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MOLECULAR DETECTION OF VIRULENCE GENES FOR *LISTERIA MONOCYTOGENES* ORGANISM ISOLATED FROM RAW MILK AND SOME LOCALLY MADE MILK PRODUCTS

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ABSTRACT

This study was designed to find out the prevalence of *Listeria monocytogenes* in raw milk and some dairy products and the molecular identification of their virulence genes. One hundred and twenty samples in all, including milk, Kareish cheese, and yogurt (40 of each), were gathered randomly from various sources for this study and examined for the presence of *Listeria monocytogenes*. Eight distinct strains of *L. monocytogenes* were identified and molecularly examined for 16 srRNA-specific genes for *Listeria monocytogenes,* as well as the identification of many pathogenicity genes (*inlA*, *inlB*, *hlyA*, and *prfA* genes). All eight isolates harbored 16srRNA-specific genes for *Listeria monocytogenes*; seven isolates harbored both *inlA* and *inlB*; six isolates harbored *hlyA*; and five isolates harbored the *prfA* gene. The results of antimicrobial susceptibility testing showed high sensitivity to Ciprofloxacin (CP), Gentamycin (CN), Vancomycin (VA), Doxycycline (DO), and Trimethoprim-sulfamethoxazole (SXT) with 100, 87.5, 87.5, 78.5, and 62.5%, respectively, while showing high resistance to Ampicillin (AM), Amoxicillin (AX), Oxicillin (OX), Erythromycin (E), Lincomycin (L), and Ceftriaxone (CRO) with 100, 100, 100, 100, and 75%, respectively. All eight *Listeria monocytogenes* isolates were multidrug-resistant, holding a variety of antibiotic resistance indexes (MARI) in the range of 0.417–0.833. In conclusion, the study's findings highlight the necessity of implementing more stringent sanitary control procedures, particularly while processing, storing, and marketing dairy products. The pasteurization temperatures must not be less than 85 °C, where only 85% of the bacteria are destroyed by pasteurization at 71–75 °C for 15 seconds.

Keywords: *Listeria monocytogenes,* Virulence Genes, milk products, PCR

INTRODUCTION

Dairy products and milk are highly nutritious. These food components are therefore, ideal for the growth of microbes, particularly harmful bacteria. The dairy industry, farms, and processing facilities are popular places to find Listeria species (Sarfaz *et al.*, 2017).

Listeriosis is a well-known foodborne illness that mostly affects people, especially those who are elderly, immune-compromised, or pregnant (Buchanan *et al.*,

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2017). The main culprit behind listeriosis in both people and animals is *Listeria monocytogenes* which is one of the most prevalent foodborne pathogens worldwide. In addition to causing a range of clinical symptoms, it poses the biggest risk to food safety and public health (Schlech, 2019).

Now is a major global public health concern as well as a source of foodborne infections, Listeriosis can result from food contamination caused by *L. monocytogenes*, which can endure freezing temperatures and stick to or are often integrated into food manufacturing processes (Yang *et al.*, 2020). Despite having a lower occurrence than other foodborne illnesses, the mortality rate from listeriosis is often high, ranging from 20 to 30 percent (Lopez-Valladares *et al.*, 2018).

A review of several worldwide epidemics of human listeriosis has been conducted (Desai *et al.*, 2019). Various food kinds have been identified as carriers of *Listeria monocytogenes*, which can spread the infection to humans and result in listeriosis (Lopez-Valladares *et al.*, 2018).

Human listeriosis outbreaks are known to be mostly spread by food (Zhu *et al.*, 2017). The use of various foods, such as meals of animal origin, meat, and dairy products, has been linked to numerous listeriosis outbreaks worldwide, according to WHO fact sheets (Dorcheh *et al.*, 2013).

A wide range of adverse conditions are tolerated by *L. monocytogenes*, such as low pH and temperature as well as high salt concentrations (Owusu-Kwarteng *et al.*, 2018). This Gram-positive facultative intracellular bacterium is a member of the Listeria genus, which has 18 species in total, only two of which are harmful (*Listeria ivanovii* and *L. monocytogenes*) (Orsi and Wiedmann, 2016).

The capacity of *L. monocytogenes* to pass through the placenta, blood-brain, and digestive barriers is essential to its basic dissemination from the gastrointestinal tract, and a variety of virulence factors mediate its infection, where the pathogenicity of *Listeria monocytogenes* is known to be significantly influenced by a variety of Listeria determinants. Additionally, osmotic stress, pH, oxygen availability or lack, and temperature all influence pathogenicity opportunities (Lopes-Luz *et al.*, 2021).

