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Genetic Characterization and Antibiogram Profiles of *Listeria* species Isolated from Poultry and Poultry Handlers

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Abstract

Up to now, there has been limited information regarding the circulation of pathogenic *Listeria* species in poultry and their contact handlers. The present study was, therefore, planned to evaluate the potential existence of *Listeria* spp. in different poultry species and their products as well as their contact workers at Dakahlia Governorate, Egypt and to address the antimicrobial profile of *Listeria* isolates using classical microbiological techniques and multiplex PCR targeting three virulence-associated genes (*iap*, *hly*A, and *act*A). The study included cloacal swabs from different poultry species (n =200), poultry carcasses (n = 25), poultry products (25 samples each of chicken luncheon and frozen chicken breast fillets), chicken eggs (n = 50) and stool specimens from the contact workers (n = 25). Findings showed that 60 samples out of 350 (17.14%) were found to be positive to *Listeria* spp., which were identified as follow: *L. innocua* (28.3%), *L. ivanovii* (28.3%), *L. monocytogenes* (15%) and *L. grayi* (15%), *L. seeligeri* (10%) and *L. welshimeri* (3.3%). *L. monocytogenes* showed high resistance to nalidixic acid (100%) followed by neomycin (85.1%), streptomycin (80%), cefotaxime (65%) and penicillin g (58.3%). Results could likely suggest that poultry and poultry products had a significant role in dissemination and transmission of virulent and multidrug resistant *L. monocytogenes* which represent a potential risk particularly in the absence of strict hygienic practices and preventive measures. A constant monitoring of pathogenic *L. monocytogenes* is thus urgently needed to avoid the dissemination of such pathogenic strains in food production chains.

KEYWORDS
Listeria monocytogenes, Antibiotics resistance, Multiplex PCR, Poultry, public health.

INTRODUCTION

Poultry production continues to grow globally, both in developed and in the developing countries whereas, fresh chicken meat and chicken products is universally popular and become the consumers' first choice (Kralik *et al*., 2018). In Egypt, broilers are raised on deep litter, which can be easily contaminated with pathogenic microorganisms as *L. monocytogenes* (Dahshan *et al*., 2016). However, little is known about the prevalence of *Listeria* spp. especially zoonotic *L. monocytogenes* throughout poultry and poultry products.

Listeria monocytogenes is a member of the *Listeria* genera that belong to the firmicutes division, it is a Gram-positive food borne pathogen and cause severe human diseases. *Listeria* species especially *Listeria monocytogenes* is present in different environmental sources (e.g., water, soil, decaying vegetation, sewage, slaughterhouse waste and silage) (Şanlıbaba *et al*., 2018). Cattle, sheep, goats, poultry, insects, ticks and crustaceans can serve as carriers for *Listeria* species, whereas these species have been isolated from the intestinal content of healthy animal and from stools of healthy humans (Matle *et al*., 2020). Diverse food products including dairy products, ready-to-eat foods, fish, and fish products have been linked to *L. monocytogenes* outbreaks because of their ubiquitous characters (Abdeen *et al*., 2021). The pathogenicity of *L. monocytogenes* has been controlled by several virulence factors which are responsible for entrance and replication within the host cells as well as microbial adherence and invasion (Matereke and Okoh, 2020). It has been shown that actin polymerization protein (*act*A) is a bacterial surface protein and responsible for intracellular movement of the *L. monocytogenes* within the cytoplasm (Matereke and Okoh, 2020). Furthermore, the invasion-associated protein (*iap*) is an extracellular protein p60 that is encoded by *iap* gene and listeriolysin O (LLO encoded by *hly*A) have been found as a key role in virulence and pathogenicity of this bacterium (Soni *et al*., 2014).

L. monocytogenes is often susceptible to different classes of antibiotics; however, the excessive and uncontrolled use of antibiotics in humans and poultry could likely lead to spread and dissemination of antibiotic resistance among food borne bacteria, including *L. monocytogenes* (Wilson *et al*., 2018). In Egypt, there has been limited data regarding the potential existence of *L. monocytogenes* in poultry, poultry products and contact workers. Therefore, the present study was set to provide updated information regarding the circulation of *Listeria* spp., including the pathogenic *L. monocytogenes* in poultry, poultry products and contact workers.

