Prevalence and molecular studies on *Listeria monocytogenes* isolated from chicken in El-Gharbia Governorate

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A B S T R A C T

Listeria monocytogenes is the causative agent of listeriosis and food-borne disease that can lead to meningitis and bacteremia. *L. monocytogenes* is the most pathogenic species in the genus Listeria. A total of 400 random samples of muscles, liver, spleen and kidneys (100 samples each) were taken from 100 diseased chickens. All samples were obtained from different poultry farms and markets in El-Gharbia Governorate to estimate the prevalence of *Listeria monocytogenes* in chicken. The bacteriological examination of the samples resulted; 53(13.25%) isolates as 11, 21, 8 and 13 from muscles, liver, spleen and kidneys respectively. The antimicrobial sensitivity test showed that the isolated *L. monocytogenes* were sensitive to sulfamethoxate-trimethoprim (91.6%) followed by ampicillin, gentamycin, vancomycin and chloromphenicol (83.3%) followed by ciprofloxacin (66.6%) followed by erythromycin and tetracycline (50.0%), while the isolated strains were completely resistant to cephalothin. All *L. monocytogenes* strains were virulent where all of them were positive to CAMP test and Anton's test. The PCR results for six studied strains of isolated *L. monocytogenes* showed that all genes (*16S rRNA; inlA; prfA and hlyA*) were detected (100.0%) while *plcA* gene was not detected.

Keywords: *Listeria monocytogenes*, chicken, PCR, virulence genes.

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1. INTRODUCTION

Listeria monocytogenes is the causative agent of listeriosis, a food-borne disease that can lead to meningitis and bacteremia (Mackiw *et al*., 2016).

Listeria spp. are considered as an important cause of zoonoses infecting many types of animals such as domestic pets, avian species, rodents, livestock, fish, amphibians and arthropods. The approximate fatality rate is 30% that may increase up to75% in high risk groups, such as neonates, pregnant women and immune-compromised adults (Jalali and Abedi, 2007).

The genus Listerias presently composed of 17 different species: *L. monocytogenes, L. seeligeri, L. ivanovii, L. welshimeri, L. marthii, L. innocua, L. grayi, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. rocourtiae, L. weihenstephanensis, L.*

grandensis, L. riparia and L. booriae (Orsi and Wiedmann, 2016).

Morphologically, *L. monocytogenes* is a gram-positive small, ranging from 1 to 2 μm in length and 0.5 μm in diameter, rod-like shape bacteria with rounded ends. Cells are usually in single units but can be clustered in short chains (3-5 or more) arranged in a V or Y disposition as well as in palisades. It is considered motile showing tumbling motility due to the production of peritrichous flagella when grown below 30 ºC, due to flagellin being produced and assembled in flagella at cell surface. If grown at 37 ºC, flagellin production is reduced to residual (Ryser and Marth, 2007).

L. monocytogenes are classified into13 different serotypes based on somatic (O) and flagellar (H) antigens. 1/2a, 1/2b, 1/2c, and 4b are the four main pathogenic serotypes from which 1/2a, 1/2b and 4b are responsible for 98% of human Listeriosis (Jacquet *et al*., 2002).

L. monocytogenes usually shed in all the secretions and excretions of the infected birds. Disease is transmitted through ingestion of contaminated feed, water, litter and soil. Infection can also follow inhalation or wound contamination. In birds, generally, the infection is subclinical and incubation period is not reported (Kurazono *et al*., 2003).

Signs of Listeriosis infection in birds, if seen, are suggestive of septicemia and may include depression and listlessness, emaciation, diarrhea and per acute/sudden death can occur at times (Akanbi *et al*., 2008).

Listeria is sensitive to a wide range of antibiotics: ampicillin, amoxicillin, tetracycline, chloramphenicol, -lactam antibiotics together with an aminoglycoside, trimethoprim and sulphamethoxazole are recommended (Altuntas *et al*., 2012).

Further identification of the pathogenic *L. monocytogenes* a set of genes are responsible for the virulence activity of *L. monocytogenes* must be detected (Shen *et al*., 2013). The virulence genes of *L. monocytogenes* are *inlA, inlB, hlyA, prfA, plcA, plcB, mpl* and *actA* (Karthikeyan *et al*., 2015). *inlA* is a gene that responsible for the survival and invasion of *L. monocytogenes* of intestinal epithelium (Liu *et al*. 2007) While *inlB* responsible for hepatocyte colonization (Kirkan *et al*., 2005) and the well-recognized virulence gene is *hlyA* which encodes the Listeriolysin O (Gouws and liedemann 2005) and finally the regulatory gene for those virulent genes *prfA* which encodes the promoter protein for *inlA, inlB* and *hlyA* (Scortti *et al*., 2007).

