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Molecular characterization of listeria monocytogenes isolated from raw milk and some dairy products at local markets in Damanhour city, Egypt

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A B S T R A C T

Consumption of milk and dairy products has been involved with several outbreaks of *L. monocytogenes*. The current study was conducted to investigate the prevalence rate of *listeria monocytogenes* in milk and some dairy products. A total of 225 samples of; raw milk (75), pasteurized milk (50), ice cream (50) and Ras cheese (50) were collected randomly within Damanhour city, El-Behira governorate, Egypt, from different retail outlets, supermarkets, and other markets outlets. Out of 225 samples, 29 (12.88%) were positive for presence of different *Listeria* species. The incidence rate of *Listeria* spp. in the samples of raw milk, pasteurized milk, ice cream and Ras cheese were 10 (13.33%), 6 (12%), 7 (14%) and 6 (12%), respectively. The most prevalent isolated species of *listeria* examined in this study were *L. innocua* and *L. monocytogenes*. The biochemically identified isolates of *L. monocytogenes* (16) were molecularly identified by PCR for detection of three different virulence genes (*iap*, *hylA* and *actA*); the results showed that *iap* gene was demonstrated in all isolates (100%); *hylA* and *actA* were detected in 83.3 and 66.7% of isolates from raw milk; 66.7 and 66.7% of isolates from pasteurized milk; 80 and 80% of isolates from ice cream; 100 and 50% of isolated from Ras cheese samples. Concerning antibiotic resistance, 16 isolates of *L. monocytogenes* were tested against 14 antibiotics discs. All isolates of *L. monocytogenes* were resistant to Kanamycin (100%) and Nalidixic acid (93.75%), meanwhile, most of the isolates showed sensitivity against Ciprofloxacin (87.50%) and Ampicillin (68.75%). In conclusion, the study findings emphasize the critical need for applying strict and proper hygienic measures especially during stages of processing, storage and marketing of milk and dairy products.

Keywords: Listeria spp., *Listeria monocytogenes*, Dairy products, multiplex PCR

1. Introduction

The high nutritional value of milk and its products favor the multiplication of several microorganisms, including pathogenic bacteria (Kasalica et al, 2011). Listeria spp. are gram positive, rod-shaped, non-spore forming and facultative anaerobic organisms (Odetokun and Adetunji, 2017). The genus Listeria has been divided according to 16S rRNA sequences into 17 species with 4 subtypes (Anonymus 2017). Classic Listeria species (*L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L. seeligeri, L. welshimeri*) can be isolated from food. Recently, 11 recent Listeria species were identified (Barre et al., 2016). Both *L. monocytogenes* and *L. ivanovii* are the most popular pathogenic species within this bacteria genus. *L. monocytogenes* can cause illness and even death for humans and all mammals. Ruminant animals are primarily infected with *L. ivanovii* (Hellberg et al., 2013). Listeria species are widely distributed in soil, sewage, surface water, animal feed, food processing equipment, farm, urban and suburban settlements (Korsak and Szuplewska, 2016). Listeria spp. are the most frequently prevalent in the milk processing environment.

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Also, dairy products may become contaminated with *L. monocytogenes* during subsequent stages of production (Seyoum et al., 2015). Eldaly et al., (2013) confirmed that insufficient heat treatment of food enhances the multiplication of *L. monocytogenes*. Several outbreaks of *L. monocytogenes* were associated with milk and dairy products consumption as Listeria microorganism able to of multiply slowly in refrigerated food which subjected to minimal further processing heat treatment and post processing contamination (CDC, 2011; Gaulin et al., 2012).

Post-pasteurization contamination of milk or defects during pasteurization (inadequate temperature, technical errors) are related with *L. monocytogenes* incidence in pasteurized milk. Therefore, the occurrence of *L. monocytogenes* in milk and dairy products could be due to failure in the pasteurization process or post-pasteurization contamination (Lee et al., 2019). Seyoum et al., (2015) reported high incidence of listeria species in pasteurized milk (60%).

L. monocytogenes is characterized by an antimicrobial resistance which is associated with the presence of a plasmid, conjugated genes, and chromosomal gene mutation (Poros-Gluchowska and Markiewicz, 2003). Also, resistance of *listeria monocytogenes* to antibiotics associated with misuse of antibiotics (Rahimi et al., 2012).

