



## Phenotypic and genotypic characterization of listeria species isolated from poultry and milk products

Ashraf A. Abd El-Tawab<sup>1</sup>, Wafaa, M.M Hassan<sup>2</sup>, Fatma I El-Hofy<sup>1</sup> and Haidy, T.Zaki<sup>2</sup>

<sup>1</sup> Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Benha University

<sup>2</sup> Animal Health Research

### ABSTRACT

*Listeria monocytogenes* has become increasingly important as a food-associated pathogen. It can cause a rare but serious disease called listeriosis with high fatality rates (20–30%) compared with other foodborne microbial pathogens. To estimate the incidence and levels of *Listeria* spp. in different food sources, A total of 200 random samples which collected from different sources 65 samples isolated from poultry , 45 from poultry byproducts (Frozen chicken meat balls (kofta), Frozen chicken burger and frozen chicken sausages), 20 from pasteurized milk ,30 from milk products (Feta cheese ,cream cheese )and 40 from poultry eggs .Only 7 of total 200 samples (14%) that collected from suspected isolates and detected on Oxford agar, PALCAM agar and ALOA agar were suspected to be *Listeria* spp. In poultry, poultry byproducts, pasteurized milk, milk byproducts and poultry eggs, listeria percentage were 0/65(0%), 4/45(1.8), 0/20(0%), 0/30(0%), 3/40(1.2%) isolates, respectively. The in-vitro antimicrobial sensitivity test showed that the isolated *L. monocytogenes* were sensitive to Sulphamethoxazole /trimethoprim, Gentamycin, Ceftazidime and Cephazoline followed by Sulphamethoxazole, ceftriaxone , weak sensitivity to Penicillin . While they were resistant to Erythromycin, Clindamycin, Ampicillin. The results of virulence tests for isolated listeria strains appeared that all of *L. monocytogenes* were virulent strains as all of them were positive to CAMP test; showed narrow zone of  $\beta$ -hemolysis on sheep blood agar .The PCR results for *Listeria* isolates showed that all strains are *L. monocytogenes* and have *Inl A* and *hly A* genes positive as virulence genes while negative *Inl B*, *iap* and *prf A* genes.

**Keywords:** *L. monocytogenes*, poultry, milk.

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### 1. INTRODUCTION

Veterinarians, medical doctors and people involved in food science know listeriosis by various names (circling disease, meningioencephalitis disease, cheese sickness) but few know who Gustav Hülphers was because he did not preserve his bacterial strains, which he named bacillus hepatis, later

recognized as *Listeria monocytogenes* (McLauchlin, 2004& Hülphers, 2004).

*Listeria monocytogenes* is a Gram-positive rod-shaped non-capsulated bacterium that form single short chains (Theivagt and Friesen, 2006), facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 mm in

width and 1–1.5 mm in length (Vazquez-Boland *et al.*, 2001 and Vera *et al.*, 2013).

Taxonomically, it is divided into six species (i.e. *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi*), of which only *L. monocytogenes* and *L. ivanovii* are pathogenic. While *L. monocytogenes* infects both man and animals, *L. ivanovii* is principally an animal pathogen that rarely occurs in man (Robinson *et al.*, 2000).

The organism can survive at varying temperatures ranging from 4 to 37C (Janakiraman, 2008). The bacterium can tolerate a wide range of pH and temperatures. Optimum growth occurs at 30-37C but the organism can multiply at 4-45C. A typical tumbling motility is observed around 25C. It can grow at pH 4.5-9.6, although the growth is minimal at low pH and low temperatures (OIE, 2014).

Listeriosis is often noticed along with other poultry diseases such as *coccidiosis*, *infectious coryza*, verotoxic *E.coli*, *salmonellosis*, *campylobacteriosis* and parasitic infections, signifying the opportunistic nature of the organism (Adzitey *et al.*, 2012)

Egg and egg products have never been caused listeriosis but is most frequently isolated from egg shells and in the environment of laying hens. (Chemaly *et al.*, 2008) *L. monocytogenes* can survive 90 days on stored egg at 5C and for 15 days at 10C (Gandhi and Chikindas, 2007).

