

Listeria Species in Broiler Poultry Farms: Potential Public Health Hazards

Hesham Dahshan^{1*}, Abdallah Mohamed Amin Merwad², and Taisir Saber Mohamed³

¹Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia Governorate, Egypt

²Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Egypt

³Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt

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*Corresponding author
Phone: +20-552383261;
Fax: +20-552283683;
E-mail: Dahshanhesham@gmail.com;
Dr_hisham2001@yahoo.com

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Broiler meat production worldwide has been plagued by lethal food-poisoning bacteria diseases, including listeriosis. A fatality rate of 15.6% was recorded in human beings in the EU in 2015. During 2013, a total of 200 poultry farm samples, including litter, chicken breast, farm feed, and drinking water, were collected to generate baseline data for the characterization of the genus *Listeria* in broiler poultry farms. *Listeria* spp. were detected in a total of 95 (47.5%) poultry farm samples. The isolates of *Listeria* spp. included *L. innocua* (28.5%), *L. ivanovii* (12.5%), *L. welshimeri* (4.5%), and *L. monocytogenes* and *L. seeligeri* (1% each). *Listeria* spp. contamination rates were higher in farm feed (70%), followed by litter (52.5%), chicken breasts (42.2%), and drinking water (10%). Almost all *Listeria* spp. isolates were resistant to more than three classes of antibiotics (multidrug resistant). Besides this, we observed a significant resistance level to penicillin and fluoroquinolone drugs. However, lower resistance levels were recorded for broad-spectrum cephalosporins. The *inlA*, *inlC*, and *inlJ* virulence genes were detected in almost all of the *L. monocytogenes* isolates. Thus, food safety management approaches and interventions at all stages of the broiler rearing cycle were needed to control cross-contamination and the zoonotic potential of listeriosis.

Keywords: Poultry farm, *Listeria* spp., antibiotic resistance, virulence genes, health hazards

Introduction

Egypt's modern poultry industry began in 1964 with the establishment of the National General Poultry Company, which is government-owned, to provide Egypt's fast growing human population with high-quality, affordable animal protein. In 1965, the Government encouraged the expansion of the rural small-scale family and private poultry sectors to fulfill the growing demand for poultry products. Unfortunately, the livestock population of Egypt has been plagued by lethal food-poisoning bacteria diseases, including listeriosis. However it is considered a rare form of food poisoning and one that is treatable. It is, nevertheless, a serious type of food poisoning that is particularly dangerous for pregnant women, the elderly, and anyone with poor immunity. According to the recent European Community Report, 1,763 confirmed human cases of listeriosis were

recorded in the European Union (EU) in 2015, and the reported case fatality rate was 15.6% [13].

Listeria spp. are ubiquitous and have been isolated from poultry, animals, different types of foods, and environments worldwide [9]. Among the different species of the genus *Listeria*, *Listeria monocytogenes* is the causative agent of listeriosis [27]. Although poultry flocks are rarely reported as a vector of *Listeria* outbreaks, the relatively high prevalence of *L. monocytogenes* in chicken farms presents a potential risk. In Egypt, some studies have already investigated the prevalence of *L. monocytogenes* in chicken carcasses or fresh chicken meat [1, 2]. However, scanty data are available on *Listeria* spp. prevalence, including *L. monocytogenes*, in chicken farm samples, especially environmental samples such as litter, drinking water, and poultry feed.

Owing to the severity and case fatality rate of listeriosis, treatment with antimicrobial agents is essential for the

resolution of the infection. Therefore, the emergence of antibiotic resistance in the genus *Listeria* (especially, *L. monocytogenes*) may have public health consequences. This consideration led to an EU-wide ban on the use of antibiotics as growth promoters in animal feed [6]. Moreover, an announcement of the US Food and Drug Administration from April 2012 suggests stopping application of antibiotics for growth promotion on a voluntary basis (www.fda.gov). In spite of all admitted bans, multidrug-resistant isolates of *L. monocytogenes* are still reported in poultry, animals, foods, and in human case samples. To the best of our knowledge, there are very few data on the prevalence of multidrug-resistant *Listeria* spp. from poultry farm environmental samples worldwide. Accordingly, in Egypt's modern poultry industry, monitoring of multidrug-resistant *Listeria* spp. is crucial.