Contamination of milk and its products with different species of Listeria constitutes serious health problems for consumers, so, the aim of the present study is molecular identification and antimicrobial resistance profile of *Listeria monocytogenes* which has been isolated from milk and some dairy products produced in Damanhour city, El-Behira governorate, Egypt**.**

2. Materials and Methods

2.1. Collection of samples:

A total of 225 random samples represented by raw milk (75), pasteurized milk (50), ice cream (50) and Ras cheese (50) were purchased from different markets and dairy shops located in Damanhour city, El-Behira governorate, Egypt. All collected samples were separately collected in clean polyethylene bag and transferred without undue delay in an icebox to the Food analysis central Lab, Benha University for further examination.

2.2. Isolation and identification of the Listeria species according to ISO 11290-1 (2017):

For milk, ice cream and Ras cheese; 25 ml or g of each product sample was aseptically homogenized in Listeria half Fraser broth (225 ml, Oxoid) which was supplemented with Listeria selective enrichment (Oxoid). Homogenization was applied for 2-4 minutes in a stomacher followed by incubation for 48 hours at 30 °C. Accurately, 1 ml of the primary enrichment was added to Fraser broth (10 ml) and incubated for 48 hours at 30 °C. A loopful of the previously incubated Fraser broth was inoculated into Oxford agar media and incubated under the same incubation condition. Characteristic colonies (2 mm darker greenish sheen with black halo and sunken centers) were subcultured onto tryptone soy agar which was supplemented with yeast extract (TSAYE, 0.6%) then incubated at 37º C for 24 hours.

All separated colonies were biochemically characterized according to (Aygun and Pehlivanlar, 2006).

2.3. Detection of Listeria monocytogenes virulence genes by multiplex PCR technique: -

L. monocytogenes were tested for the incidence of invasive associated protein gene (iap), Listeriolysin O (hlyA) and Actin polymerization protein gene (actA).

***2**.3.1. DNA Extraction of L. monocytogenes using QIA amp kit (Shah et al., 2009):*

All isolated strains of *L. monocytogenes* were grown in Brain Heart Infusion (BHI) broth overnight at 37ºC, then the mixture was heated for 20 minutes at 100 ºC. Accurately, the culture (50-200 µl) was kept at -40ºC till use. In PCR reaction mixture, 5 µl of the lysate was used as DNA template.

2.3.2. Amplification reaction of L. monocytogenes according to Kaur et al., (2007):

The amplification reaction was performed on the Master Thermal Cycler (Eppendorf, Hamburg, Germany). A multiplex PCR was applied for detection of three virulence genes (iap, hlyA and actA). The PCR reaction volume was set up 50 µl. The optimized reaction mixture was: 10 µl PCR buffer (consisting of Tris–HCl, pH 8.3(100 mmol l); 500 mmol KCl; 0.01% gelatin and 15 mmol MgCl2), 7.5 mmol MgCl2, 1 mmol dNTP mix and 10 lmol of both forward and reverse primer for each gene, 5 µl of cell lysate, 5 U of Taq DNA polymerase and sterilized milliQ water.

Primer used in this study in table (1).

PCR cycling conditions included an initial DNA denaturation for 2 min at 95°C followed by 35 denaturation cycles (each 15 sec) at 95°C, 30 sec annealing at 60°C, extension for one minute at 72°C, final extension at 72°C for10 min and held at 4°C. The same PCR amplification cycles were used for all the virulence gene primers.

Agarose gel 1.5% electrophoresis (AppliChem, Germany, GmbH) with ethidium bromide stain in 1x TBE buffer were used for visualization of Amplified DNA fragments on UV transilluminator. A DNA Ladder (100 bp plus, Qiagen, Germany, GmbH) was applied fragment size detection.

2.4. Antibiotic Resistance of isolated Listeria monocytogenes (Jamali et al., 2013).

Antimicrobial susceptibility of 16 biochemically identified *L. monocytogenes* was examined using the single diffusion technique. Sensitivity antibiotic discs (Oxoid Limited, Basingstoke, Hampshire, UK) with different concentrations were used.