Briefly, invasiveness by virulence factors, *L. monocytogenes* expresses cell-surface and secreted proteins that enable attachment to host cells, escape from the phagocytic vacuole by Internalin A (inIA) and Internalin B (inIB) mediate the attachment of *L. monocytogenes* to the surface of host cells. Once ingested the bacterium produces listeriolysin (LLO) to escape from the phagosome and lyses the phagosomal membrane, The bacterium then

multiply rapidly in the cytoplasm and moves through the cytoplasm to invade adjacent cells by polymerizing actin to form long tails, the actin-assembly-inducing protein (ActA) which propels bacteria through the cell and into neighboring cells, (Todar, 2008).

Each step requires expression of specific virulence factors. The major virulence genes are clustered together on the chromosomes and regulated by the positive regulatory factor A protein. PrfA (Positive Regulatory Factor A (PrfA)). (Scotti *et al.*, 2007 and Freitag *et al.*, 2009).

There is an interplay between temperature and *L. monocytogenes* motility. *L. monocytogenes* can employ either multi-flagellar based motility or actin based propulsion depending on its environment. When *L. Monocytogenes* lives outside a host (<30 °C), it is flagellated and utilizes these flagella for movement. When the bacteria encounters higher temperatures, like those in a host, the flagellar expression is halted and the bacteria are non-flagellated (Williams *et al.*, 2005).

The present study was conducted to estimate the prevalence of listeria species in poultry and milk products with special interest to *L. monocytogenes*. In addition to clarify the virulence of isolated strains and to carry out the antibiotics sensitivity testing of them. In addition, detection of some virulence factors of *L. monocytogenes* by PCR technique.

## 2. MATERIALS AND METHODS

### 2.1. Samples collection:

Two hundred random samples which collected from different sources 65 samples isolated from poultry, 45 from poultry byproducts (Frozen chicken meat balls (kofta), Frozen chicken burger and frozen chicken sausages), 20 from pasteurized milk, 30 from milk products (Feta cheese, cream cheese) and 40 from poultry eggs. The samples were collected in sterile plastic bags,

kept in ice box and transferred with a minimum delay to the laboratory for studying the presence of listeria species.

#### 2.2. Bacteriological examination:

A-Primary stage: One ml of sample was inoculated into 9 ml Fraser broth 1, half Fraser broth (without supplement) and incubated aerobically at  $30 \pm 1^\circ\text{C}$  for  $24 \pm 3$  hours .B-Secondary stage : one ml of incubated broth was inoculated into 9ml Fraser broth 2 , full strength Fraser broth (with supplement) and incubated at  $37^\circ\text{C}$  for  $48 \pm 3$  hours .C-Third stage :0.1ml of incubated Fraser broth was streaked onto the following media : ALOA agar; PALCAM agar and Oxford agar plates then the plates were incubated at  $37 \pm 1^\circ\text{C}$  for 48 hours and examined after  $24 \pm 3$  hours. The listeria like colonies were picked and streaked onto Tryptic Soy agar ( Bio-life ) with 0.6% yeast extract (TSA, YA) then, incubated at  $35^\circ\text{C}$  for 48 hours. The isolates were morphologically identified by Gram stain and biochemical tests according to (Markey et al., 2013)

#### 2.3. In –Vitro anti-microbial sensitivity tests:

The isolated *L. monocytogenes* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method: antibiotic discs are Erythromycin(E), Penicillin (P), Sulphamethoxazole (SM), ceftriaxone (CRO), Gentamycin (CN), Clindamycin (DA), Ampicillin (AM), Cefazoline (KZ), Sulphamethoxazole/ trimethoprim (SXT), ceftazidime (CAZ). (Fine gold and Martin, 1982 and NCCIS, 1999)

#### 2.4. Virulence tests:

Hemolytic activity: all isolates were cultured on 5% sheep blood agar to determine their hemolytic activity. Also, they were subjected to CAMP test (Mckellar,1994) by streaking *staphylococcus aureus* strains in single

straight lines in parallel on sheep blood agar plates , the isolated listeria strains streaks perpendicularly,(1-2mm).then incubated for 24-48hours at  $35^\circ\text{C}$  ,enhanced zone of  $\beta$ -hemolysis considered as a positive reaction.