Hence, the objective of the present study was to generate baseline data for the contamination rate of the genus *Listeria* in broiler poultry farms; identify the *Listeria* spp. prevalence rate; determine the phenotypic antimicrobial resistance patterns of isolated species; and detect virulence-associated genes of *L. monocytogenes* isolates.

Materials and Methods

Sample Collection

The study was carried out in Sharkia Governorate, which has one of the major sources of animal protein, the poultry sector, in Egypt. Ten broiler poultry farms in Sharkia district were selected for investigation by a simple random sampling method. A total of 200 samples were collected, 20 samples/farm, during the summer of 2013. Samples for each comprised litter ($n = 8$), raw chicken meat carcass ($n = 8$), poultry feed ($n = 2$), and drinking water ($n = 2$). From each broiler farm, pooled samples of litter, feed, and water were taken. The litter sample was collected in a zigzag manner from different areas of the farm floor, then pooled to form one uniform sample, and packed separately in sterile plastic bags. Feed samples were obtained from feeders in front of the birds. Meanwhile, water samples were collected in 150-ml sterile glass bottles from drinkers. Chicken meat carcass samples were collected from raw chicken breasts. The region of the breast was favored

because a new consumer report study found that about 97% of all chicken breasts contain harmful bacteria (<http://www.allgov.com>). A piece of approximately 30 g from the breast was obtained from each carcass. After that, all collected samples were immediately transported under cooled conditions to the laboratory and stored in the dark at 4°C prior to analysis.

Detection of *Listeria* spp.

Detection of *Listeria* spp. was carried out using ISO11290-1 protocol [19]. Half Fraser broth and Half Fraser supplement (Oxoid, UK) were used as a primary selective enrichment medium. Briefly, 25 g of each sample was pre-enriched and then enriched by the secondary selective Fraser broth (containing Fraser broth base and Fraser supplement) for 24 h at 37°C. Finally, a loopful of enrichment broth culture was streaked onto Chromogenic *Listeria* agar (ISO) (CM1084; Oxoid) and incubated at 37°C for 24 h. Plates were examined for turquoise blue colonies with or without precipitation zones. The presumed colonies were purified on tryptone soya yeast glucose agar. The purified colonies were verified by biochemical tests and the Microbact *Listeria* 12L system (MB1128; Oxoid).

Phenotypic Detection of Antimicrobial Resistance in *Listeria* spp. Isolates

Antibiotic susceptibility testing was done for all recovered isolates via standard disc diffusion on Mueller–Hinton agar (Oxoid 337) using the Kirby-Bauer method [4]. The antimicrobials tested were ceftriaxone (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), amoxicillin/flucloxacillin (10 µg), amoxicillin/clavulanate (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), and ofloxacin (5 µg). All antibiotic discs were provided by Oxoid. After incubating the inoculated plate, the susceptibility of the isolates to each antimicrobial agent was assigned according to break points recommended previously [10, 21]. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *E. coli* ATCC 35218 were used as quality control strains [8].

Detection of Virulence Genes in *L. monocytogenes* Isolates

After confirming the *L. monocytogenes* isolates via biochemical identification kit, the isolates were identified for the presence of virulence markers (*inlA*, *inlC*, and *inlJ*) using multiplex PCR as described by Liu *et al.* [22]. The primers and PCR conditions are presented in Table 1.

Table 1. Primers and PCR conditions used for *L. monocytogenes* virulence gene detection.