In the agar plate technique, nutrient agar was used as a substrate for growth of the bacterium tested for its antibiotic resistance. The surface of nutrient agar was uniformly inoculated with the bacterial culture. The antibiotic discs were distributed on the surface of plate inoculated with L. monocytogenes. The plates were incubated at 25°C for 2-7 days and examined for *L. monocytogenes* growth area around the discs. Complete inhibition zones were measured and interpreted.

Therefore, the antimicrobial susceptibility analysis was applied according to the guidelines stipulated Clinical and Laboratory Standards Institute (CLSI, 2018). Accordingly, the concentrations of the antimicrobial discs and the diameters of the obtained inhibition zones are demonstrated in table (2)

3.2. Statistical analysis:

Data analysis was performed by Statistical Package for Social Science (SPSS version 16, 2008).

3. Results and Discussion

The high prevalence rate of *Listeria* spp. in milk and its products is considered an important hazard on dairy industry and public health (Scallan et al., 2011). Many listeriosis outbreaks all over the world are related to consumption of milk and its products. In the USA, listeriosis was firstly reported in 1983 after pasteurized milk consumption (Cartwright et al., 2013).

The results in Table (3) illustrated that the highest incidence rate of *listeria* species was observed in ice cream samples 14% followed by raw milk 13.33%, and 12 % for both pasteurized milk and Ras cheese samples. The highest prevalence of different *Listeria* spp. in ice cream could be originated from raw milk contamination, supplies with contaminated water, the ingredients added with low quality, lack of proper hygienic practices during handling processing and lack of pasteurization step in case of ice cream produced at small scale.

A nearly similar prevalence of *listeria* species in raw milk was obtained by Saha et al., (2015) who reported that incidence was 13.46%. Higher incidence percentage: 54% and 45% in raw milk was reported by Hossein et al., (2013), and Hesham et al., (2017), respectively. Lower incidence of listeria species 7.5%, 7.33% and 5.49 % in raw milk was reported by El Hag et al., (2020); Haggag et al., (2019) and Shamloo et al., (2015), respectively.

Higher incidence rate of listeria species in pasteurized milk was demonstrated by Seyoum et al., (2015) who reported an incidence rate of 60%. Lower prevalence was reported by Waghamare et al., (2012) who found that the prevalence rate of listeria species was 4% in pasteurized milk. In contrast to the postulated findings, Listeria spp. couldn't be detected in examined samples of pasteurized milk in studies applied by Şanlıbaba and Tezel (2018); Owusu-Kwarteng et al., (2018); Muthulakshmi et al., (2018)

Higher rate of incidence of listeria in ice cream (45%) was reported by Garedew et al., (2015) while, lower incidence rate (3%) was reported by Abd El-Tawab et al., (2015). Contrary to the recorded findings, Listeria spp. couldn't be detected ice cream samples examined by Kevenk and Gulel (2016); Akrami-Mohajeri et al., (2018); Mohamed et al., (2020) studies. In addition, Mohamed et al., (2020) couldn't isolated listeria species from Ras cheese samples.

The abovementioned result in Table (3) illustrated that the most prevalent listeria isolates from raw milk samples was *L. innocua* 41.7% followed by *L. monocytogenes* 35.29%. In the examined pasteurized milk samples, the incidence rate of listeria isolates was, L. monocytogenes 37.5% followed by *L. innocua* 25%, *L. seeligeri* 25%; from examined ice cream samples, *L. monocytogenes* incidence rate was 41.67% followed by *L. innocua* 33.33% finally from examined Ras cheese samples, *L. innocua* incidence rate was 42.86% followed by *L. monocytogenes* 28.57% and *L. ivanovii* 28.57% .

Listeria innocua has the highest incidence rate between listeria species recovered from examined raw milk followed by *L. monocytogenes*, this result was agreed with Meshref et al., (2015) who reported that L. innocua has the highest incidence (35.71%) among listeria species recovered from the samples of raw milk in Beni-suef, Egypt.

The most prevalent listeria species isolated from samples of pasteurized milk samples was L. monocytogenes. This result agreed with Seyoum et al. (2015) who found that the most prevalent listeria species isolated from pasteurized milk was *L. monocytogenes* (20%), L. innocua (15.4%) and L. ivanovii (9.2%). *L. monocytogenes* was the most prevalent listeria species isolated from ice cream samples. This finding disagreed with El-Shinaway et al., (2017) who found that the most prevalent listeria species isolated from samples of ice cream was *L. grayii*.