#### 2.5. Genotypic detection of isolated *L. monocytogenes* and some virulence in them using polymerase chain reaction (PCR):

PCR using six sets of primers was used for genotypic detection of *L. monocytogenes* strains and five virulence genes of *L. monocytogenes*. The virulence genes were 16SRNA; internalin A (inlA); internalin B (inlB); heamolysin (hlyA); invasion associated protein (iap) and positive regulatory factor A (prfA).

It was applied on seven isolated *L. monocytogenes* following QIAamp® DNA Mini Kit instructions (Catalogue no. 51304),Emerald Amp GT PCR master mix (Takara) Code No. RR310A and Agarose 1% (Sambrook et al., 1989).The PCR condition have specific sequence and amplify specific products as shown in Table (2). Temperature and time condition of the primers during PCR are shown in table (3) according to specific authors and Emerald Amp GT PCR master mix (Takara) kit.

### 3. RESULTS

Of the total 200 different samples analyzed, *L. monocytogenes* was detected in 7 samples with prevalence of 14% ; represented as in poultry, poultry byproducts, pasteurized milk, milk byproducts and poultry eggs, listeria percentage were 0/65 (0%), 4/45 (1.8), 0/20(0%), 0/30(0%), 3/40 (1.2%) isolates, respectively. as Table (1) The isolated colonies grow well on ALOA agar producing blue-green colonies surrounded by opaque halo and on PALCAM agar gives grey-green with black center and black halo against cherry-red background and on Oxford agar gives grayish colonies surrounded by black

halos. They were Gram – positive bacilli or coccobacilli; motile showing Umbrella – shaped motility. On biochemical reactions, they produce acid from L-rhamnose, dextrose and but not with D-xylose and mannitol. The results of virulence tests showed that, all isolated *L. monocytogenes* strains showed narrow zone of  $\beta$ -hemolysis on 5% sheep blood agar, positive in CAMP test and showed as arrow-shaped zone of weak enhanced hemolysis at junction of tested strains and *S. aureus* strain.

The result of in -vitro sensitivity test showed that, the isolated *L. monocytogenes* were sensitive to Sulphamethoxazole / trimethoprim (100%), followed by Gentamycin, Ceftazidime and Cephazoline (71.4%), followed by Sulphamethoxazole, ceftriaxone (42.8%), weak sensitivity to Penicillin (14.2%). While the isolated strains were resistant to Erythromycin, Ampicillin and Clindamycin as Fig (1)

Table 1: Total number and percentage of positive samples for listeria isolated from examined samples.

Samples	Number of samples	Number of positive samples	Positive percentage		
			%1	%2	%3
Poultry	65	zero	zero	zero	zero
Frozen wings	35	zero	zero	zero	zero
Frozen breast	30	zero	zero	zero	zero
Poultry products	45	4	8.9	57.1	2.0
Frozen chicken meat balls (kofta)	10	2	4.4	28.5	1.0
Frozen chicken burger	15	1	2.2	14.2	0.5
frozen chicken sausages	20	1	2.2	14.2	0.5
Pasteurized Milk	20	zero	zero	zero	zero
Milk products	30	zero	zero	zero	zero
Feta cheese	10	zero	zero	zero	zero
Cream cheese	20	zero	zero	zero	zero
Poultry eggs	40	3	7.5	42.8	2.5
Total	200	7	3.5	100.0	3.5

<sup>1</sup> Percentage in relation to total number of samples in each raw.

<sup>2</sup> Percentage in relation to total number of positive samples (7).

