Bacteriological and Molecular studies of Listeria species in milk and milk products at El-Kaliobia Governorate

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ABSTRACT

A total of 200 random samples of raw milk, Fita cheese, Kariesh cheese and ice cream (50 samples each) were collected from small retails and different supermarkets in El-Kaliobia Governorate to estimate the prevalence of Listeria species in such products with special interest to *L. monocytogenes*. The bacteriological examination of the samples resulted; 13(6.5%) isolates of Listeria species were recovered from 200 samples, includes 10 *L. monocytogenes* (5.0%) and 3 *L. gray*i (1.5%). Moreover, the other 4 species (*L. ivanovii; L. innocua; L. seeligeri* and *L. welshimeri*) were not isolated from all samples (Listeria strains). The in-vitro antimicrobial sensitivity test showed that the isolated *L. monocytogenes* were sensitive to amoxicillin; gentamycin; enrofloxacin; kanamycin and ampicillin. While they were resistant to Nalidixic acid, streptomycin and tetracycline. The results of virulence tests for isolated Listeria strains appeared that all of *L. monocytogenes* strains were virulent strains as all of them were positive to CAMP test; showed narrow zone of β-hemolysis on sheep blood agar and were positive for Anton's test. Meanwhile, *L. gray*i strains were non-virulent, as none of them could produce hemolysin (CAMP test negative) and negative for Anton's test. The PCR results for *L. monocytogenes* showed that all genes (16S rRNA; inlA; inlB; hlyA and prfA) were detected in five studied strains (100.0%) i.e., all studied strains were *L. monocytogenes* and all of them were virulent strains.

Keywords: Milk products, bacteriological evaluation, *L. monocytogenes*.

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1. INTRODUCTION

Milk and dairy products are excellent sources of essential nutrients and casein, a major milk protein. Because of their high nutritional value, they are very suitable for development of microorganisms, including pathogenic bacteria as Listeria species (Farber and Peterkin, 1991; Kasalica et al.,2011 and El-Marnissi et al., 2013) resulting in listeriosis in both human and animals (Ryser and Marth, 2007). *L. monocytogenes* has been involved in many outbreaks and sporadic cases of diseases primarily associated with the consumption of pasteurized milk, cheeses made from unpasteurized milk and other dairy based products that serve as good medium for the growth and survival of many pathogenic organisms in both industrialized and developing countries (Makino et al., 2005 and Manfreda et al., 2005). The outbreaks most often occurred from consumption of raw milk and dairy products because the Listeria organism capable of slow multiplication in refrigerated foods (Fleming et al., 1985). Important characteristics of *L. monocytogenes* are its ability to grow at temperatures of 1-44°C, at pH values of 5.0 and above, in high salt concentrations, and are relative resistance to freezing and drying (Lovett, 1989).

Members of the genus Listeria are short rods, aerobic to facultative anaerobic, Gram- positive, not forming spores and

capsules, distributed individually and in form of short chains, sometimes in form of the letters V and Y. In direct smear, they can be coccoid, and therefore mistaken with streptococci (Todar, 2009). Genus Listeria includes *L. monocytogenes; L. ivanovii; L. innocua; L. seelgeri; L. welshimeri* and *L. grayi. L. monocytogenes* is pathogenic for humans and animals, and *L. ivanovii* is mainly pathogenic for animals, primarily sheep. Other species are considered non-pathogenic. *L. monocytogenes* is intracellular pathogen and produces weak beta- hemolysis on blood agar. (McLaughlin, 1987; McLauchlin, 1990; Schuchat et al., 1991 and Low et al., 1993). The virulence factors of *L.monocytogenes* include Listeriolysin O (hlyA); internalin A (inlA); internalin B (inlB); a phosphatidylinositol- specific phosphor-lipase C and a lecithinase, aids in intracellular invasiveness and others aid in detoxifying cytotoxic oxidants as catalase and superoxide dismutase. The genes coding for many of the virulence factors are clustered together on the chromosome and regulated by the Positive regulatory factor (prfA) gene (Mengaud et al., 1991; Portnoy et al., 1992; Renzoni , 1999 ; Gedde et al., 2000 and Michael ,2005). There is a link between animals and their role as a source of infection for human either as a result of occupational contact with infected animals, during lambing or calving, or after consumption of contaminated animal products as meat, milk, cheese, ice cream and yoghurt (Conly and Johnston, 2008 and El-Marnissi et al., 2013) resulting in septicemia; papular exanthema; encephalitis and abortion or stillbirth of pregnant women (Gupta et al., 2003 and Hassan et al., 2005) . As the level of contamination of both milk and its products with Listeria species constitutes serious problems for consumers, so, the present study was conducted to estimate the prevalence of Listeria species in milk, Fita cheese, Kariesh cheese and ice cream at El-Kaliobia Governorate with special interest to *L.monocytogenes*. In addition to clarify the virulence of isolated strains and to carry out the antibiotic sensitivity testing of them. In addition, detection of some virulence factors of *L.monocytogenes* by PRC technique.