The most prevalent listeria species recovered from examined Ras cheese samples was *L. innocua* then *L. ivanovii* and *L. monocytogenes*. Contrary to these results, *L. monocytogenes* couldn't be detected in Ras cheese samples examined by Mohamed et al., (2020). Rahimi et al. (2010) revealed higher prevalence rate of *L. monocytogenes* in the examined milk samples (72.4%). Lower incidence rate (2.1%) of *L. monocytogenes* in raw milk was demonstrated by Durmaz et al., (2015) (2.1%) and Seyoum et al. (2015) (2.04%). On the contrary, *Listeria monocytogenes* couldn't be recovered from raw milk samples in study performed by Aygun and Pehlivanlar (2006).

Lower incidence rate of *L. monocytogenes* in examined ice cream was reported by Garedew et al., (2015) 15%. On the other hand, *L. monocytogenes* failed to isolate from ice cream samples in Akya et al. (2013); Metwally and Ali (2014) and Akrami-Mohajeri et al., (2018) studies.

According to Egyptian Standards, (2005), which stipulated that milk and dairy products should be free from *L. monocytogenes*, there are 13.33, 12, 14, and 12% of examined raw milk, pasteurized milk, ice cream and Ras cheese samples exceeding that permissible limit, respectively (Table 4).

Three main virulence genes were detected in 16 biochemically identified *L. monocytogenes* isolates by multiplex PCR technique (Table 5). The iap gene occurrence rate was in 100% of isolates from raw milk, pasteurized milk, ice cream and Ras cheese samples; hylA and actA were detected in 5 /6 (83.3%) and 4 /6 (66.7%), respectively in raw milk; 2 /3 (66.7%) and 2/3 (66.7%), respectively in pasteurized milk; 4/5 (80%) and 4/5 (80%), respectively in ice cream; 2 /2 (100%) and 1/2 (50%), respectively in Ras cheese sample.

In the current study hylA was detected in 83.3 and 80 of examined raw milk and ice cream samples. These results nearly agreed with Abd El Tawab et al. (2015) who reported that the hlyA gene was detected in 5 (100%) of *Listeria monocytogenes* isolates from samples of raw milk and ice cream samples. Also, Nayak et al. (2015) isolated listeriolysin O (hlyA) virulence gene in *L. monocytogenes* isolates of raw milk.

All the biochemically identified strains (16) of *L. monocytogenes* were examined for three virulence associated genes iap, hylA and actA using multiplex PCR technique (Photo 1); iap, hylA and actA was found in 7 isolates *of L. monocytogenes* at 131, 456 and 839 bp, respectively; iap and hylA was detected in 5 isolates; Finally, iap and actA was detected in 3 isolates. In the current study, the detected virulence genes were the most common genes in detection of *L. monocytogenes* virulence (Osaili et al., 2011).

The main virulence genes of *L. monocytogenes* are hlyA, inlA, prfA, plcA, inlB, plcB, actA, and mpl (Almeida et al., 2017). hlyA gene encoded Listeriolysin O (LLO) which is present only in virulent strains of the listeria species Suriyapriya et al. (2016). In many studies the hlyA gene is employed to differentiate *L. monocytogenes* from other Listeria spp. and other related microorganisms (Norton and Batt, 1999). The surface protein actin A (ActA) is responsible for intracellular mobility through polymerization of actin and cell-to cell invasion and adhesion (Travier et al., 2013).

Findings of antimicrobial susceptibility testing are presented in Table (6). The results revealed an increased resistance rates to kanamycin 100%, Nalidixic acid (93.75%), Streptomycin (81.25%), Neomycin (81.25%), Amikacin (62.50%) and oxytetracycline (56.25%) were observed. On the other hand, 87.50, 68.75, 62.50, 56.25, 56.25 and 50% of strains were susceptible to Ciprofloxacin, Ampicillin, Cefotaxime, enrofloxacin, Sulphamethoxazole and gentamycin, respectively. Our study results reported that most isolates of the *L. monocytogenes* were sensitive to sulfamethoxazole, gentamicin and trimethoprim because these antimicrobial agents are not widely used in veterinary field (Harakeh et al., 2009).