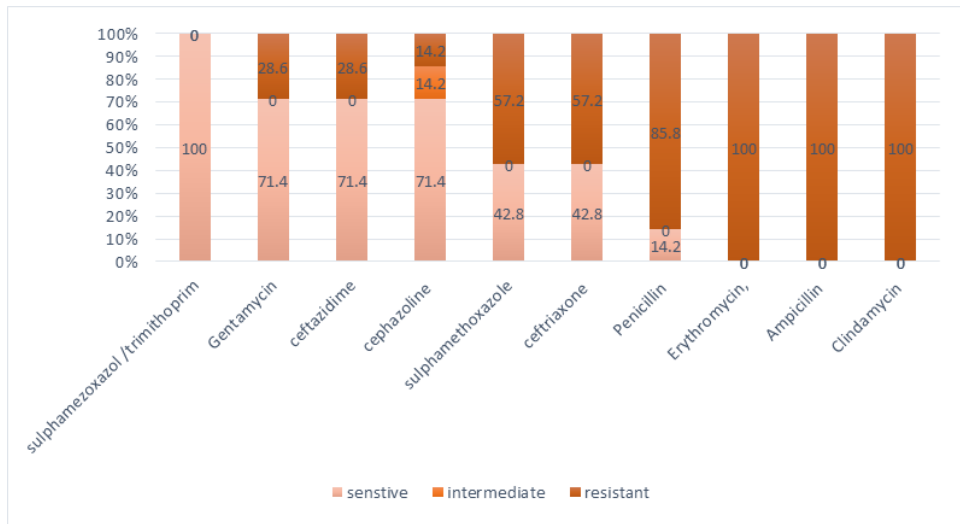
<sup>3</sup> Percentage in relation to total number of collected samples(200).

Table 2: Oligonucleotide primers sequences sources.

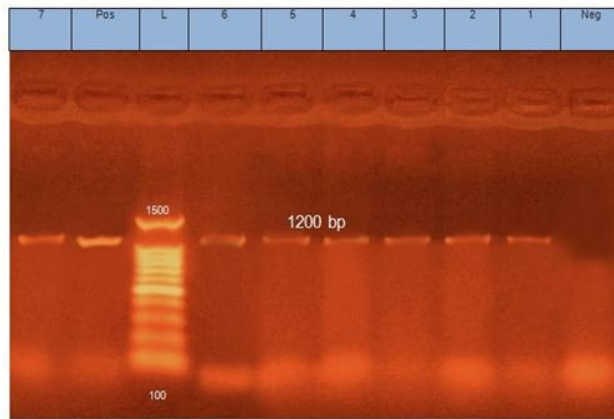
Primer	Sequence	Amplified product	Reference
<i>16S rRNA</i>	ggA CCg ggg CTA ATA CCg AAT gAT AA TTC ATg TAg gCg AgT TgC AgC CTA CTG CTT GAG CGT TCA TGT CTC ATC	1200 bp	Kumar et al., 2015
<i>Iap</i>	CCC C CAT GGG TTT CAC TCT CCT TCT AC	131 bp	Soni et al., 2014
<i>prfA</i>	TCT-CCG-AGC-AAC-CTC-GGA-ACC TGG-ATT-GAC-AAA-ATG-GAA-CA	1052 bp	Dickinson et al., 1995
<i>inlA</i>	ACG AGT AAC GGG ACA AAT GC CCC GAC AGT GGT GCT AGA TT	800 bp	Liu et al., 2007
<i>inlB</i>	Ctggaaagtttgatttgggaaa tttcataatgccatcatcact	343 bp	
<i>hlyA</i>	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT CCYTTTTATGTACCCAAYGA	174 bp	Deneer and Boychuk, 1991

Table 3: Cycling conditions of the different primers during cPCR.

