baladi	Cloacal swab	AMC - S - E -DA – DO	*act*A-*iap*-*inl*A	0.45
35	Baladi	organs	C-AMC-S-SXT	*hly*A-*inl*A-inlB	0.36
36	Baladi	organs	C-AMC	*hly*A-*iap*-*inl*A-inlB	0.18
37	Baladi	organs	C-AMC-S	*act*A-*inl*A	0.27
38	Baladi	organs	S – DA	*act*A-*hly*A-*iap*-*inl*A-inlB	0.18
39	Baladi	Cloacal swab	AMC - S - E -DA – DO	*act*A--*iap*-*inl*A	0.45
40	Baladi	organs	C - AMC – S	*act*A-*inl*A	0.27
41	Baladi	organs	AMP- CN - S – DA	*act*A-*hly*A-*iap*-*inl*A-inlB	0.45
42	Baladi	Cloacal swab	AMP- AMC - S - E - DA – DO	*act*A-*iap*-*inl*A	0.54

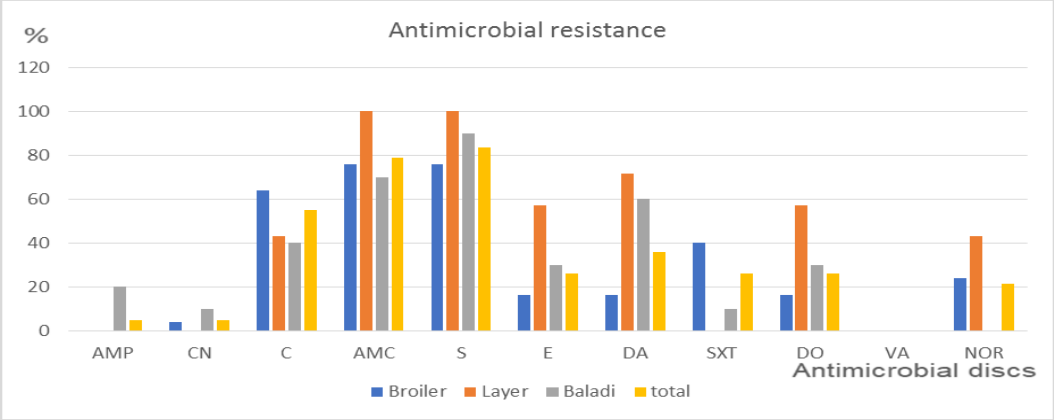

Figure 1. Antimicrobial profile of *L. monocytogenes* isolated from different chicken breeds. Erythromycin(E), Gentamycin (CN), Danofloxacin (DA), Ampicillin (AMP), Sulphamethoxazole/trimethoprim (SXT), Chloramphenicol (C), Streptomycin (S), Doxycycline (DO), Amoxycillin/Clavulanic Acid (AMC), Vancomycin (VA), Norfloxacin (NOR).