Depending on the source, location, and sample type, virulence genes are more prevalent in *L. monocytogenes* than in other species of Listeria (Matle *et al.*, 2019). The virulence genes of *L. monocytogenes* strains have been demonstrated to be the cause of their pathogenicity (Koopmans *et al.*, 2023; Wiktorczyk-Kapischke *et al.*, 2023), especially those discovered on the Islands of Listeria Pathogenicity (LIPIs) (Lopez-Valladares *et al.*, 2018). In addition to sustaining the infectious life cycle and surviving in the context of food processing, the virulence genes in the LIPI-1 and LIPI-3 clusters play several functions in *L. monocytogenes'* pathogenicity (Cotter *et al., 2008).* Specifically, *inlA, inlB,* and *inlC* are involved in the attachment of the virulence genes to the host cell and their internalization. *Htp* allows for intracellular replication, the *ActA* gene facilitates *L. monocytogenes* cellular motility, and the *plcA, plcB,* and *hlyA* genes are in charge of vacuole release (Koopmans *et al.*, 2023; Wiktorczyk-Kapischke *et al.*, 2023). *L. monocytogenes* may also carry several genetic markers for antibiotic resistance in addition to extra-related virulence factors, including invasion-associated protein (iap) (Owusu-Kwarteng *et al.*, 2018).

Antimicrobial resistance in microorganisms has emerged as a global issue. The abuse of antibiotics in humans, the widespread use of antibiotics in animals as a human reserve, and the prolonged use of antibiotics in feed to promote development are the main causes of the resistance observed in human medicine (Collignon and McEwen, 2019). Much research showed that feeding animals antibiotics favors the development

of resistant foodborne bacteria that could contaminate human food, where the biggest global concern at the moment is antimicrobial resistance. Drugs that lower morbidity and mortality linked to severe and potentially fatal illnesses become less effective because of threatening human health (Angulo *et al.,* 2009).

A public health indicator, particularly for high-risk groups, is the presence of food-borne multidrug-resistant *L. monocytogenes*. Learning about the relevance of laws governing food safety and the drugs used on both humans and animals is highly advised (Garedew *et al.,* 2015). To address the growing rates of morbidity and mortality, the World Road Map (WRM) on Antimicrobial Resistance (AMR) was developed by the World Health Organization (WHO) (Friedman *et al.,* 2016). The public's health is seriously endangered by both antibiotic resistance and the appearance of antibiotic-resistant genes (ARGs) in *L. monocytogenes*, especially in regard to the food industry. Several cases of resistant *L. monocytogenes* strains in milk and milk products have been reported (Kayode and Okoh, 2022).

The current study set out to determine the prevalence of *L. monocytogenes* in raw milk and a few locally made dairy products, such as Kareish cheese and yogurt, that are sold in and around Assiut City since the level of contamination of milk and its products with this pathogen poses serious risks to consumers. Furthermore, molecular identification of some of their virulence genes is being done to learn more about the existence of multiple antidrug resistance (MAR) in *Listeria monocytogenes*.

MATERIALS AND METHODS

Samples collection:

A total of 120 samples of raw milk, Kareish cheese and yoghurt (40 of each) were collected randomly from street vendors, dairy shops, and supermarkets in Assiut City. The samples were collected hygienically under aseptic conditions and safety precautions. The samples were promptly delivered to the lab after being stored in an ice box (2-4^{o}C) for isolation and identification of *Listeria monocytogenes*. The raw milk samples were mixed thoroughly and tested for heat treatment by Storch test according to Lampert (1975) before being subjected to examination.