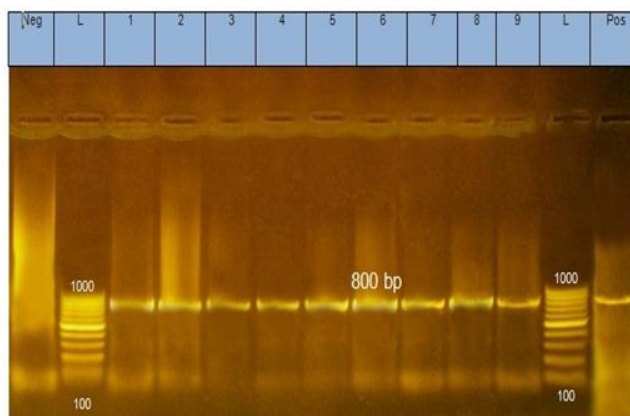
Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
<i>16S rRNA</i>	94°C	94°C	60°C	72°C	35	72°C
<i>Iap</i>	5 min.	30 sec.	1 min.	1 min.		12 min.
<i>prfA</i>	94°C	94°C	60°C	72°C	35	72°C
	5 min.	30 sec.	30 sec.	30 sec.		7 min.
<i>inlA</i>	94°C	94°C	50°C	72°C	35	72°C
	5 min.	30 sec.	50 sec.	1 min.		10 min.
<i>inlB</i>	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	45 sec.	45 sec.		10 min.
<i>hlyA</i>	94°C	94°C	50°C	72°C	35	72°C
	5 min.	30 sec.	30 sec.	30 sec.		7 min.



**Fig.1.** In-vitro antimicrobial sensitivity test for isolated *L.monocytogenes*.

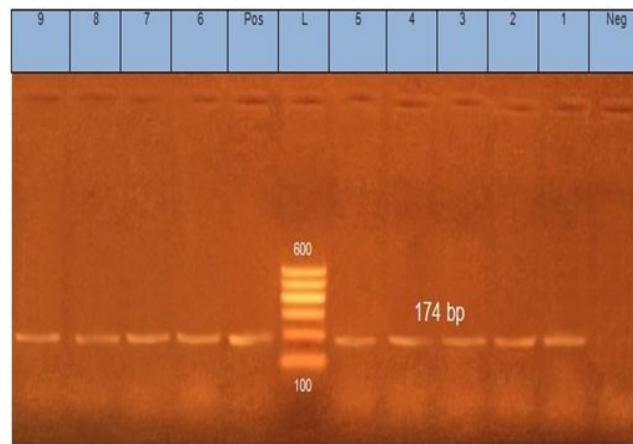


**Fig.2.** 16SrRNA genes . Lane L:100-1500bp Ladder. Neg: Negative control . Pos. : positive control at (1200bp) . Lanes 1 to 7 :*L. monocytogenes* (16SrRNA) gene.

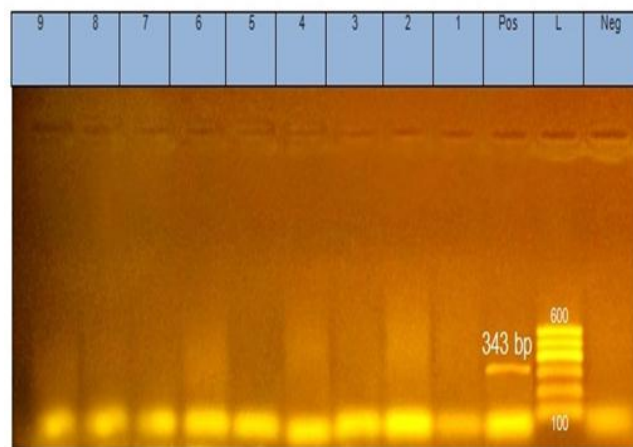


**Fig.3.** inlA gene. Lane L:100-1000bp Ladder. Neg: Negative control. Pos: Positive control at (800bp) . Lanes 1 to 7 : *L. monocytogenes* (inlA) gene positive.