2. MATERIAL AND METHODS

2.1. Collection of the samples:

A total of 400 random samples of muscles, liver, spleen and kidneys (100 samples each) were taken from100 diseased chickens during November 2016 to April 2017. All samples were obtained from different poultry farms and markets in El-Gharbia Governorate. The examined samples were collected separately under hygienic measure as possible and transferred directly in ice box to the laboratory (Animal health research laboratory in Tanta) for bacteriological examination.

2.2. Bacteriological examination:

Listeria monocytogenes was isolated from the examined samples according to 11290-1:1996/Amd.1:2004 (ISO, 2005).

Approximately 25 grams of samples were added to 225 ml half-Fraser broth (Oxoid) in a 500ml flask and mixed well by shaking. The enrichment broth was incubated at 30°C for 24 hours. Then, 1ml from the half-Fraser broth was transferred into 9 ml of Fraser broth (Oxoid) and incubated at 37°C for 48 hours.

Loopful of incubated Fraser broth was streaked onto PALCAM agar media then the plates were incubated at 37±1°C for 48 hours and examined after 24±3 hours and the positive one was further streaked onto Oxford agar and ALOA agar plates then the plates were incubated at 37±1°C for 48 hours and examined after 24±3 hours. The Listeria like colonies were picked and streaked onto Tryptic Soy agar with 0.6 % yeast extract (TSA,YA) then, incubated at 35°C for 48 hours, the isolates were morphologically identified by Gram' stain according to Quinn *et al*., (2011) and biochemical tests (Catalase reaction, Oxidase test, Nitrate reduction test, Urease test, Methyl red test (MR), Vages-proskauer test (VP), Carbohydrate fermentation test, Esculin test Gelatin hydrolysis test and Motility tests) were applied according to Markey *et al.,* (2013).

2.3. Virulence tests:

2.3.1. Hemolytic activity:

All isolates were cultured onto 5% sheep blood agar to determine their hemolytic activity. In addition to, they were subjected to CAMP test (Christie-Atkins-Munoh-Peterson), (McKellar, 1994) by streaking of *Staphylococcus aureus* strains in single straight lines in parallel on sheep blood agar plates, the isolated listeria strains streaked perpendicularly, with quite touching (1-2mm). After incubation for 24-48 hours at 35°C, a positive reaction consists of an enhanced zone of β-hemolysis.

2.3.2. The biological characters:

Anton's test (Quinn *et al.,* 2002) by instillation 2-3 drops of listeria suspension into the conjunctiva of rabbits.

2.4. In-Vitro anti-microbial sensitivity test (CLSI, 2014):

All *L. monocytogenes* isolates were tested for their antimicrobial resistance/susceptibility pattern by disc diffusion technique according to (CLSI, 2014). By using antimicrobial discs as fluoroquinolones represented by ciprofloxacin; β –lactam represented by ampicillin; cephalosporins represented by cephalothin; aminoglycosides represented by gentamycin; tetracycline represented by tetracycline; phenicols represented by chloromphenicol; sulphonamides and trimethoprim represented by sulfamethoxate-trimethoprim; macrolides represented by erythromycin; glycopeptide represented by vancomycin (Oxoid).

2.5. 2.5. Genotypic detection of isolated L. monocytogenes and some virulence genes:

PCR using five sets of primers (Table 1) was used for genotypic detection of *L. monocytogenes* strains and their virulence

genes. These genes were *16SrRNA* gene; internalin A *(inlA);* Positive regulatory factor *(prfA);* Listeriolysin O, haemolysin *(hlyA)* and Phospholipase A (*plcA*). It was applied on six random isolates of *L. monocytogenes*, following QIA amp® DNA Mini Kit instructions (Catalogue no. M501DP100), Emerald Amp GTPCR master mix (Takara) with Code No. RR310A and 1.5 % agarose gel electrophoreses (Sambrook *et al.*, 1989).