Isolation and characterization of *Listeria monocytogenes* according to ISO 11290-1 (2017):

Primary enrichment: Using a Stomacher (BagMixer, Buch & Holm A/S, Interscience, 78860 St Nom, France), each sample was homogenized aseptically at a rate of about 25 ml/g in 225 mL of Listeria enrichment broth base. (CM 0862, Oxoid Ltd., Basingstoke, UK) with selective enrichment supplement (SR 0141, Oxoid Ltd., Basingstoke, UK) in Stomacher bags (Seward Ltd., Worthing, UK) for 30 seconds. The samples were then incubated at 30 °C for 24 hours. Secondary Enrichment: 0.1 ml of the incubated broth was added to 10 ml of Fraser broth, and the combination was incubated for 24 hours at 37 °C. A loopful of the incubated Fraser broth was streaked onto OXFORD Agar (Oxoid, United Kingdom) and Listeria Chromogenic Agar Base according to Ottaviani and Agosti (ALOA) agar plates (Oxoid, United Kingdom). The plates were then incubated at 37 °C for 24 hours, and after 24 ± 3 hours, they were examined. Following selection, as per ISO 11290-1 (2017), the colonies that exhibited characteristics of listeria were streaked onto Tryptic Soy Agar (TSA) and incubated for a whole day at 37 °C. Gram staining and biochemical tests were used to identify the isolates.

Biochemical Identification of the isolates:

Based on morphological characteristics on ALOA agar, *L. monocytogenes* was biochemically identified (Merck, Poland). Colonies of *L. monocytogenes* typically have a turbidity zone surrounding them and are blue or turquoise. For identification and

characterization, up to five (5) suspected colonies from each positive plate have been streaked on tryptic soy agar (Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract (Oxoid, Basingstoke, UK) (TSA-YE) and incubated at 35 °C for 24 hours (Alsheikh *et al.,* 2013). The colonies from TSA-YE plates were verified and identified using the following assays: hemolysis test, rhamnose, mannitol, maltose, and sucrose fermentation tests, motility, catalase, oxidase, and gram stain. PCR was then performed. *Listeria monocytogenes* was identified using 16S rRNA gene (Abdeen *et al.,* 2021).

Antibiotic sensitivity testing:

For the isolates of *L. monocytogenes*, the phenotypic antibiotic drug susceptibility testing was conducted using the conventional Kirby-Bauer disc diffusion technique (Bauer *et al.,* 1966). The results were interpreted using the Clinical Laboratory Standards Institute's (Sigma-Aldrich, USA) CLSI (2022). Using sterile swabs, the bacterial suspension was spread out on Mueller-Hinton agar plates (Himedia) after being adjusted to a 0.5 McFarland standard. Twelve different antibiotic disks (Oxoid Ltd., Basingstoke, UK) representing nine distinct categories of antibiotics were used. The antibiotics included were Ampicillin (AM), Amoxicillin (AX), Oxicillin (OX), Gentamycin (CN), Erythromycin (E), Vancomycin (VA), Doxycycline (DO), Trimethoprim-sulfamethoxazole (SXT), Lincomycin (L), Ciprofloxacin (CP), Ceftiofur (EFT), and Ceftriaxone (CRO). The results were interpreted in accordance with the Clinical and Laboratory Standards Institute's (CLSI) 2022 guidelines. The term "multidrug-resistant strains" (MDR) refers to strains that exhibit resistance to three or more distinct antibiotic classes (Magiorakos *et al.,* 2012). In this investigation, isolates exhibiting moderate susceptibility were categorized as resistant. The formula developed by Singh *et al.* (2010) was used to compute the index of multiple antibiotic resistance (MARI). MARI is equal to the number of antibiotics to which the isolate was exposed and the number of drugs to which it was resistant.

Molecular detection of *16S rRNA* and some virulence genes of *L. monocytogenes*:

"PCR was applied in the Reference Lab of the Animal Health Research Institute in Doki, Giza, Egypt, for veterinary quality control on poultry production." All eight isolates of *L. monocytogenes* were subjected to polymerase chain reaction (PCR) to identify the characteristic *16S rRNA* gene and some virulence genes of *L. monocytogenes* (*inlA, inlB, hlyA* and *prfA*) (Cao *et al.,* 2018).

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. All DNA samples were stored at -20 °C until tested using specific primers supplied by Metabion (Germany), as shown in Table A. PCR amplification and analysis of the PCR products were performed as previously described by Sadek and Koriem (2022).

Behavior of *Listeria monocytogenes* in pasteurized milk during Refrigerator Storage:

1- The preparation of the examined bacteria:

The well-characterized and isolated strain of *Listeria monocytogenes* that carries the 16S r RNA virulence gene was used. The following method was used to create bacterial dilutions: A 0.6% yeast extract inoculation of the *Listeria monocytogenes* strain was applied to Tripticase soy broth, and the mixture was then incubated at 35 °C for 24 to 48 hours. Following incubation, sterile peptone buffer was used to serially dilute one milliliter of the culture. The suspension was then adjusted using the pour plate technique to the point 0.5 of the McFarland standard turbidity growth, as per Farber *et al.* (1992) and Malaka *et al.* (2019). The normal strain suspension was the previous one, and 1

milliliter could contain around 8 × 10^6 CFU/ml. The suspension may be utilized right away or kept at 4 °C until needed.