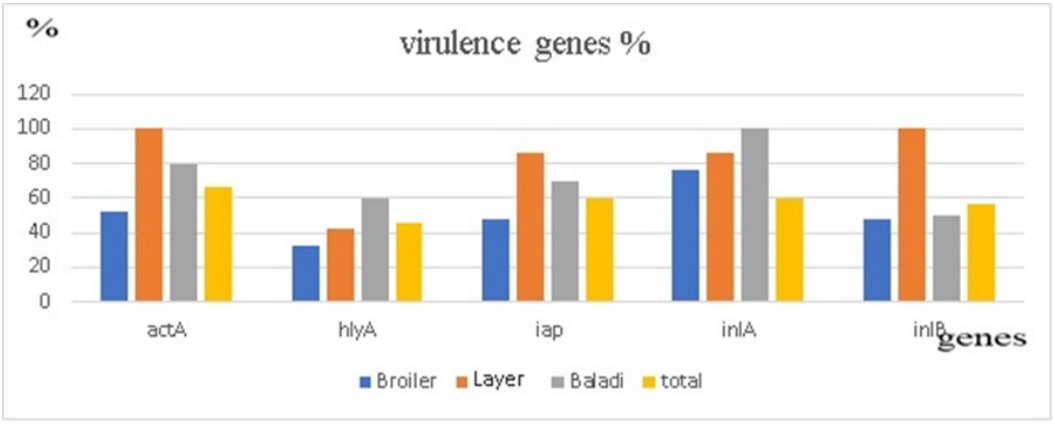

Figure 2. Percentage of virulence genes among different chicken breeds

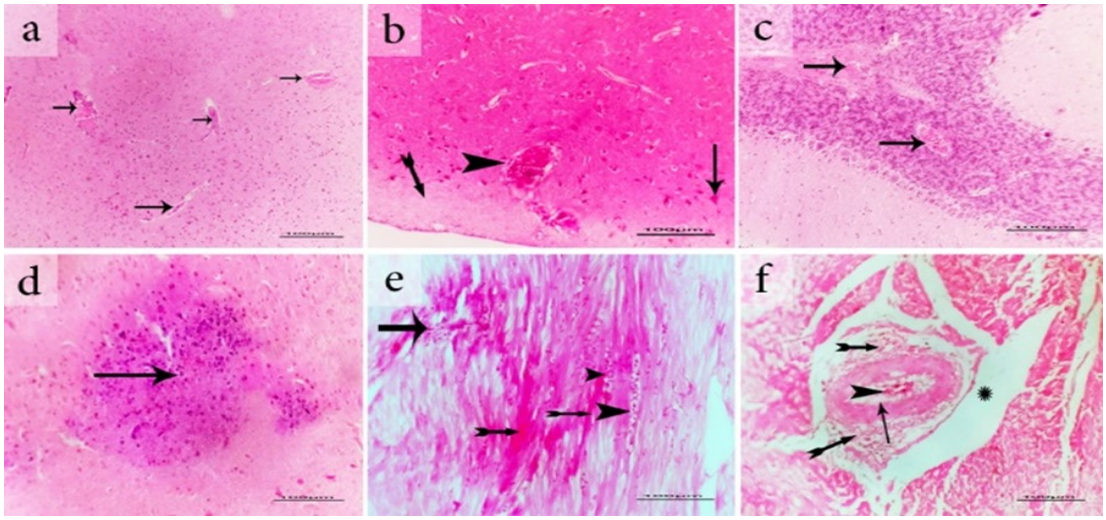

Figure 3. Photomicrograph of H&E-stained sections of chicken brain and heart infected with *Listeria monocytogenes* showing a) diffuse congestion of cerebral blood vessels (arrows). b) demyelination of some neurons (arrow) with submeningeal encephalomalacia (tailed arrow) and severe congestion (arrowhead). c) diffuse congestion of cerebellar blood vessels (arrows). d) encephalitis with focal inflammatory cells infiltrating the parenchyma (arrow). e) myocarditis with inflammatory cellular infiltration (arrow), diffuse vascular congestion (arrows head) and hyaline degeneration of some cardiac muscle fibers (tailed arrows). f) perivascular fibrosis (tailed arrows) and edema (asterisk) with vascular degenerations represented in endotheliosis (arrow) and congestion (arrowhead).

DISCUSSION

Live birds may be a significant vector for contaminating the processing environment and spreading Listeria to consumers, according to farm research (**Rothrock et al. 2017**). Additionally, *L. monocytogenes* can be found in both broilers and laying hens (**Ricke et al. 2023**). *Listeria monocytogenes* infection can be fatal, so it is important to seek veterinary care immediately if you observe any of these signs or symptoms in your birds. Additionally, it is important to note that the clinical signs may be similar to those of other diseases, such as avian salmonellosis and Newcastle disease **(Jamshidi & Zeinali 2019).** Therefore, it is important to have a veterinarian diagnose the cause of the illness so that appropriate treatment can be provided.

Among the examined birds (n = 550), *L. monocytogenes* could be detected in 7.6% (n = 42/550) of total samples, 8.3% (n = 25/300) of broiler chickens, 4.6% (n = 7/150) of layer chickens and 10% (n = 10/100) of Baladi chickens. Investigations of *L. monocytogenes* in broilers have ranged from as low as 5% (**Milillo et al. 2012**) to 26.5% (**Esteban et al. 2008**) and 30% (**Abdel Aziz and Mohamed, 2020**), while in layers they have varied between 1.6% (**Schwaiger et al, 2010**) and 4.7% (**Çokal1 et al. 2022**) and 31% (**Aury et al.,2011**). To the best of our knowledge, *L. monocytogenes* has not been detected in Baladi chickens yet. This variation may be attributed to the considerable relationship between hygienic standards and bacterial contamination. The lower the sanitary measures practiced in poultry farms, the more bacterial contamination, and consequently, the higher the isolation rate.