The abovementioned results agreed with Şanlıbaba et al., (2018) who reported that strains of *L. monocytogenes* isolated from food products exhibit resistance to amoxicillin, kanamycin and levofloxacin, Also, Girma and Abebe (2018) reported *L. monocytogenes* isolates from samples of raw milk exhibited resistance to nalidixic acid, followed by tetracycline, chloramphenicol and streptomycin. Aksoy et al., (2018) cleared that *L. monocytogenes* have high resistance to trimethoprim-sulfamethoxazole.

Our results indicated that *L. monocytogenes* was sensitive to Ciprofloxacin and gentamycin. This result agrees with Sreeja et al., (2016) reported that ciprofloxacin was significantly effective in interfering with L. monocytogenes growth. Gohar et al., (2017) recorded sensitivity of all *L. monocytogene*s strains isolated from raw milk to ciprofloxacin and gentamicin. On contrary to the postulated results, Saha et al., (2015) reported that the highest resistant of *L. monocytogenes* recorded against Ciprofloxacin (100%).

Wang et al., (2013) recommended a combination of gentamicin and, amoxicillin or ampicillin to overcome human listeriosis.

4. Conclusion

It is concluded that raw milk and some dairy products present in Damanhour markets may be a threat to the consumer health. People with high risk factors to get listeriosis should avoid consumption of such products. This also reflects the important need for monitoring the potential sources of *L. monocytogenes*. Application of HACCP is important for listeria control during production and processing of dairy products and could decrease contamination of these products with Listeria species. The incidence of antimicrobial-resistant Listeria strains is a serious alarm to the public health danger. The high awareness of limited use of antibiotics is a critical step to limit the risk of development of multidrug-resistant bacteria.

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Table 1 Primer used in this study.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
iap (F)	5′ ACAAGCTGCACCTGTTGCAG ′3	131	Swetha et al. (2013)
iap (R)	5′ TGACAGCGTGTGTAGTAGCA ′3		
hlyA (F)	5′GCAGTTGCAAGCGCTTGGAGTGA ′3	456	
hlyA (R)	5′ GCAACGTATCCTCCAGAGTGATCG ′3		
actA (F)	5′ CGCCGCGGAAATTAAAAAAAGA ′3	839	Suarez and Boland (2001)
actA (R)	5′ACGAAGGAACCGGGCTGCTAG ′3		

Table 2 Antimicrobial discs and interpretation of their action on the isolated pathogens.

Antimicrobial agent	Content (ug)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Neomycin (N)	30	12 or less	13-16	17 or more
Ampicillin (AM)	10	13 or less	14-17	18 or more
Cefotaxim (CF)	30	17 or less	18-22	23 or more
Ciprofloxacin (CP)	5	15 or less	15-19	20 or more
Erythromycin (E)	15	13 or less	14-22	23 or more
Cephalothin (CN)	30	14 or less	15-17	m18 or more
Gentamicin (G)	10	12 or less	13-14	15 or more
Enrofloxacin (EN)	5	11 or less	12	13 or more
Kanamycin (K)	30	13 or less	14-17	18 or more
Amikacin (AK)	30	12 or less	13-15	16 or more
Streptomycin (S)	10	11 or less	12-14	15 or more
Oxytetracycline (T)	30	14 or less	15-18	19 or more
Nalidixic acid (NA)	30	13 or less	14-18	19 or more
Sulfamethoxazole (SXT)	25	10 or less	11-15	16 or more

Table (3): The frequency of Listeria species in raw milk and examined dairy products.