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MATERIALS AND METHODS

Samples collection and preparation

The study comprised 350 samples collected from different sources including cloacal swabs from different poultry species (n =200), poultry carcasses (n = 25), poultry products (25 samples each of chicken luncheon and frozen chicken breast fillets), chicken eggs (n = 50) and stool specimens from the contact workers (n = 25). The cloacal swabs were collated from different poultry species including broilers (n = 40), layers (n = 40), ducks (n = 40), pigeons (n = 40), and turkeys (n= 40). The samples were collected from different localities in Dakahlia governorate, Egypt in the period between November 2020 and June 2021. All samples were collected in sterile plastic cups and placed into individual sterile bags and were sent immediately in insulated coolers to the laboratory of Hygiene and Zoonoses, Faculty of Veterinary Medicine, Mansoura University for bacteriological examination. All procedures were approved by Mansoura University Ethical Committee.

Isolation and identification of Listeria

Isolation and identification of *Listeria* was performed according to the standard protocol provided by (ISO 2017). The collected swabs from poultry, eggs and human stool samples were inserted into 9 ml of tryptone soya broth (TSB) tubes and incubated at 30°C for 24 h. For poultry carcasses and poultry products 25 g from each sample was added to 225 ml TSB and incubated at 30°C for 24 h. Ten ml from the initial suspension were transferred to a sterile tube containing 90 ml of *Listeria* enrichment broth base with selective supplement (Oxoid, SR0141) and incubated at 30 °C for 48 h. A loopful from enriched broth was streaked on *Listeria* selective agar (Oxford formulation) with *Listeria* selective supplement (Oxoid, SR0140). Then incubated at 37°C for 48 h. Suspected colonies (gray-green colonies surrounded by black zone) were picked up and sub-cultured on tryptone soya agar (Jajere *et al*., 2014), slopes then incubated at 37°C for 24 h. (Roberts *et al*., 1995). The suspected colonies were then identified according to Hitchins (2001) using a panel of biochemical tests (oxidase, catalase, and motility, hemolysis on blood agar, CAMP, and fermentation of rhamnose, xylose, and mannitol).

Molecular characterization

All biochemically suspected *L. monocytogenes* isolates were tested by multiplex PCR targeting three virulence-associated genes (*iap*, *hly*A, and *act*A) as previously described by Kaur *et al*. (2007).

DNA extraction

Genomic bacterial DNA was extracted by GeneJET purification kit (Fermentas) based on the manufacturer`s guidelines.

Multiplex PCR

Multiplex PCR was carried out using Master cycler, Eppendorf, Hamburg, Germany. The used primers sequences and their corresponding amplicon sizes are listed in Table 1. The reaction was performed in a final volume of 50 µl: 10 µl PCR buffer (100 mmol Tris–HCl, pH 8.3; 500 mmol KCl; 15 mmol MgCl2 and 0.01 percent gelatin), 1 mmol dNTP mix, 7.5 mmol MgCl2, 10 mmol forward and reverse primers of each set, 5 U Taq DNA polymerase, 5 µl of cell lysate and completed to 50 µl with DNA/RNA free water with the following thermal profile: Initial denaturation of genomic DNA at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds, and extension at 72°C for one minute, followed by a final extension for 10 minutes at 72°C. Agarose gel electrophoresis using 1.5 % agarose stained with ethidium bromide in 1x TBE buffer was performed to visualize the amplified DNA fragments, using a UV transilluminator.

Antibiotic sensitivity assay

Antimicrobial susceptibility of the recovered *Listeria* spp. to neomycin (N30), ciprofloxacin (CF30), doxycycline (DO30), erythromycin (E15), meropenem (M10), gentamycin (G10), cephalothin (CN30), penicillin–G (P10), sulphamethoxazol (SXT25), streptomycin (S10), tetracycline (T30), nalidixic acid (NA30), cefotaxime (CF30), ampicillin (AM10) and amikacin (AK30) was tested using disc diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2017).