2. MATERIAL AND METHODS

2.1. Samples collection:

Two hundred random samples of raw milk, Fita cheese, Kariesh cheese and ice cream (50 samples each) were collected from small retails and different supermarkets at El-Kaliobia Governorate 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 broth1, half Fraser broth (without supplement) and incubated aerobically at 30±1 ºC for 24±3 hours. B- Secondary stage: One ml of incubated broth was inoculated into 9ml. Fraser broth2, full strength Fraser broth (with supplement) and incubated at 37 ºC for 48±3 hours. C- Third stage: 0.1 ml 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±1 ºC for 48 hours and examined after 24±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°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 test:

The isolated *L.monocytogenes* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Martin, 1982 and NCCLS, 1999).

2.4. Virulence tests:

A- Hemolytic activity: All isolates were cultured onto 5% sheep blood agar to

determine their hemolytic activity. In addition to, they were subjected to CAMP (Christie-Atkins- Munoh-Peterson) test (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 (1-2 mm). After incubation for 24-48 hours at 35°C, a positive reaction consists of an enhanced zone of β- hemolysis.

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

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

PCR using five sets of primers was used for genotypic detection of *L.monocytogenes* strains and four virulence genes that may play a role in virulence of *L.monocytogenes*. These genes were 16S rRNA gene; internalin A (inlA); internalin B (inlB); Listeriolysin O, haemolysin (hlyA) and Positive regulatory factor (prfA). It was applied on five random isolated *L.monocytogenes*, following QIAamp® DNA Mini Kit instructions (Catalogue no. M501DP100), Emerald Amp GT PCR mastermix (Takara) with Code No. RR310A and 1. 5% agarose gel electrophoreses (Sambrook et al., 1989). The PCR condition have specific sequence and amplify a specific product as shown in Table (3). Temperature and time conditions of the primers during PCR are shown in Table (4) according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit

3. RESULTS

The bacteriological examination of studied samples revealed that, Listeria spp. were isolated from 11 positive samples out of 200 ones (5.5%); represented as 3 positive samples (1.5% and 27.3%) from raw milk; Kariesh cheese and ice cream samples followed by 2 (1.0% and 18.2%) from Fita cheese samples (Table, 1). Mixed isolates were present in raw milk samples only. Moreover, 13 (6.5%) isolates of Listeria species were recovered from 200 samples, includes 10 *L. monocytogenes* (5.0%) and 3 *L. grayi* (1.5%). *L.monocytogenes* was isolated with an incidence of 76.9% (3 from each samples of raw milk; Kariesh cheese and ice cream (23.1%) and 1(7.7%) from Fita cheese). Meanwhile, *L. grayi* was isolated with an incidence of 23.1% (2 from raw milk samples (15.4%) and 1(7.7%) from Fita cheese only), percentages were calculated in relation to total number of 13 isolated Listeria species as shown in Table (2). Moreover, the other 4 species (*L. ivanovii; L. innocua; L. seeligeri and L. welshimeri*) could not isolated from all samples.