Virulence gene	Primers	PCR conditions	Amplicon Size (bp)
<i>inlA</i>	F-(ACG AGT AAC GGG ACA AAT GC) R-(CCC GAC AGT GGT GCT AGA TT)	- Initial denaturation at 94°C for 2 min - Annealing and extension: 30 cycles of	800
<i>inlC</i>	F-(AAT TCC CAC AGG ACA CAA CC) R-(CGG GAA TGC AAT TTT TCA CTA)	94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min	517
<i>inlJ</i>	F-(TGT AAC CCC GCT TAC ACA GTT) R-(AGC GGC TTG GCA GTC TAA TA)	- Final extension at 72°C for 10 min	238

Results and Discussion

Prevalence of *Listeria* spp. in Broiler Poultry Farm Samples

Although broiler poultry production has been one of the fastest growing industries in Egypt and people rely on poultry sectors as a primary animal protein source, there are limited reports documenting the prevalence and characterization of *Listeria* spp., including *L. monocytogenes*, in poultry farm environmental samples. Our results revealed that out of 200 tested samples, 95 (47.5%) were contaminated with *Listeria* spp., out of which 42, 37, 14, and 2 were isolated from broiler poultry farm litter, raw chicken meat carcass, farm feed, and farm drinking water, respectively (Table 2). The *Listeria* spp. isolates included *L. monocytogenes* ($n = 2$, 1%), *L. innocua* ($n = 57$, 28.5%), *L. ivanovii* ($n = 25$, 12.5%), *L. welshimeri* ($n = 9$, 4.5%), and *L. seeligeri* ($n = 2$, 1%). All recovered *Listeria* isolates were confirmed using the Microbact *Listeria* 12L system.

A higher prevalence of *Listeria* spp. indicates a significant public health hazard associated with litter, feeder, drinking systems, and/or the consumption of contaminated poultry or poultry products. The isolation rate of *L. monocytogenes* was in line with a few studies that have reported a low prevalence from 0 to 4.3% [11, 24]. However, with other countries such as France [3, 7] and Thailand [20], the rate was lower. A higher prevalence of 5.7% was observed in raw chicken meat in China [28]. Moreover, *L. monocytogenes* was isolated (based on PCR) from northern Spain at a prevalence of 26.5% [14] and 32% [7]. The differences in the prevalence of *L. monocytogenes* in poultry farm samples in different countries might be attributed to the sensitivity of bacteriological detection methods. The presence of *L. monocytogenes* in poultry farms even in low prevalence threatens public health and it may cause several diseases in children, pregnant women, the immune-compromised, and elderly.

L. innocua was the predominant *Listeria* spp. in our study. This finding is comparable to the results reported by other investigators [15, 23]. *L. innocua* is important because it is

closely related to *L. monocytogenes* and they are genetically similar. Despite that *L. innocua* is non-pathogenic in character, it was recognized as a reservoir of antibiotic resistance (AB^R) for *L. monocytogenes* [5].

Although *L. ivanovii* is found almost exclusively in ruminants (mainly sheep), it was isolated from broiler poultry farm samples as it was the second predominant spp. (12.5%) in our study (Table 2). Early studies even document the link between ruminant listeriosis and contaminated poultry litter [12, 25]. The contamination of broiler farm samples with *L. ivanovii* might be due to the nature of the litter material used that is mainly from pasture-raised animals (such as wood shavings, hay, or chopped rice straw), which provides the suitable environment for *Listeria* spp. cross-contamination [18]. Furthermore, broiler poultry farms in Egypt have very poor biosecurity practices, mainly because the farms are built very close to each other in cluster farm formation (large flock size), and poor rodent and vector controls and hazardous waste disposal, which may provide more chances for introduction of *Listeria* spp. or any other pathogen to the flocks. Therefore, more attention is required as *L. ivanovii* is an enteric opportunistic human pathogen.

Broiler Poultry Farm Sample Contamination Rate

Our findings demonstrated a high contamination rate of *Listeria* spp. in broiler feed (70%, 14/20), followed by broiler farm litter, raw chicken meat carcass, and broiler farm drinking water in which *Listeria* spp. prevalence was 52.5% (42/80), 46.2% (37/80), and 10% (2/20), respectively (Fig. 1). In Egyptian broiler poultry farms, feed is formed mainly from ingredients mixed on the farm by the farmer using a small mechanical simple crushing and mixing unit (vertical mixing). This trend is having many production problems and is related to poultry feed contamination. Besides this, part of this contamination could have also originated from bird feces that may have contaminated the manual hanging feeders and from litter contamination due to maladjusted feeders. Therefore, it is not surprising to

Table 2. Prevalence of *Listeria* spp. isolated from broiler poultry farms, Egypt.