The antibiogram profile of the 42 *L. monocytogenes* isolates against eleven antibiotics displayed high resistance to streptomycin (83.3%), amoxicillin-clavulanic acid (78.5%), chloramphenicol (70.5%), erythromycin (64.6%), amoxicillin (64.6%), gentamicin (58.7%) and vancomycin (54.7%). Conversely, low resistance was seen against gentamycin and ampicillin (4.7% for each) with no resistance to vancomycin. Our findings corroborate earlier studies (**Akrami-Mohajeri et al. 2018; Abdeen et al. 2021, Abuhatab et al. 2022**) which showed high resistance of *L. monocytogenes* isolates against streptomycin; amoxicillin/clavulanic acid, and chloramphenicol and **Rahimi et al. (2010**) who detected that *Listeria* spp. were sensitive to vancomycin and gentamycin. The rapid increase of *Listeria* spp. antimicrobial resistance against the most prevalent antibiotics used to treat human and animal listeriosis may be due to antibiotic misuse. The intrinsic resistance of *L. monocytogenes* against these antibiotics is because of the lack or low affinity of the enzyme in the final step of cell wall synthesis (**Al-Nabulsi et al. 2015**).

Additionally, *Listeria monocytogenes* can become resistant to antimicrobial agents through the acquisition of three types of movable genetic elements, namely self-transferable plasmids, mobilizable plasmids, and conjugative transposons (**Moreno et al. 2014**). However, there is an increasing number of reports of *L. monocytogenes* spontaneously acquiring resistant genes through mutations.

Multiple drug resistance (MDR), also known as multidrug resistance or multiresistance is the antimicrobial resistance shown by a species of microorganism to at least one antimicrobial drug in three or more antimicrobial categories (**Magiorakos et al. 2012**). In our study, 59.5% of isolates were MDR, similar to that reported by **Jamshidi et al. (2019**). The Multiple Drug Resistance (MDR) Index values of 69.0% (29/42) of isolates in our investigation were >0.2. Our results were higher than **Al -Mayahi et al. (2020)** who reported that 15.4% of isolates were >0.2 and lower than **Mpondo et al. (2021)** who recorded that MARI values of all L. monocytogenes ranged between 0.87 and 1. In general, a MARI value >0.2 indicates that bacteria have been exposed and originate from an environment where antibiotics are potentially misused and the 'high-risk' source of contamination (**Khan et al. 2015**).

Several virulence genes have been found to play a vital role in the pathogenicity of *L. monocytogenes*, including *actA, hlyA, inlA, inlB,* and *iap* (**Khelef et al. 2006**). We used PCR technique to confirm bacteriologically isolated

L. monocytogenes by amplifying the 16S rRNA gene and screening for the five virulence genes of *L. monocytogenes*. Amplification of the 16S rRNA gene was found in all isolates and the *inlA* and *actA* genes were mostly detected in 83.3% and 66.6% of the examined samples, respectively. Previous reports have shown an increase in the prevalence of virulence genes in *L. monocytogenes*; the *inlA* virulence gene was detected in almost all of the *L. monocytogenes* isolates in Japan (**Sasaki et al. 2014**). In India, all *L. monocytogenes* isolates possess the *actA, hlyA* and *iap* virulence-related genes (**Kaur et al. 2018**). Additionally, the *iap* gene was detected in all isolates and *actA* in most of them (**Abuhatab et al. 2022**) in Egypt.

Our histopathological lesions were detected in both the brain and heart which were in complete accordance with **Gu et al. (2015)** who found lesions in the visceral organs characterized by monocyte infiltration, localized encephalitis, and meningitis. This was in partial accordance with **Crespo et al. (2013)** who found severe myocarditis with no lesions in the brain which could be attributed to the difference in the severity of the infection and immune status of birds.

CONCLUSION

This study provided a comprehensive picture of the prevalence, antibiotic resistance and virulence determinants of *L. monocytogenes* in various chicken breeds in Egypt. There was a slight difference in overall prevalence between the three breeds, with prevalence decreasing in layers. The situation of antibiotic resistance of *L. monocytogenes* in poultry farms presents a potential risk particularly in the absence of preventive measures and strict hygienic practices at human-poultry interface. Thus, ongoing monitoring of antimicrobial resistant pattern and virulence genes in *L. monocytogenes* in chickens is essential, as it allows us to gain a better understanding of future risks and treatment of the microorganism.

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