The isolated colonies grow well and showed green-blue colonies surrounded by an opaque halo on ALOA agar and gray green colonies with black depressed button center and black hollow surrounded them on PALCAM agar and black colonies with dimpled centers on Oxford agar. They were Gram - positive bacilli or coccobacilli; motile showing umbrella pattern motility. 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 in vitro sensitivity test (Fig., 1) showed that, the isolated *L.monocytogenes* were sensitive to amoxicillin and gentamycin (80.0%) followed by enrofloxacin; kanamycin and ampicillin (70.0%; 70.0% and 60.0% respectively).While the isolated strains were resistant to Nalidixic acid, streptomycin and tetracycline.

The results of virulence tests proved that, all isolated *L.monocytogenes* strains showed narrow zone of Beta hemolysis on 5% sheep blood agar, 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 hr followed by keratitis in all rabbits (Anton's test positive). Meanwhile, *L. grayi* strains were non-virulent, as none of them could produce haemolysin (CAMP test negative) and negative for Anton's test.

The PCR results for *L. monocytogenes* showed that, all genes (16S rRNA; inlA; inlB; hlyA and prfA) were detected in five studied strains (100.0%) i.e., all studied strains were *L. monocytogenes* and all of them were virulent strains. The 16 S rRNA gene was amplified in five (100.0%) *L. monocytogenes* strains giving product of 553 bp as shown in Fig. (2). i.e., all studied strains were *L. monocytogenes*. The inlA gene was amplified in five (100.0%) *L. monocytogenes* strains giving product of 800 bp as shown in Fig. (3).The prfA gene was amplified in five (100.0%) *L. monocytogenes* strains giving product of 1052 bp as shown in Fig. (3).The inlB gene was amplified in five (100.0%) *L. monocytogenes* strains giving product of 343 bp as shown in Fig. (4).The hlyA gene was amplified in five (100.0%) *L. monocytogenes* strains giving product of 174 bp as shown in Fig. (4).

Table (1): Total number and Percentage of positive samples for Listeria isolation from the examined samples

Samples	Number of samples	Number of positive samples	Positive percentage		
			%[1]	%[2]	%[3]
Raw milk	50	3	6.0	27.3	1.5
Fita cheese	50	2	4.0	18.2	1.0
Kariesh cheese	50	3	6.0	27.3	1.5
Ice cream	50	3	6.0	27.3	1.5
TOTAL	200	11	5.5	100.0	5.5

[1]Percentage in relation to total number of samples in each row. [2] Percentage in relation to total number of positive samples (11). [3] Percentage in relation to total number of collected samples (200)

Table (2): Incidence of Listeria species strains isolated from the examined samples

Listeria species	*L.monocytogenes*			*L. grayi*			*TOTAL*		
Samples	No.	%*	%**	No.	%*	%**	No.	%*	%**
Raw milk	3	6.0	23.1	2	4.0	15.4	5	2.5	38.4
Fita cheese	1	2.0	7.7	1	2.0	7.7	2	1.0	15.4
Kariesh cheese	3	3.0	23.1	0	0.0	0.0	3	1.5	23.1
Ice cream	3	6.0	23.1	0	0.0	0.0	3	1.5	23.1
TOTAL	10	5.0	76.9	3	1.5	23.1	13	6.5	100.0

* Percentage in relation to total No. of each examined sample (50 & 200 for total).
** Percentage in relation to total No. of isolated Listeria species (13).

Table (3): Oligonucleotide primers sequences Source

Primer	Sequence	Amplified product	Reference
16S rRNA	CCT TTG ACC ACT CTG GAG ACA GAG C AAG GAG GTG ATC CAA CCG CAC CTT C	553 bp	Lantz *et al*., 1994
prfA	TCT-CCG-AGC-AAC-CTC-GGA-ACC TGG-ATT-GAC-AAA-ATG-GAA-CA	1052 bp	Dickinson *et al*., 1995
inlA	ACG AGT AAC GGG ACA AAT GC CCC GAC AGT GGT GCT AGA TT	800 bp	Liu *et al*., 2007
inlB	CTGGAAAGTTTGTATTTGGGAAA TTTCATAATCGCCATCATCACT	343 bp	Kirkan *et al*., 2006
hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT	174 bp	Deneer and Boychuk, 1991