Table (1): Oligonucleotide primers sequences

Primer	Sequence	Amplified product	Reference
16S rRNA	F- GGA CCG GGG CTA ATA CCG AAT GAT AA R- TTC ATG TAG GCG AGT TGC AGC CTA	1200 bp	Kumar *et al.*, 2015
prfA	F- TCT-CCG-AGC-AAC-CTC-GGA-ACC R- TGG-ATT-GAC-AAA-ATG-GAA-CA	1052 bp	Dickinson *et al*., 1995
inlA	F- ACG AGT AAC GGG ACA AAT GC R- CCC GAC AGT GGT GCT AGA TT	800 bp	Liu *et al*., 2007
hlyA	F- GCA-TCT-GCA-TTC-AAT-AAA-GA R- TGT-CAC-TGC-ATC-TCC-GTG-GT	174 bp	Deneer and Boychuk, 1991
plcA	F- ACA AGC TGC ACC TGT TGC AG R- TGA CAG CGT GTG TAG TAG CA	1484 bp	Soni *et al.*, 2014

3. RESULTS

A total of 53 (13.25%) isolates of *Listeria monocytogenes* were recovered from 400 samples includes 11(2.75%) isolates from muscles, 21(5.25%) from liver, 8 (2%) from spleen and 13 (3.25%) from kidneys (Table 2).

The isolated colonies grow well and showed gray green colonies with black depressed button center and black hollow surrounded them on PALCAM agar, black colonies with dimpled centers on Oxford agar and green-blue colonies surrounded by an opaque halo on ALOA agar.

They were Gram - positive bacilli or coccobacilli; were motile at room temperature on Semisolid trypticase soy agar with yeast extract and showing the characteristics umbrella growth and the templing motility.

The Biochemical reactions showed that all strains were catalase (+); oxidase (-) and produce acid with dextrose, L-rhamnose but not with mannitol, D-xylose and sucrose.

The results of virulence tests proved that, all isolated *L. monocytogenes* strains were CAMP test positive with zone of β-hemolysis at the junction of tested strains and *S. aureus* strains. In addition, all of them produced purulent conjunctivitis within 24-48 hours followed by keratitis in all rabbits (Anton's test positive).

The results of antimicrobial sensitivity tests for the isolated *L. monocytogenes*

(Table 3) showed that the isolated *L. monocytogenes* (n=12) were sensitive to sulfamethoxate-trimethoprim (91.6%) followed by ampicillin, gentamycin, vancomycin and chloromphenicol (83.3%) followed by ciprofloxacin (66.6%) followed by erythromycin and tetracycline (50.0%), while the isolated strains were completely resistant to cephalothin.

The PCR results for *L. monocytogenes* showed that, all genes (*16SrRNA; inlA; inlB; hlyA* and *prfA*) were detected in all six studied strains (100.0%) while *plcA* gene was not detected. i.e., all six studied strains were *L. monocytogenes* and were virulent strains.

The (*16SrRNA; inlA; prfA; hlyA* were amplified at 1200, 800, 1052, 174 bp respectively for the six isolates of *L. monocytogenes* (figures 1-4) but *plcA* gene was not detected (100%) (Figure 5).

Table (2): Prevalence of *Listeria monocytogenes* isolated from different organs in chicken samples

Samples	Number of samples	Number of positive samples	Positive percentage %
Muscles	100	11	2.75
Liver	100	21	5.25
Spleen	100	8	2
Kidneys	100	13	3.25
Total	400	53	13.25

Table (3): Results of antimicrobial sensitivity test for isolated *Listeria monocytogenes* strains (n=12)

Antimicrobial Family	Antimicrobial disc	Sensitive Isolates		Intermediate Isolates		Resistant Isolates	
		Number	%	Number	%	Number	%
Fluoroquinolones	Ciprofloxacin(CIP)	8	66.6	4	33.3	0	0
β-lactam	Ampicillin(AMP)	10	83.3	1	8.3	1	8.3
Cephalosporins	Cephalothin(KF)	0	0	0	0	12	100
Aminoglycosides	Gentamycin (CN)	10	83.3	1	8.3	1	8.3
Tetracycline	Tetracycline (TE)	6	50	0	0	6	50
Phenicols	Chloromphenicol C))	10	83.3	0	0	2	16.6
Sulphonamides and trimethoprim	Sulfamethoxate-Trimethoprim SXT))	11	91.6	0		1	8.3
Macrolides	Erythromycin (E)	6	50	2	16.6	4	33.3
Glycopeptide	Vancomycin (VA)	10	83.3	1	8.3	1	8.3