2- Survival of *Listeria monocytogenes* in pasteurized milk:

A milk sample purchased from a dairy shop was transported to the lab using an ice box and verified to be clear of *Listeria monocytogenes*. The sample was then divided into five treatment groups and stored at 4 °C in a sterile whirlpool bag. One milliliter of the previously made *Listeria monocytogenes* suspension was combined with 100 milliliters of milk and placed into appropriate sterile jars, except the fifth group, which received unpasteurized milk as a negative control and was free of strain suspension. One jar of fresh milk served as a positive control, and the fresh milk that was to be pasteurized was subjected to High-Temperature Short Time (HTST) pasteurization, which involved heating the milk at three distinct temperatures (75°C, 85°C, and 95°C) for 15 seconds at a time. Every jar is kept at 4°C in the refrigerator. Moreover, the counts of *Listeria monocytogenes* for one day, one week, and two weeks were investigated.

3- Microbiological analyses:

* Aerobic recovery: Ten folds serial dilution was done then the listeria plates of five groups were incubated aerobically at 35°C for 24-48h.

* Anerobic recovery: Placed the previous listeria plates of five groups in an anaerobic jar (Model 3640; National Appliance Co., Portland, OR) according to Knabel *et al.* (1990) at 43°C for 24- 48h.

Statistical analysis:

To ascertain whether there was a significant difference between them, The Chi-square test and the one-sample t-test were used to statistically assess the data, a "P" value of less than 0.05 was regarded as statistically significant. The data was analyzed using GraphPad Prism 9.5.1 (GraphPad Software Inc., San Diego, CA, USA). The Shapiro-Wilk test was utilized to determine if the distribution of the data was normal or non-normal. The Kruskal-Wallis test was used in comparative statistics to compare the means of non-parametric variables (data distribution without normality) (Lantz *et al*., 2016).

Table (A): Primer pairs and PCR condition used in genotypic characterization of *L. monocytogenes.*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extensio		
16S rRNA	GGA CCG GGG CTA ATA CCG AAT GAT AA / TTC ATG TAG GCG AGT TGC AGC CTA	1200	94˚C 5 min.	94˚C 30 sec.	60˚C 1 min.	72˚C 1 min.	72˚C 12 min.	**Kumar *et al.* (2015)**
prfA	TCT-CCG-AGC-AAC-CTC-GGA-ACC / TGG-ATT-GAC-AAA-ATG-GAA-CA	1052	94˚C 5 min.	94˚C 30 sec.	50˚C 50 sec.	72˚C 1 min.	72˚C 10 min.	**Dickinson *et al.* (1995)**
inlA	ACG AGT AAC GGG ACA AAT GC / CCC GAC AGT GGT GCT AGA TT	800	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Liu *et al.* (2007)**
inlB	CTGGAAAGTTTGTATTTGGGAAA / TTTCATAATCGCCATCATCACT	343	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA / TGT-CAC-TGC-ATC-TCC-GTG-GT	174	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Deneer and Boychuk (1991)**

RESULTS

Totally, eight positive samples (6.7%) out of 120 examined raw milk, yoghurt, and Kareish cheese samples were *Listeria monocytogenes* positive, representing 5 (12.5%) out of 40 raw milk and 3 (7.5%) out of 40 Kareish cheese, while yoghurt samples were negative for *Listeria monocytogenes* (Table 1). All eight *Listeria monocytogenes* strains, mainly from raw milk and Kareish cheese, were multidrug-resistant (Tables 2 and 3). Gene-specific polymerase chain reaction (PCR) tests were performed on all eight strains of *Listeria monocytogenes* to identify the characteristic 16S rRNA-specific gene and some virulence genes of *L. monocytogenes* genes (*inlA, inlB, hlyA, prfA*). All eight strains were confirmed positive by the 16S rRNA-*specific* gene for *Listeria monocytogenes,* seven strains harbored both *inlA* and *inlB,* six strains harbored *hlyA,* and five strains harbored *prfA* genes (Table 4).