Statistical analysis

Data were transferred to Microsoft excel sheet for analysis, descriptive analysis was carried out using percentages. Antimicrobial resistance rates were analyzed using non-parametric tests (chi-square test) using statistical package for social sciences (SPSS, Inc., version 22.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

The presence of microorganisms in food products at retail outlets is considered a serious flaw in quality control procedures (El-Demerdash and Raslan, 2019). In Egypt, *L. monocytogenes* is predominantly transmitted to humans through infected meat and poultry products (Abd El-Malek *et al*., 2010). The ability of the bacterium to proliferate in the refrigerator or under chilled conditions in a variety of food products makes it difficult to control. In the present study, *Listeria* spp., was identified in 17.14% (60/350) from the examined samples. Of which,15.5% (31/200) were recovered from the cloacal swabs; while 22.5%, 22.5%, 12.5%, 10%, and 10% were isolated from broilers, layers, ducks, pigeons, and turkeys, respectively. The most predominant *Listeria* spp. identified in the examined cloacal swabs were *L. ivanovii* (n=

Table 1. Oligonucleotide primers sequence used for PCR with amplicon sizes.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
iap (F)	5′ ACAAGCTGCACCTGTTGCAG ′3	131	Swetha *et al*. (2012)
iap (R)	5′ TGACAGCGTGTGTAGTAGCA ′3		
*hly*A (F)	5′ GCAGTTGCAAGCGCTTGGAGTGAA ′3	456	
*hly*A (R)	5′ GCAACGTATCCTCCAGAGTGATCG ′3		
*act*A (F)	5′ CGCCGCGGAAATTAAAAAAAGA ′3	839	Suárez *et al*. (2001)
*act*A (R)	5′ ACGAAGGAACCGGGCTGCTAG ′3		

10, 32.3%), *L. innocua* (n= 9, 29%) and *L. monocytogenes* (n=6, 19.4%). Findings from this study agreed with that reported by Petersen and Madsen (2000) who identified *Listeria* spp. in 17% out of fecal samples yielding an overall prevalence of 14% (10/71) among 71 Danish broiler flocks. In contrast, a lower prevalence of *Listeria* spp. (4.7%) was reported in Tokyo by Iida *et al*. (1991).

L. monocytogenes was identified in 19.4% (6/31) from the examined cloacal swabs (Tables 2 and 3). A high rate of occurrence of *L. monocytogenes* from poultry cloacal swabs was previously reported in France (31.7%) (Aury *et al*., 2011) and in Nigeria (91.5%) (Ishola *et al*., 2016). However, several recent studies have failed to isolate *L. monocytogenes* from broilers cloacal swabs (Iannetti *et al*., 2020). In another study, Rothrock *et al*. (2017) reported that *L. monocytogenes* was identified among broiler flocks with a range from 0 to 32%. The circulation of *Listeria* spp., especially *L. monocytogenes* in poultry with variable results makes poultry a potential source for *Listeria* spp. and *L. monocytogenes*.

In this study, *Listeria* species were identified in 15 samples of poultry products out of 75 (20%), in which 32% (8/25) came from chicken carcasses (raw meat), 16% (4/25) from chicken luncheon and 12% (3/25) came from frozen chicken breast fillets (Table 2). A high rate of occurrence of *Listeria* species was recorded previously in Egypt (Dahshan *et al*., 2016) from raw chicken meat although *L. monocytogenes* could not be determined from frozen chicken breast fillets in the present study. Several other studies have revealed different detection rates of *L. monocytogenes* in raw chicken meat (AL-Jobori *et al*., 2016; Ahmed *et al*., 2017), who identified *L. monocytogenes* in raw chicken with the percentage of 10% and 8%, respectively. In addition, 16% of the examined luncheon samples (4/25) were found positive to *Listeria* spp. Of which, two isolates were identified as *L. monocytogenes*. The obtained results were lower than those reported by Mahmoud *et al*. (2019), who isolated *Listeria* spp. from eight out of 20 luncheon samples (40%). A comparatively low result (4%) was also reported by Mohamed *et al*. (2016), who isolated *Listeria* spp. from 25 luncheon samples. In contrast, some authors have failed to detect *Listeria* spp. from the luncheon samples (Meshref *et al*., 2015). The low contamination level demonstrated in luncheon samples might be explained by the exposure of luncheon to a high temperature during the manufacturing, which in turn led to thermal inactivation of *Listeria* spp.