Table (4): Cycling conditions of the different primers during cPCR

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

4. DISCUSSION

Listeria species in milk and dairy products are very important for human health. *L. monocytogenes* has been involved in several outbreaks and sporadic cases of listeriosis associated with the consumption of pasteurized milk and other dairy products (Van Kassel et al., 2004 and Makino et al., 2005). Therefore, this study was conducted to estimate the prevalence of Listeria species in milk, Fita cheese, Kariesh cheese and ice cream at El-Kaliobia Governorate with special interest to *L. monocytogenes* and bacteriological characterization of

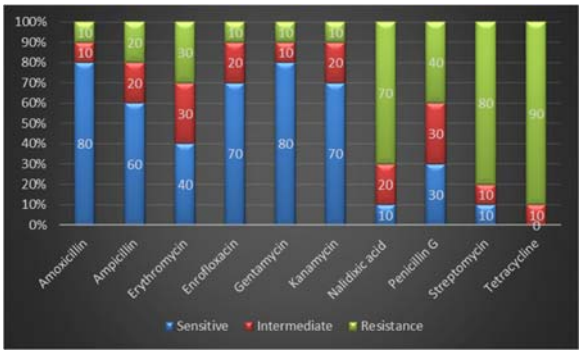

Fig. (1): In-Vitro anti-microbial Sensitivity test for isolated *L.monocytogenes* strains

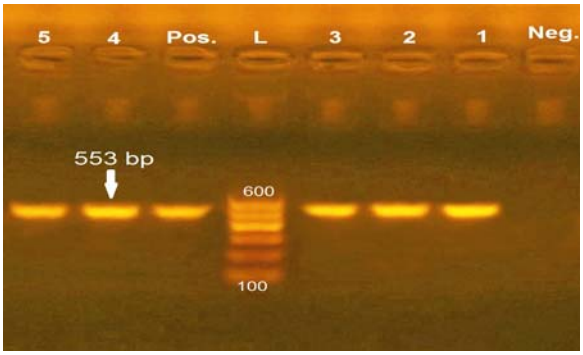

Fig. (2): 16S rRNA genes. Lane L: 100 – 600 bp Ladder. Neg.: Negative control. Pos.: Positive control (at 553 bp). Lanes 1 to 5: L. monocytogenes (16S rRNA) gene positive

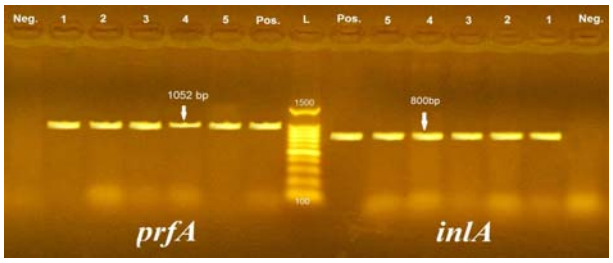

Fig. (3): internalin A (inlA) genes. Lane L: 100 – 1500 bp Ladder. Neg.: Negative control.Pos.: Positive control (at 800 bp). Lanes from right side 1 to 5: L.monocytogenes ((inlA) gene positive. Lane L: 100 – 1500 bp Ladder. Neg.: Negative control. Pos.: Positive control (at 1052 bp). Lanes from left side 1 to 5: L.monocytogenes ((prfA) gene positive.

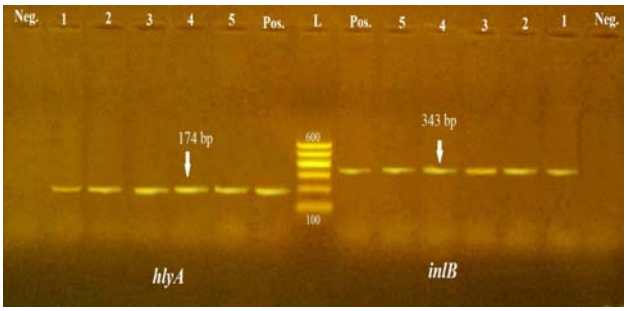

Fig. (4): internalin B (inlB) genes. Lane L: 100 – 600 bp Ladder. Neg.: Negative control. Pos.: Positive control (at 343 bp). Lanes from right side 1 to 5: L. monocytogenes (inlB) gene positive Lane L: 100 – 600 bp Ladder. Neg.: Negative control. Pos.: Positive control (at 174 bp). Lanes from left side 1 to 5: L. monocytogenes ((hlyA) gene positive