Table 1: Occurrence of *Listeria monocytogenes* in raw milk and some dairy products.

Type of samples*	Number of samples tested	Positive Samples		Negative Samples	
		No	%	No	%
Raw milk	40	5	12.5	35	87.5
Kareish	40	3	7.5	37	92.5
Yoghurt	40	0	0	40	100
Total	120	8	6.7	112	93.3

* No statistical differences were found between types of the examined samples

Table 2: Antibiotic resistance of *Listeria monocytogenes* (n = 8).

Antibiotics	Antimicrobial Classes	Resistant **		sensitive	
		No	%	No	%
Ampicillin (AM) 10µg (µg)	β-lactamase	8	100	0	0
Amoxicillin (AX)25 µg	β-lactamase	8	100	0	0
Oxicillin(OX)1 µg	β-lactamase	8	100	0	0
Gentamicin (CN)10 µg	Aminoglycosides	1	12.5	7	87.5
Erythromycin (E)15 µg	Macrolides	8	100	0	0
Vancomycin (VA)30 µg	Glycopeptides	1	12.5	7	87.5
D0xycycline (DO)30 µg	Tetracycline	1	12.5	7	87.5
Trimethoprim-Sulfamethoxazole (SXT-25) 1.25/23.75 µg	Sulfonamides	3	37.5	5	62.5
Lincomycin (L)2 µg	Lincosamide	8	100	0	0
Ciprofloxacin (CP) 5µg	Fluoroquinolones	0	0	8	100
Ceftiofur (EFT) 30µg	Cephalosporin	4	50	4	50
Ceftriaxone (CRO) 30µg	Cephalosporin	6	75	2	25

** High significance difference among resistance of different types of antibiotics ($p< 0.0001$, $\chi 2 = 63.09$).
% was calculated based on the total number of isolates of *L. monocytogenes* (n = 8).

Table 3: Antibiogram profiles and Multiple Antibiotic Resistance (MARI) index for *Listeria monocytogenes* isolates. (n = 8).

Isolate No.	Antimicrobial resistance patterns	No of antibiotics	MARI**	virulence genes
1	AM, AX, OX , E, L, EFT, CRO	7	0.583	*InlA, inlB, hlyA*
2	AM, AX, OX, E, DO, L, CRO	7	0.583	*InlA, hlyA, prfA*
3	AM, AX, OX, E, SXT, L, CRO	7	0.583	*InlB, hlyA, prfA*
4	AM, AX, OX, E, CRO	5	0.417	*InlA, inlB, prfA*
5	AM, AX, OX, E, SXT, L, EFT, CRO	8	0.667	*InlA, inlB, hlyA*
6	AM, AX, OX, E, L, CRO	6	0.500	*InlA, inlB, hlyA*
7	AM, AX, OX, CN, E, VA, SXT, L, EFT, CRO	10	0.833	*InlA, inlB, hlyA, prfA*
8	AM, AX, OX, E, L, CRO	6	0.500	*InlA, inlB, prfA*

** High significance differences among MARI were found ($p< 0.0001$, $t =13.11$).

Table 4: PCR results for detection of 16S rRNA and some virulence genes (*inlA, inlB, hlyA* and *prfA*) of *L. monocytogenes* genes.

Sample No.	16S rRNA	*inlA*	*inlB*	*hlyA*	*prfA*
1	+	+	+	+	-
2	+	+	-	+	+
3	+	-	+	+	+
4	+	+	+	-	+
5	+	+	+	+	-
6	+	+	+	+	-
7	+	+	+	+	+
8	+	+	+	-	+

Table 5: *Listeria monocytogenes* behavior in pasteurized milk during storage in the refrigerator.

Isolation time after pasteurization	Aerobic incubation*				Anaerobic incubation*			
	Positive control*	At 75°C (T1) *	At 85°C (T2) *	At 95°C (T3) *	Positive control*	At 75°C (T1) *	At 85°C (T2) *	At 95°C (T3) *
Zero time	$8x10^6$ cfu/ml intial count	$6x10^4$ cfu/ml	-ve	-ve	$4x10^6$ cfu/ml	$3x10^4$ cfu/ml	-ve	-ve
1st day	$23x10^6$ cfu/ml	$7x10^6$ cfu/ml	-ve	-ve	$16x10^6$ cfu/ml	$9x10^5$ cfu/ml	-ve	-ve
One week	$12x10^7$ cfu/ml	$2x10^6$ cfu/ml	-ve	-ve	$18x10^7$ cfu/ml	$33x10^6$ cfu/ml	-ve	-ve
Two week	$6x10^8$ cfu/ml	$3x10^7$ cfu/ml	-ve	-ve	$14x10^8$ cfu/ml	$19x10^7$ cfu/ml	-ve	-ve

positive control: without pasteurization negative control group: was negative

T1: pasteurization at 75 for 15 sec. T2: pasteurization at 85 for 15 sec. T3: pasteurization at 95 for 15 sec.