% According to the total no of *L. monocytogenes* isolates

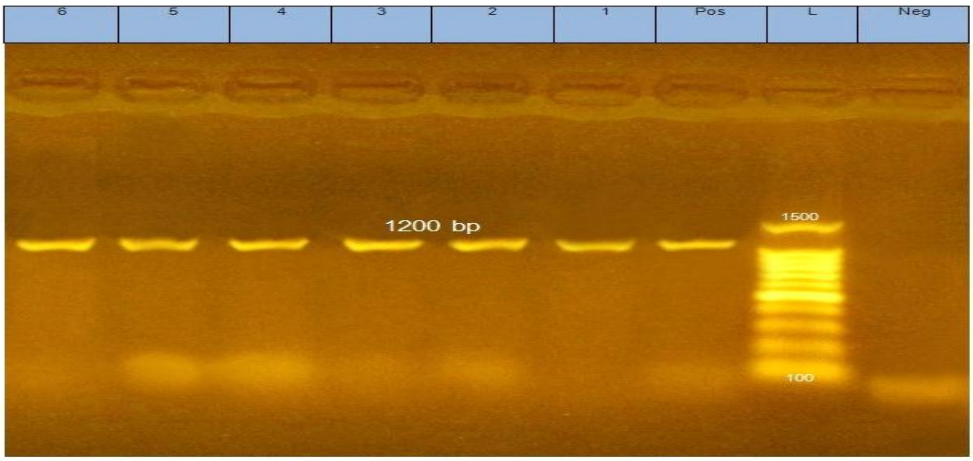

Figure (1): Agarose gel electrophoresis of *16SrRNA* genes.
Lane L: 100-1500bp Ladder. Neg.: Negative control. Pos.: Positive control (at 1200bp). Lanes from 1 to 6: L. *monocytogenes* (*16S rRNA*) gene positive at 1200bp.

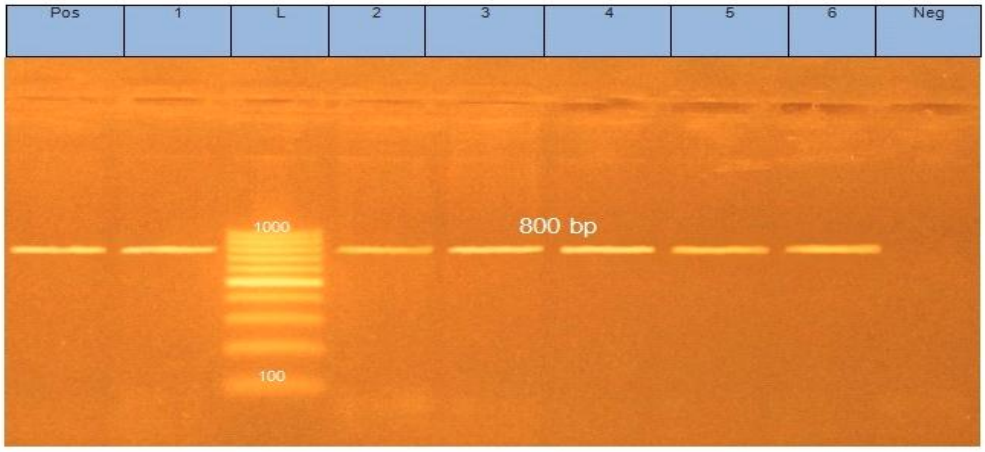

Figure (2): Agarose gel electrophoresis of internalin A (*inlA*) genes.
Lane L: 100-1000bp Ladder. Neg.: Negative control. Pos.: Positive control (at 800bp). Lanes from 1 to 6: *L. monocytogenes*(*inlA*) gene positive at 800bp.

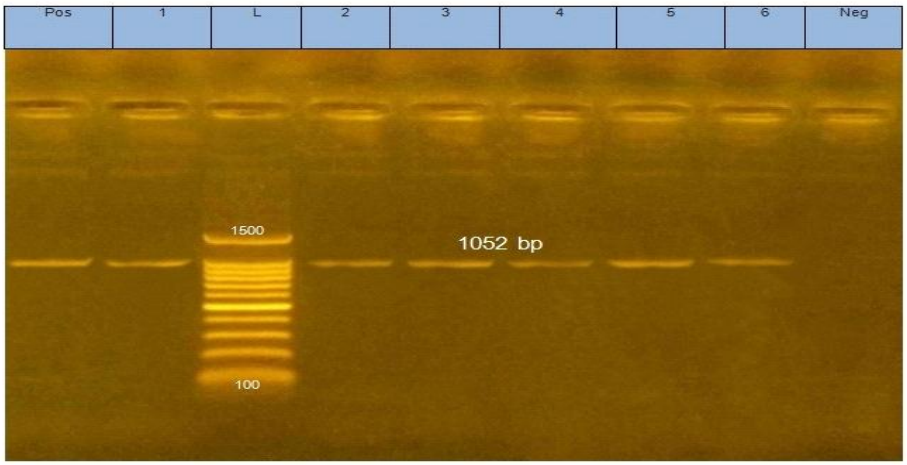

Figure (3): Agarose gel electrophoresis of Positive regulatory factor (*prfA*) genes. Lane L: 100-1500bp Ladder. Neg.: Negative control. Pos.: Positive control (at 1052bp). Lanes from 1 to 6: *L. monocytogenes* (*prfA)* gene positive at 1052bp.