them. The results of Listeria spp. isolation (Table 1) revealed that, 11 out of 200 samples were positive for isolation (5.5%); represented as 3 positive samples (1.5% and 27.3%) from each type of samples of raw milk, Kariesh cheese and ice cream samples followed by 2 (1.0% and18.2%) from Fita cheese samples. Mixed isolates were present in raw milk samples only. These results came in accordance with that obtained by (EL-Malt and Abd El – Hameed, 2009 and El- Marnissi et al., 2013).

The results of bacteriological examination of examined samples revealed that, a total of 13(6.5%) isolates of Listeria species were recovered from 200 samples, includes 10 *L.monocytogenes* (5.0%) and 3 *L. grayi* (1.5%). *L.monocytogenes* was isolated with an incidence of 76.9% (3 from each samples of raw milk; Kariesh cheese and ice cream (23.1%) and 1(7.7%) from Fita cheese). Meanwhile, *L. grayi* was isolated with an incidence of 23.1% (2 from raw milk samples (15.4%) and 1(7.7%) from Fita cheese only), percentages were calculated in relation to total number of 13 isolated Listeria species as shown in Table (2). Nearly similar results were recorded by Abd El –Aal and Atta, 2009; El- Marnissi et al., (2013) and Ning et al., (2013). Meanwhile, these results disagreed with those recorded by Massa et al. (1990); Morales et al. (1995) and Pednekar et al. (1997) who failed to isolate *L. monocytogenes*. Moreover, the other 4 species (*L.ivanovii; L. innocua ; L. seeligeri* and *L. welshimeri*) were not isolated from all samples .These results finding go hand in hand with the finding of Farber et al. (1988) and Lund et al. (1991). Meanwhile, they disagreed with the finding of Silva et al. (1998); EL-Malt and Abd El -Hameed, 2009) who isolated them from milk and dairy product samples.

The results of antibiotic sensitivity tests for the isolated *L.monocytogenes* (Fig., 1) showed that, the isolated *L.monocytogenes* were sensitive to amoxicillin and gentamycin (80.0%) followed by enrofloxacin; kanamycin and ampicillin (70.0%; 70.0% and 60.0% respectively).While the isolated strains were resistant to Nalidixic acid, streptomycin and tetracycline. Nearly similar results were recorded by Hof et al. (1997); Al- Sadie et al. (1998); Drevets et al. (2004); Maarouf et al. (2007); Ennaji et al., (2008) and Garedew et al., (2015).

The pathogenicity of Listeria spp. is closely associated with their haemolytic activities (Cossart et al., 1989; Gedde et al, 2000 and Maarouf et al., 2007). The results of virulence tests for isolated Listeria appeared that, all *L. monocytogenes* were positive to CAMP test and showed narrow zone of β-haemolysis on sheep blood agar. The same results were recorded by Cossart et al. (1989); Portnoy et al. (1992) and McKellar (1994) who cited that all virulent *L. monocytogenes* produce a haemolysin (Listeriolysin O) which lyses the host vacuole, allowing it to grow in the host cytoplasm. For CAMP test, McKellar (1994) suggested that *S. aureus* sphingomyelinase sensitized the RBC membrane prior to complete lyses with *L. monocytogenes*. Also, all isolated strains were positive for Anton's test. This was in agreement with Sneath et al. (1986); Shafie and Amer (2002) and Maarouf et al. (2007). Therefore, all the isolated *L.monocytogenes* strains were virulent strains as all of them produced haemolysin (CAMP test); as well as Anton's test and catalase test were positive. Similar results were reported by Conner et al. (1989); McKellar (1994); Vazquez - Boland et al (2001) and Maarouf et al. (2007). Meanwhile, *L. grayi* strains were non-virulent, as none of them could produce haemolysin (CAMP test negative) and negative for Anton's test.