* significant difference among Aerobic and Anaerobic incubation and between different temperatures of pasteurization ($p=0.000259$).

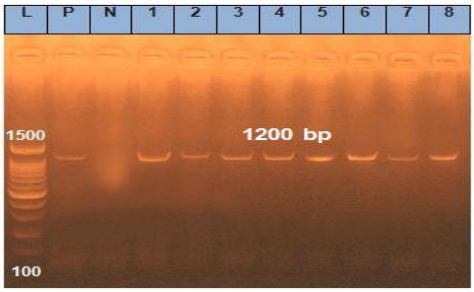

Photo (1): Amplification profiles for the *Listeria monocytogenes* 16S rRNA gene. Lane L, DNA ladder marker (100 bp). Lane P is the control positive *Listeria monocytogenes* 16S rRNA (1200 bp). Lane N, control negative. Lanes 1, 2, 3, 4, 6, 7, and 8 positive isolates for the 16S rRNA gene.

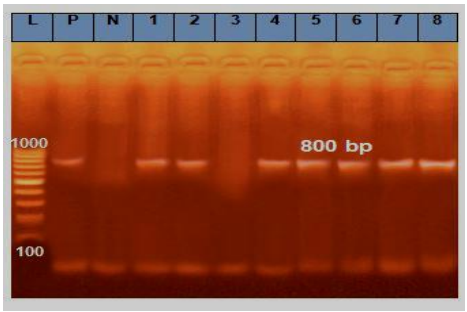

Photo (2): Amplification profiles for the *Listeria monocytogenes inlA* gene. Lane L, DNA ladder marker (100 bp). Lane P is the control positive *inlA* gene. (800 bp). Lane N, control negative. Lanes 1, 2, 4, 6, 7, and 8 positive *Listeria monocytogenes inlA* gene. Lane 3: negative *Listeria monocytogenes inlA* gene.

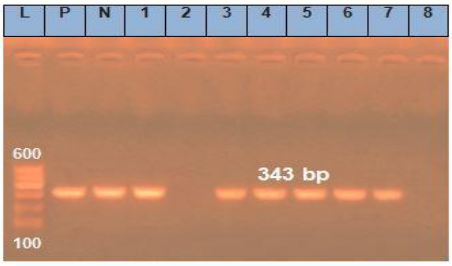

Photo (3): Amplification profiles for the *Listeria monocytogenes inlB* gene. Lane L, DNA ladder marker (100 bp). Lane P is the control positive *inlB* gene (343 bp). Lane N, control negative. Lanes 1, 3, 4, 6, and 7 positive *Listeria monocytogenes inlB* genes. Lanes 2 and 8 negative *Listeria monocytogenes inlB* genes.

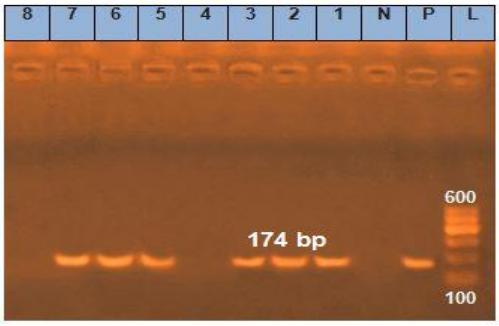

Photo (4): Amplification profiles for the *Listeria monocytogenes hlyA* gene. Lane L, DNA ladder marker (100 bp). Lane P is the control positive *hlyA* gene (174 bp). Lane N, control negative. Lanes 1, 2, 3, 5, 6, and 7 are positive *Listeria monocytogenes hlyA* genes. Lanes 4 and 8 negative *Listeria monocytogenes hlyA* genes.