In the present study, the detection rate of *Listeria* spp. among the examined eggs samples was 24% (12/50 (Table 2), in which 36% (9/25) were recovered from eggshells, while 12% (3/25) were identified in eggs contents. However, *L. monocytogenes* was not determined from the examined egg samples. Different isolation rates were previously identified in raw egg from France and Egypt (Rivoal *et al*., 2010; Amin, 2017); whereas *L. monocytogenes* was determined in 17.3% and 6.7% from eggs samples, respectively. It becomes clear that eggshells had higher contamination rate than egg contents, these results could be attributed to the possibility of bacterial contamination of the shell after laying because shell is the first line for penetration whereas, intrinsic and extrinsic variables influence bacterial penetration (Amin, 2017).

In the present study, two samples out of 25 (8%) were positive for *Listeria* spp. in the examined stool specimens of poultry handlers (Table 2). Similar isolation rates were reported in Egypt by Abd El-Malek *et al*. (2010), 7.14 %; Al-Ashmawy *et al*. (2014), 5% and EL-Naenaeey *et al*. (2019), 8% from children, dairy handlers and pregnant women, respectively. In addition, high results of *L. monocytogenes* were mentioned by Abuhatab (2018), 11.1%; Abd EL-Aziz and Mohamed (2020); 12.5% from aborted women and patients attending the outpatient clinics of Beni-Suef University Hospital, Egypt, respectively. In contrary, low results were observed by Awadallah and Suelam (2014) who isolated *L. monocytogenes* in a percentage of 2.5% of stool samples collected from persons eating RTE meat. The current study indicated that *L. innocua* and *L. ivanovii* were the predominant species, representing 28.3% out of the identified *Listeria* spp. This finding was comparable to those reported by other researchers (Dahshan *et al*., 2016; Meshref *et al*., 2015) in percentage of 28.5 and 22.2, respectively. *L. innocua* is closely related to *L. monocytogenes* and both are genetically similar (Gwida *et al*., 2020). Other *Listeria* species, as *L. ivanovii*, *L. welshimeri*, and *L. seeligeri*, have also been found in environmental farm samples or chicken feces but their detection remains rare (Iida, 1991).

In this study, *L. monocytogenes* strains were serotyped (Table 3). The results indicated that the highest occurrence of serogroup 4b in chicken luncheon, turkeys' cloacal swabs and layers' cloacal swabs, followed by serogroup 1/2a in eggshells, layers' cloacal swabs and ducks' cloacal swabs. The obtained results were in harmony with those reported in Brazil and Iran (Almeida *et al*., 2017;

Table 2. Recovery rate of *Listeria* spp. and *L. monocytogenes* isolated from the examined samples.

Type of examined samples	Source of samples	No. of examined samples	+ve *Listeria* spp.	+ve *L. innocua*	+ve *L. ivanovii*	+ve *L. monocytogenes*	Chi-square test
Cloacae swabs	Broilers	40	9 (22.5 %)	4/9	4/9	0/9	$(\chi2) = 119.980$ P < 0.05*
	Layers	40	9 (22.5 %)	2/9	1/9	3/9	
	Ducks	40	5 (12.5%)	2/5	2/5	1/5	
	Pigeons	40	4 (10%)	1/4	1/4	1/4	
	Turkeys	40	4 (10%)	0/4	2/4	1/4	
	Total	200	31 (15.5%)	9/1	10/31	6/31 (19.4%)	
Poultry products	Chicken carcasses	25	8 (32%)	2/8	2/8	0/8	$(\chi2) = 29.240$ P < 0.05*
	Chicken luncheon	25	4 (16%)	1/4	1/4	2/4	
	Frozen chicken breast fillets	25	3 (12%)	2/3	1/3	0/3	
Eggs	Eggshells	25	9 (36%)	2/9	2/9	1/9	$(\chi2) = 16.400$ P < 0.05*
	Eggs contents	25	3 (12%)	1/3	0/3	0/3	
Human	Stool specimens	25	2 (8%)	0/2	1/2	0/2	
Total		350	60 (17.14%)	17 (28.3%)	17 (28.3%)	9 (15%)	