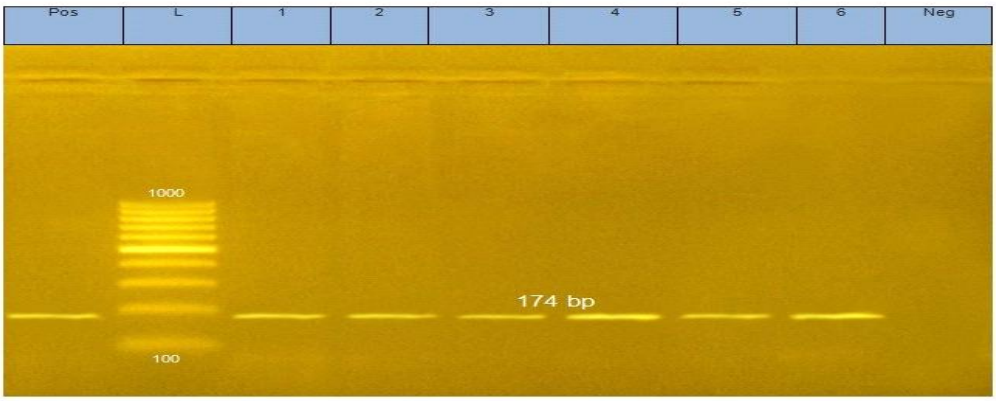

Figure (4): Agarose gel electrophoresis of Listeriolysin O, haemolysin (*hylA*) genes. Lane L: 100-1000 bp Ladder. Neg.: Negative control. Pos.: Positive control (at 174 bp). Lanes from 1 to 6: *L. monocytogenes* (*hylA*) gene positive at 174bp.

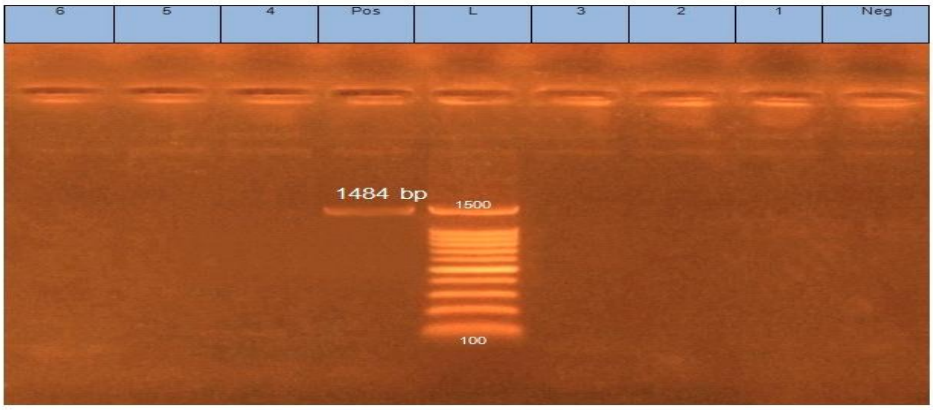

Figure (5): Agarose gel electrophoresis of Phospholipase A (*plcA*) genes. Lane L: 100-1500bp Ladder. Neg.: Negative control. Pos.: Positive control (at 1484bp). Lanes from 1 to 6: *L. monocytogenes* (*plcA*) gene negative.

4. DISCUSSION

In the present study a total of 400 examined chicken samples, 53 isolates (13.25%) of *Listeria monocytogenes* were isolated (Table 2). This is nearly similar to Osaili *et al.,* (2011), Swetha *et al.,* (2013), Saludes *et al.,* (2015) and AL-Jobori *et al.,* (2016) who isolated *L. monocytogenes* from chicken by (18%), (16%), (19%) and (11%) respectively.