Examined samples	No. of samples	No. of positive samples for *listeria* species	*Listeria species*				
			L. monocytogenes	*L. innocua*	*L. ivanovii*	*L. seeligeri*	*L. welshimeri*
Raw milk	75	10 (13.33 %)	6 (35.29%)	7 (41.17 %)	1 (5.89%)	1 (5.89 %)	2 (11.76%)
Pasteurized milk	50	6 (12%)	3 (37.5%)	2 (25%)	0 (0%)	2 (25%)	1 (12.5%)
Ice cream	50	7 (14%)	5 (41.67%)	4 (33.33 %)	1 (8.33%)	0 (0%)	2 (16.67%)
Ras cheese	50	6 (12%)	2 (28.57%)	3 (42.86 %)	2 (28.57 %)	0 (0%)	0 (0%)
Total	225	29 (12.88 %)	16 (7.11%)	16 (7.11%)	4 (1.78%)	3 (1.33 %)	5 (2.22%)

Table (4): Prevalence of Listeria monocytogenes isolated from examined milk and dairy products samples in Comparison with Egyptian Standards.

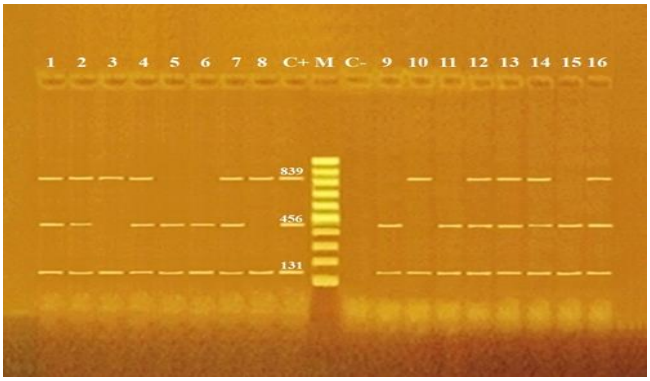
Products	No. of examined samples	Egyptian Standards	Samples do not conform with Egyptian Standards	
			No.	%
Raw milk	75	Nil (ES:154-1/2005)	10	13.33
Pasteurized milk	50	Nil (ES:1616/2005)	6	12
Ice cream	50	Nil (ES:1185-1/2005)	7	14
Ras cheese	50	Nil (ES:1007-5/2005)	6	12

Table (5): Prevalence of virulence genes of Listeria monocytogenes isolated from examined milk and dairy products samples.

Products	No. of examined Isolates	Virulence genes					
		iap		*hylA*		*actA*	
		No.	%	No.	%	No.	%
Raw milk	6	6	100	5	83.3	4	66.7
Pasteurized milk	3	3	100	2	66.7	2	66.7
Ice cream	5	5	100	4	80	4	80
Ras cheese	2	2	100	2	100	1	50
Total	16	16	100	13	81.3	11	68.8

Table (6): Antimicrobial susceptibility profile of Listeria monocytogenes isolated from examined milk and dairy products samples (n=16).

Antimicrobial agents	Sensitivity disc content (µg)	Susceptible		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Kanamycin(K)	30	-	-	-	-	16	100
Nalidixic acid (NA)	30	-	-	1	6.25	15	93.75
Streptomycin (S)	10	1	6.25	2	12.50	13	81.25
Neomycin (N)	30	3	18.75	-	-	13	81.25
Amikacin (AK)	30	4	25	2	12.50	10	62.50
Oxytetracycline (T)	30	2	12.50	5	31.25	9	56.25
Cephalothin (CN)	30	6	37.5	3	18.75	7	43.75
Erythromycin (E)	15	7	43.75	3	18.75	6	37.50
Sulphamethoxazole (SXT)	25	9	56.25	1	6.25	6	37.50
Enrofloxacin (EN)	5	9	56.25	2	12.5	5	31.25
Gentamycin (G)	10	8	50	4	25	4	25
Cefotaxime (CF)	30	10	62.50	2	12.50	4	25
Ampicillin (AM)	10	11	68.75	3	18.75	2	12.50
Ciprofloxacin (CP)	5	14	87.50	1	6.25	1	6.25



Photograph (1): Agarose gel electrophoresis of multiplex PCR of *iap* (131 bp), *hylA* (456 bp) and *actA* (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*, *hylA* and *actA* genes.
Lane C-: Control negative.
Lanes 1, 2, 4, 7, 12, 14 & 16: Positive *L. monocytogenes* strains for *iap*, *hylA* and *actA* genes.
Lanes 5, 6, 9, 11 & 15: Positive *L. monocytogenes* strains for *iap* and *hylA* genes.
Lanes 3, 8 & 10: Positive *L. monocytogenes* strains for *iap* and *actA* genes.