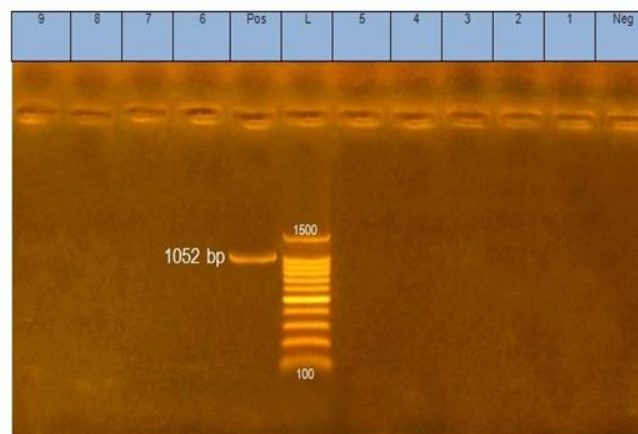
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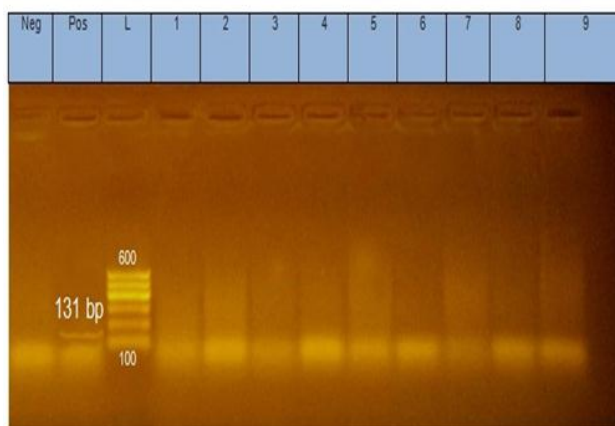
**Fig.4.** *hlyA* gene. Lane L: 100-600bp Ladder. Neg: Negative control. Pos: Positive control at (174bp). Lanes 1 to 7 : *L. monocytogenes* (*hlyA*) gene positive.



**Fig.5.** *inlB* gene. Lane L: 100-600bp Ladder. Neg: Negative control. Pos: Positive control at (343bp). Lanes 1 to 7 : *L. monocytogenes* (*inlB*) gene negative.



**Fig.6.** *prfA* gene. Lane L: 100-1500bp Ladder. Neg: Negative control. Pos: Positive control at (1052bp). Lanes 1 to 7 : *L. monocytogenes* (*prfA*) gene negative.



**Fig.7.** *iap* gene. Lane L: 100-600bp Ladder. Neg: Negative control. Pos: Positive control at (131bp). Lanes 1 to 7 : *L. monocytogenes* (*iap*)gene negative.

#### 4. DISCUSSION

Most human listeriosis cases appear to be linked to consumption of ready-to-eat (RTE) food products (Lunde'n *et al.*, 2004). In particular, *L. monocytogenes* contamination of fermented dairy products made from raw milk may be due either to use of contaminated raw milk or to post processing contamination from environmental sources (Kells and Gilmour, 2004).

Listeriosis is of great public health concern because of its high mortality (20 to 30%) and its common source epidemic potential. The most important aspect in food hygiene is the ability of the bacteria to survive in a wide range of temperatures and to make biofilms on various environmental surfaces, which serve as natural habitats or reservoirs (Duggan and Phillips, 1998).

Listeriosis is caused by members of the genus *Listeria*, which has now 17 species. However, only two species are considered pathogenic.

*L. monocytogenes* is considered pathogenic to human beings and several animal species, whereas *L. ivanovii* is pathogenic especially to ruminants but

occasionally to humans (McLauchlin & Martin, 2008).