The chi-square association of recovery rate of *Listeria*spp.in different sources. *: Statistically significant at $p \leq 0.05$

Table 3. Virulence, antimicrobial resistance profile and serotyping of *L. monocytogenes* strains

No	Source	Virulence genes	Antimicrobial resistance profile	MAR index	Serotype
1	Chicken luncheon	*iap*, *hyl*A, *act*A	NA, N, S, CF, P, DO, CN, T, CP, E, AK, SXT, M, G	1	4b
2	Chicken luncheon	*iap*, *act*A	NA, N, S, CF, P, DO, CN, T, CP, E, AK, SXT	0.857	7
3	Eggshells	*iap*, *act*A	NA, N, S, CF, P, DO, CN, T, CP, E	0.714	1/2a
4	Layers' cloacal swabs	*iap*, *hyl*A, *act*A	NA, N, S, CF, P, DO, CN, T, CP	0.643	1/2a
5	Layers' cloacal swabs	*iap*, *hyl*A, *act*A	NA, N, S, CF, P, DO, CN	0.5	1/2c
6	Turkeys' cloacal swabs	*iap*, *hyl*A, *act*A	NA, N, S, CF, P, DO, CN	0.5	4b
7	Ducks' cloacal swabs	*iap*, *hyl*A	NA, N, S, CF	0.286	1/2a
8	Layers' cloacal swabs	*iap*, *hyl*A, *act*A	NA, N, S	0.214	4b
9	Pigeons' cloacal swabs	*iap*, *act*A	NA	0.071	1/2b

Ranjbar and Halaji 2018), where serotypes 4b, 1/2a, 1/2b and 1/2c, were the main identified serotypes detected in humans and in food sources. The ability of *L. monocytogenes* to form biofilm is highly related to lineage II rather than lineage I strain, which helps also to explain the high frequency of serotypes 1/2a or 3a within the isolates assessed in the study done by Carvalho *et al.* (2019).

Treating human listeriosis can be a challenging task since *L. monocytogenes* can invade nearly all cell types. However, antibiotics have long been used successfully to treat human listeriosis. In general, ampicillin or penicillin G in combination with an aminoglycoside such as gentamicin is the antibiotic of choice for the treatment of human listeriosis. Trimethoprim in combination with sulfamethoxazole-trimoxazole is considered second-line therapy. In addition, tetracycline, erythromycin, and vancomycin have been used to treat human listeriosis. However, the development of bacterial resistance to *L. monocytogenes* has accelerated significantly (Gómez *et al*., 2014).

In this study, fourteen antibiotics were used to evaluate the sensitivity of the recovered *L. monocytogenes* (n=9) to antibiotics (Table 3). All isolated *L. monocytogenes* expressed resistance to most of the tested antibiotics where isolates originating from chicken luncheon, eggshells and layers' cloacal swabs exhibited multiple antibiotic resistances. However, some of the identified *L. monocytogenes* strains from pigeons and layers cloacal swabs were sensitive to gentamicin, meropenem, sulphamethoxazole, and amikacin. *L. monocytogenes* showed high resistance to nalidixic acid (100%) followed by neomycin (85.1%), streptomycin (80%), cefotaxime (65%) and penicillin g (58.3%). These results agreed with that reported in Turkey by Kalender (2003), who found that all tested isolates (n=24) which recovered from chicken cloacal swabs and minced beef were sensitive to gentamicin and 66.7% of the tested isolates were sensitive to sulphamethoxazole. In addition, Abd El-Tawab *et al.* (2018) showed that *L. monocytogenes* were sensitive to sulfamethoxate-trimethoprim followed by ampicillin, gentamycin, vancomycin and chloramphenicol then ciprofloxacin pursued by erythromycin and tetracycline, while the isolated strains were completely resistant to cephalothin. In contrast, several authors have reported that *L. monocytogenes* isolated from raw meat and meat products were all resistant to penicillin and sulphamethoxazole (Ndahi *et al*., 2014).