Poultry farm samples	No. of samples	<i>Listeria</i> spp.	<i>L. monocytogenes</i>	<i>L. innocua</i>	<i>L. ivanovii</i>	<i>L. welshimeri</i>	<i>L. seeligeri</i>
Litter	80	42 (52.5%)	2 (2.5%)	28 (35%)	9 (11.2%)	3 (3.7%)	N/A
Raw chicken meat	80	37 (46.2%)	N/A	25 (31.2%)	9 (11.2%)	1 (1.2%)	2 (2.5%)
Feed	20	14 (70%)	N/A	4 (20%)	5 (25%)	5 (25%)	N/A
Water	20	2 (10%)	N/A	N/A	2 (10%)	N/A	N/A
Total	200	95 (47.5%)	2 (1%)	57 (28.5%)	25 (12.5%)	9 (4.5%)	2 (1%)

N/A: None.

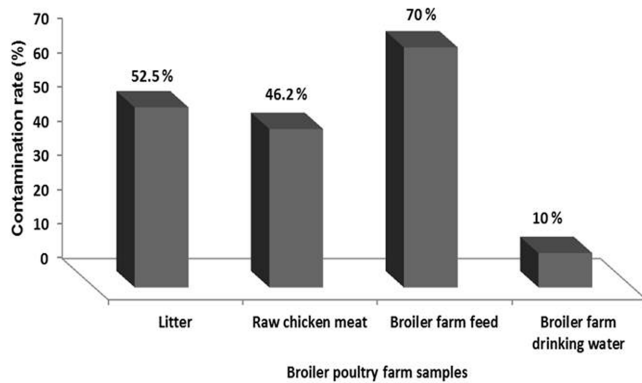


Fig. 1. Contamination rate of *Listeria* spp. in different farm samples, Egypt.

find that the prevalence rates of *Listeria* spp. in broiler feed and litter samples were higher than other farm sample types. Among the analyzed categories, raw chicken meat carcass showed relatively higher level of contamination with *Listeria* spp., preceded by feed and farm litter samples, with a prevalence that reached 46.2% (Fig. 1). This could strengthen the fact that cross-contamination in broiler poultry farms must be considered in developing strategies by food safety and regulatory authorities to control *Listeria* spp. in broiler farms, and to avoid contamination of poultry meat and poultry products that are intended to be consumed.

Antimicrobial Susceptibility

The antimicrobial susceptibility of 95 *Listeria* spp. isolates to eight antibiotics was investigated using the disc diffusion method (Table 3). The results indicate high levels of resistance to amoxicillin/clavulanate (40%), followed by norfloxacin (38%), amoxicillin/flucloxacillin (35%), ofloxacin (32%), and ciprofloxacin (25%). However, lower resistance levels were recorded for ceftazidime (22%), followed by cefoxitin

(15%) and ceftriaxone (14%). Despite the fact that isolates of *L. monocytogenes*, as well as strains of other *Listeria* spp., are susceptible to a wide range of antibiotics [17], almost all *Listeria* spp. isolates in this study were resistant to more than three classes of antibiotics (multidrug resistant). Unfortunately, it is obvious from our results that all *L. monocytogenes* isolates (100%) were resistant to penicillin group (amoxicillin/clavulanate and amoxicillin/flucloxacillin) drugs, and to our best knowledge, the treatment of choice for human listeriosis remains the administration of ampicillin or penicillin G. Acquisition of these trends of resistance would represent a major therapeutic problem in clinical settings, as it is now established that food-borne transmission constitutes the main route of acquisition of listeriosis [16]. The observed high rate of resistance to penicillin drugs might be ascribed to mis- or overuse of the drugs, as they are relatively cheaper and readily available to the local community.