Ability of *L. monocytogenes* to cause disease depends upon the expression of virulence factors and immune status of individuals. Usually individuals having weakened cell-mediated immunity are more susceptible to *L. monocytogenes*. (Lecuit *et al.*, 2004)

The result of isolated samples as in Table (1) revealed that *L. monocytogenes* was detected in 7 samples out of 200 samples (14%) represented as in poultry, poultry byproducts, pasteurized milk, milk byproducts and poultry eggs, listeria percentage were 0/65(0%), 4/45(1.8), 0/20(0%), 0/30(0%), 3/40(1.2%) isolates, respectively that disagreed with that recorded by (Alsheikh *et al.*, 2013 and Zeinali *et al.*, 2017)

In this research *listeria monocytogenes* was found in Frozen wings of poultry, frozen breast of poultry by percentage (0%) that totally disagreed with (Reiter *et al.*, 2005)

The result of in vitro sensitivity test showed that the isolated *L. monocytogenes* were highly sensitive to Sulphamethoxazole /trimethoprim (100%), followed by Gentamycin Ceftazidime and Cephazoline



(71.4%), followed by Sulphamethoxazole, ceftriaxone (42.8%), weak sensitivity to Penicillin (14.2%). While the isolated strains were resistant to Erythromycin, Ampicillin and Clindamycin. And these results came in accordance with those recorded by (Zeinali *et al.*, 2017) and disagreed with (Hof, 2004; Altuntas *et al.*, 2012).

The hemolytic activities of listeria species determine its pathogenicity (Maarouf *et al.*, 2007). The result of virulence tests for isolated listeria showed that ,all *L. monocytogenes* were positive to CAMP test and showed narrow zone of  $\beta$ -hemolysis in sheep agar, the similar results were reported by (Marrouf *et al.* , 2007)

The PCR results for *L.monocytogenes* isolated showed that(*16srRNA* ;*inl A* and *hlyA*) genes were detected in all seven strains. While *inl B*; *iap* and *prfA* genes are not detected in all isolated that dissimilar to that reported by (Xiaolong *et al.*, 2017)

Regarding to the occurrence of *16SrRNA* genes in *L. monocytogenes* isolates , the obtained result revealed that , it was amplified in all seven tested isolates 100% ,that agreed with those recorded by (Gelbicova and Karpiskova, 2012 and Ciolacu *et al.*, 2015)

The result of PCR for amplification of internalin A (*inlA*) gene in *L. monocytogenes* (Fig.3) showed that , *inlA* gene was amplified in all seven tested isolates giving product of 800bp,that similar recorded by (Gelbicova and Karpiskova ,2012 and Ciolacu *et al.* ,2015).

The result of PCR for amplification of internalin B (*inlB*) gene in *L. monocytogenes* (Fig.5) showed that, *inlB* gene wasn't amplified in all seven tested isolates, that disagreed with (Shen *et al.*, 2000)

The result of PCR for amplification of listeriolysin O (hemolysin,*hlyA*) gene in *L. monocytogenes* (Fig.4) showed that , *hlyA* gene was amplified in all seven tested isolates giving product of 174bp,that similar recorded by (Schuerch *et al.*, 2005; Gelbicova and Karpiskova ,2012; Khan *et al.*, 2014; and Ciolacu *et al.* ,2015).

The result of PCR for amplification of positive regulatory factor gene (*prfA*) in *L. monocytogenes* (Fig6) showed that, *prfA* gene wasn't amplified in all seven tested isolates, that dissimilar with (Holko *et al.*, 2002; Gelbicova and Karpiskova, 2012and Ciolacu *et al.*,2015).

The result of PCR for amplification of Invasion-associated protein (*iap*) gene in *L. monocytogenes* (Fig7) showed that *iap* gene wasn't amplified in all seven tested isolates, that dissimilar with Schuerch *et al.* (2005).

At the last, we can conclude from the present work that *L. monocytogenes* are mainly food born pathogen that could contaminate poultry products ,milk ,milk products and poultry eggs causing listeriosis. The isolated *L. monocytogenes* were sensitive to Sulphamethoxazole / trimethoprim (100%), followed by Gentamycin, Ceftazidime and Cephazoline (71.4%), and resistant to Erythromycin, Ampicillin and Clindamycin. all the isolated *L. monocytogenes* were CAMP Positive and produce  $\beta$ -zone of hemolysis .In PCR result assured that all seven strains are *listeria monocytogenes* and have *InlA* and *hlyA* virulence genes and lack *inlB* , *prfA* and *iap* genes.

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