In Egypt, Tahoun *et al.* (2017) found that *L. monocytogenes* had a significant resistance to tetracycline (81%) and ciprofloxacin (66.7%), but had high susceptibility to ampicillin, erythromycin, and trimethoprim sulfamethoxazole. While in another recent study from Egypt, Abd EL-Aziz and Mohamed (2020) found that *L. monocytogenes* isolated from chicken meat were highly resistant to penicillin (100%), ampicillin (100%), and tetracycline (88.8%). The isolates in that study also showed resistance to erythromycin (66.6%), amoxicillin-clavulanic (55.5%), vancomycin (22.2%), and sulfamethoxazole/trimethoprim (11.1%).

The distributions of different virulence genes as *iap*, *hyl*A and *act*A in the identified *L. monocytogenes* strains were shown in Table 3 and Fig. 1. The *iap* gene was presented in all *L. monocytogenes* strains, while *hyl*A gene was expressed in 66.6% (6/9) and *act*A gene was demonstrated in 88.8% (8/9). In close to findings from this study, Mahmoud *et al.* (2019) identified *iap* gene in all isolates while *act*A and listeriolysin O were detected in 13 /16 (81.3%) and 12 /16 (75%), respectively. It becomes clear that most of the identified *L. monocytogenes* strains (4/9, 44.4%) exhibited MDR and expressed all the tested virulence genes. That could pose a high potential risk from the possibility of transferring drug-resistant *L. monocytogenes* to human through consumption of processed poultry and its products especially in the absence of strict hygienic practices and preventive measures.

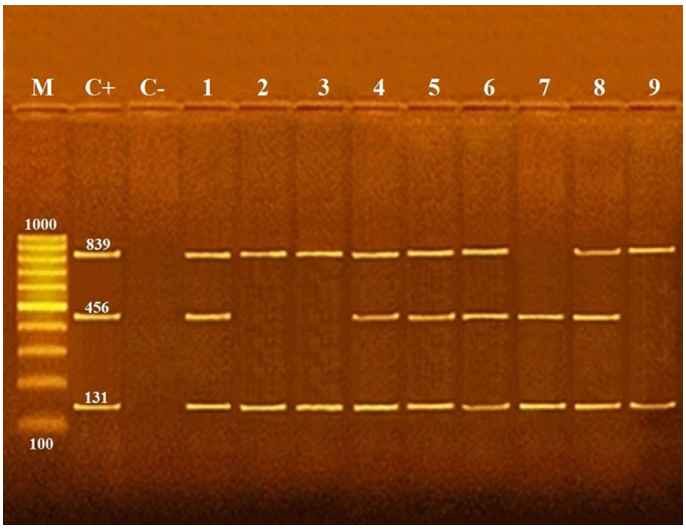

Fig. 1. Agarose gel electrophoresis of multiplex PCR for *iap* (131 bp), *hyl*A (456 bp) and *act*A (839 bp) virulence genes for characterization of *L. monocytogenes*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *L. monocytogenes* for *iap*, *hyl*A and *act*A genes. Lane C-: Control negative. Lanes 1, 4, 5, 6 & 8: Positive *L. monocytogenes* strains for *iap*, *hyl*A and *act*A genes from chicken luncheon, layers' cloacal swabs, turkeys' cloacal swabs, ducks' cloacal swabs. Lanes 2, 3& 9: Positive *L. monocytogenes* strains for *iap* and *act*A genes from chicken luncheon, eggshells, and pigeons' cloacal swabs. Lane 7: Positive *L. monocytogenes* strain for *iap* and *hyl*A genes from ducks 'cloacal swabs.

CONCLUSION

Poultry and poultry products could play a significant role in dissemination and transmission of virulent and MDR *L. monocytogenes* to human through consumption of processed poultry and its products especially in the absence of strict hygienic practices and preventive measures. Therefore, constant monitoring of pathogenic *L. monocytogenes* is urgently needed to avoid the dis-

semination of such pathogenic strains in food production chains.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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