The majority of *Listeria* isolates in our study were resistant to the tested fluoroquinolone group (norfloxacin, ofloxacin, and ciprofloxacin). Although The World Health Organization (WHO) has classified fluoroquinolone drugs as critically important in human medicine, fluoroquinolones are still introduced into the feed and water of industrially raised poultry in Egypt, primarily to make them grow faster rather than to treat disease.

In general, *Listeria* strains have been announced to be naturally susceptible and resistant to penicillins and modern cephalosporins, respectively [26]. Thus, it is surprising to note the contrary in our results (Table 3). It might be possible that alterations in the affinity of the cell walls (penicillin-binding protein 3) contribute to the differences in susceptibility to the broad-spectrum cephalosporins and penicillins. Future studies will be needed to elucidate the mechanisms contributing to the observed antimicrobial

Table 3. Antimicrobial resistance profiles of *Listeria* spp. isolated from broiler poultry farms, Egypt.

Antimicrobial agent	<i>Listeria</i> spp. (n = 95)	<i>L. monocytogenes</i> (n = 2)	<i>L. innocua</i> (n = 57)	<i>L. ivanovii</i> (n = 25)	<i>L. welshimeri</i> (n = 9)	<i>L. seeligeri</i> (n = 2)
Ceftriaxone	14 (14.7%)	1 (50%)	8 (14%)	5 (20%)	N/A	N/A
Cefoxitin	15 (15.8%)	N/A	11 (19.3%)	4 (16%)	N/A	N/A
Ceftazidime	22 (23.1%)	1 (50%)	10 (17.5%)	9 (36%)	2 (22.2%)	N/A
Amoxicillin/flucloxacillin	35 (36.8%)	2 (100%)	22 (38.6%)	8 (32%)	3 (33.3%)	N/A
Amoxicillin/clavulanate	40 (42.1%)	2 (100%)	23 (40.3%)	10 (40%)	5 (55.5%)	N/A
Ciprofloxacin	25 (26.3%)	1 (50%)	17 (29.8%)	5 (20%)	2 (22.2%)	N/A
Norfloxacin	38 (40%)	1 (50%)	22 (38.6%)	10 (40%)	5 (55.5%)	N/A
Ofloxacin	32 (33.7%)	2 (100%)	19 (33.3%)	8 (32%)	3 (33.3%)	N/A

N/A: None.

resistance patterns among *Listeria* spp.

Virulence Genes in *L. monocytogenes* Isolates

As differentiation between virulent and non-virulent *L. monocytogenes* isolates is significant for evaluating the potential hazards of this microorganism for food safety and public health, *L. monocytogenes* isolates ($n = 2$) were tested for the presence/absence of virulence genes *inlA*, *inlC*, and *inlJ*. The *inlA* gene was observed in all of the *L. monocytogenes* isolates (100%). However, *inlC* and *inlJ* were detected in only one isolate (50% each). The differences in the levels of surface-associated internalins (A/C/J) may be correlated to *L. monocytogenes* serotype. Further characterization studies are needed to highlight this correlation. The presence of internalin genes in *L. monocytogenes* isolates suggests that these isolates could potentially cause human diseases. To the best of our knowledge, this is the first study to examine *L. monocytogenes* isolates from environment samples of broiler poultry farms for the presence of surface-associated internalin genes.

In conclusion, a high contamination rate of *Listeria* spp. was demonstrated in broiler poultry farm samples in Egypt. The detection of *L. ivanovii* for the first time in poultry farms highlights the needs to control cross-contamination of farm feed and litter. Drug-resistant, including multidrug-resistant, *Listeria* spp. were found circulating among farm samples, posing high risk of infection for consumers. Moreover, an interesting aspect of the present study is the *Listeria* resistance patterns to penicillins and broad-spectrum cephalosporins. Finally, potentially pathogenic *L. monocytogenes* isolates from poultry farm environmental samples were recorded. Therefore, food safety management approaches and interventions at all stages of the broiler rearing cycle are needed to control the zoonotic potential of listeriosis.

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