The PCR results for *L.monocytogenes* showed that, all genes (16S rRNA; inlA ;inlB; hlyA and prfA) were detected in five studied strains (100.0%) i.e., all studied strains were *L.monocytogenes* and all of them were virulent strains. Similar results were decided by (Dramsi et al., 1996; Kuhn and Goebel ,1999; Jaradat et al., 2002;

Joseph and Goebel, 2007; Gelbicova and Karpiskova (2012); Shen et al., 2013 and Ciolacu et al., (2015). Regarding to the occurrence of 16 S rRNA genes in *L.monocytogenes* isolates. The obtained result revealed that, it was amplified in all tested five (100.0%) *L.monocytogenes* strains giving product of 553 bp as shown in Fig. (2). i.e., all studied strains were *L.monocytogenes*. These results came in accordance with those recorded by Holko et al., (2002); Michael et al., (2005); Schuerch et al., (2005); Liu et al., (2007); Gelbicova and Karpiskova (2012); Swetha et al., 2012 and Ciolacu et al., (2015). They reported that, PCR save time for diagnosis hence allowing a rapid identification of *L.monocytogenes* with high sensitivity and specificity. The results of PCR for amplification of internalin A (inlA) gene in *L.monocytogenes* (Fig., 3) showed that, the inlA gene was amplified in five (100.0%) strains giving product of 800 bp. Similar findings were recorded by Almeida and Almeida (2000); Jaradat et al., (2002); Joseph and Goebel (2007); Liu et al., (2007); Gelbicova and Karpiskova (2012) Shen et al., (2013) and Ciolacu et al., (2015). The results of PCR for amplification of Positive regulatory factor (prfA) gene in *L.monocytogenes* (Fig., 3) showed that, the prfA gene was amplified in five (100.0%) strains giving product of 1052 bp. Similar findings were recorded by Holko et al., (2002); Jaradat et al., (2002); Begley et al., (2005); Liu et al., (2007); Joseph and Goebel ,(2007); Seveau et al., (2007); Lemon et al., (2010); Gelbicova and Karpiskova (2012); Shen et al., 2013 and Ciolacu et al., (2015) who reported that, this gene positively regulates all virulence factors in *L. monocytogenes*. The results of PCR for amplification of internalin B (inlB) gene in *L.monocytogenes* (Fig., 4) showed that, the inlB gene was amplified in five (100.0%) strains giving product of 343 bp. Similar findings were recorded by Jaradat et al., (2002) Dongyou Liu (2006); Kirkan et al., (2006); Joseph and Goebel (2007) and Gelbicova and Karpiskova (2012). The results of PCR for amplification of Listeriolysin O, haemolysin (hlyA) gene in *L. monocytogenes* (Fig., 4) showed that, the hlyA gene was amplified in five (100.0%) strains giving product of 174 bp. Similar findings were recorded by Jaradat et al., (2002); Schuerch et al., (2005); Joseph and Goebel, (2007); Rawool et al., (2007); Gelbicova and Karpiskova (2012); Swetha et al., (2012); Khan et al., (2014); Mohamed, (2014) and Ciolacu et al., (2015).

Finally, from results of the present work it could be concluded that, Listeria species specially, *L.monocytogenes* are important pathogens could be contaminate the milk and milk products as Fita cheese and ice cream and causes a foodborne disease(listeriosis). The isolated *L.monocytogenes* were sensitive to amoxicillin; gentamycin; enrofloxacin; kanamycin and ampicillin and were resistant to Nalidixic acid, streptomycin and tetracycline. PCR results were in complete agreement with those obtained from standard cultural procedures, as, all 5 studied strains were *L. monocytogenes* and all of them had virulent genes with 100%, moreover, all the isolated *L. monocytogenes* strains produced B zone of hemolysis and CAMP test positive; as well as Anton's test and catalase test were positive.

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