These results in El-Gharbia Governorate (13.25%) are higher than recorded by Ali and Shalaby (2002) in Giza city 3.3%, Farghaly (2011) in Cairo Governorate 3.6% and Dahshan *et al.,* (2016) in Sharkia Governorate in Egypt who did not detected *L. monocytogenes* from chicken samples.

However, higher prevalence rate of *L. monocytogenes* isolated from chicken were recorded by Minamia *et al.,* (2010), Pesavento *et al.,* (2010), Kuan *et al.,* (2013) and Zeinali *et al.,* (2017) who isolated *L. monocytogenes* from chicken by (36%), (24.5%), (26.39%) and (18%), respectively.

The colonial appearance of the recovered isolates in this study grow well and showed: small 2-3 mm in diameter; gray green colonies in color with black depressed button center and black hollow surrounded them (esculin hydrolysis) on PALCAM agar that was similar to the previously recorded studied by (Osman *et al.,* (2014) and Abd El-Tawab *et al.,* (2015). Black colonies with dimpled centers on Oxford agar that was similar to the previously recorded studied by (Magalhães *et al.,* (2014) and Abd El-Tawab *et al.,* (2015). Green-blue (due to presence of β-glucosidase) regular round colonies surround by an opaque halo (due to the activity of phospholipase involved in pathogenic Listeria) on ALOA agar that was similar to the previously recorded studied by (Jeyaletchumi *et al.,* (2010a) and Abd El-Tawab *et al.,* (2015).

The results of biochemical identification showed characteristic

identical biochemical reaction to be Listeria species that was similar to the previously recorded studied by (Schmid *et al.,* (2005); Todar, (2009) and Abd El-Tawab *et al.,* (2015).

All isolated Listeria were motile at room temperature and showing the characteristics umbrella growth and the templing motility that was similar to the previously recorded studied by (Ryser and Marth, (2007); Bhunia, (2008); Todar, (2009) and Abd El-Tawab *et al.,* (2015).

The result of virulence tests for the isolated *Listeria monocytogenes* strains appeared that, all *L. monocytogenes* strains were virulent strains, where all of them were positive to CAMP test and showed narrow zone of β-hemolysis on sheep blood agar. Also, all L. *monocytogenes* isolates were positive for Anton's test. That was similar to the previously recorded studied by (Maarouf *et al.,* (2007); Todar, (2009); Fentahun and Fresebehat, (2012) and Abd El-Tawab *et al.,* (2015).

The results of antimicrobial sensitivity tests for the isolated *L. monocytogenes* (Table 3) showed that the isolated *L. monocytogenes* were sensitive to Sulfamethoxate-Trimethoprim (91.6%) followed by ampicillin, gentamycin, vancomycin and chloromphenicol (83.3%) followed by ciprofloxacin (66.6%) followed by erythromycin and tetracycline (50.0%), while the isolated strains were completely resistant to cephalothin. Nearly similar results were recorded by Conter *et al.,* (2009); Granier *et al.,* (2011) and Altuntas *et al.,* (2012).

The PCR technique can identify the pathogenic *L. monocytogenes* based on the fact that virulence varies not only among different species but also among strains of the same species. Thus, numerous studies have been conducted to identify virulence factors of isolated *L. monocytogenes* strains (Shen *et al.,* 2013; Khan *et al.,* 2014 and Ciolacu *et al.,* 2015).

So, the present study was directed mainly to genotypic detection of *L. monocytogenes* strains and virulence genes that may play a role in virulence of *L. monocytogenes* by using one of the recent developments molecular biological techniques (PCR). These genes were *16 rRNA* gene; internalin A (*inlA*); Positive regulatory factor (*prfA*); Listeriolysin O, haemolysin (*hlyA*) and Phospholipase A (*plcA*).

The PCR results for six studied strains of *L. monocytogenes* isolates showed that, the genes (*16rRNA, inlA, hlyA and prfA*) were detected (100.0%). i.e., all six studied strains were *L. monocytogenes* and all of them were virulent strains. Similar results were decided by Shen *et al.,* (2013); Abd El-Tawab *et al.,* (2015) and Ciolacu *et al.,* (2015); as well as they reported that, PCR save time for diagnosis hence allowing a rapid identification of *L. monocytogenes* with high sensitivity and specificity.

While the result of PCR for amplification of Phospholipase A (*plcA*) gene in *L. monocytogenes* (Figure 5) showed that, it was not detected in all six studied strains of *L. monocytogenes* isolates in the current study.

Finally, from results of the present work it could be concluded that, *Listeria monocytogenes* are serious pathogens could be contaminated chickens in El-Gharbia Governorates, Egypt.

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