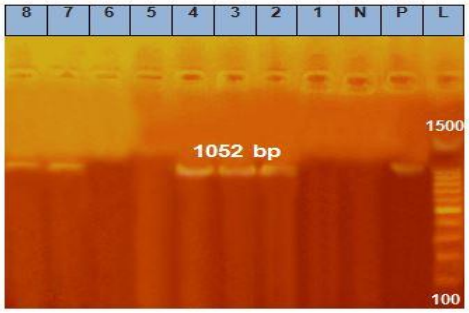

Photo (5): Amplification profiles for the *Listeria* monocytogenes *prfA* gene. Lane L, DNA ladder marker (100 bp). Lane P, the control positive *prfA* gene (1052 bp). Lane N, control negative. Lanes 2, 3, 4, 7, and 8 *Listeria monocytogenes* positive for the for the *prfA* gene. Lanes 1,5 and 6 *Listeria monocytogenes negative prfA* gene.

DISCUSSION

The World Health Organization has identified four foodborne pathogens, including *L. monocytogenes,* that may infect both people and animals and have an invasive infection rate of 20–30%. In pregnant women, severe listeriosis can cause miscarriage, fetal infection, sepsis, and meningoencephalitis (Radoshevich and Cossart, 2018). Table 1 displays the total prevalence of *Listeria monocytogenes* in 120 distinct samples, which was 6.7% (8/120). A similar finding of 6.8% (17/250) of the samples was detected by Abdeen *et al.* (2021) in Egypt, and nearly the same result of 7.2% (72/1035) of *L. monocytogenes* was detected in Portugal by Mena *et al.* (2004). However, Mary and Shrinithivihahshini (2017) found an extensive incidence of *L. monocytogenes*, 52.7% (219/541). While a lower prevalence of *L. monocytogenes* (2.17%) was detected by Sharma *et al.* (2024)**.** As a result, the degree of *L. monocytogene* contamination of food items varies greatly depending on the region and is undoubtedly influenced by sample size, food products, and/or sanitary conditions.

One major risk to the dairy business and public health is the high frequency of *Listeria monocytogenes* infections in milk and other dairy products. The findings in Table 1 demonstrated that *Listeria monocytogenes* had the greatest incidence frequency in 12.5% (5/40) of the milk samples. A substantially identical *listeria monocytogenes* prevalence of 13.46%, 12% and 13.33% was obtained in crude milk by Saha *et al.* (2015), Şanlıbaba *et al*. (2018), and Saleh *et al.* (2021). The greater frequency of *listeria monocytogenes* infections (45%, 25%, 44% and 30% in uncooked milk was documented by Hesham *et al.* (2017), Tahoun *et al.* (2017), Dapgh & Salem (2022) and Faruk *et al.* (2023) respectively. The decreased frequency of *listeria monocytogenes* (3.6%, 2.48%,5.3%, 6%, 3.8%, 7.5%, 7.33%, and 5.5%) in uncooked milk was documented by Angelidis *et al.* (2023), Su *et al.* (2023), Mohamed *et al.* (2022), Abdeen *et al.* (2021), Bouymajane *et al.* (2021), El Hag *et al.* (2021), Haggag *et al.* (2019) and Skowron *et al.* (2019), respectively. Dairy farms have the potential to contaminate raw milk. Additionally, inadequate sanitation of animals, faulty silage quality, unsanitary conditions during milking, storage, and transportation, and diseased cows on dairy farms could all be contributing factors to *L. monocytogenes* contamination (Telli *et al.*, 2016). In accordance with Egyptian Standards (2005), which stated that *L. monocytogenes* should not be present in milk or dairy products, 12.5% of the tested samples of raw milk had levels of bacteria higher than what was permitted in milk and dairy products.

The obtained findings declared that *L. monocytogenes* contaminated 7.5% (3/40) of Kareish cheese (Table 1). Nearly similar

findings 4.16%, 6.67% and 6%, were detected in Kareish cheese by Elshinaway *et al.* (2017), Şanlıbaba *et al.* (2018) and Dapgh & Salem (2022), respectively. Conversely, Metwally & Ali (2014), Meshref *et al.* (2015), & Kaptan (2016) found that Kareish cheese has an extensive frequency of Listeria spp., but Muhammed *et al.* (2013), Ismail *et al.* (2014), & Abd El Tawab *et al.* (2015) reported a lower incidence. The high prevalence of these bacteria may be brought on using unpasteurized milk, unsanitary manufacturing, and incorrect food storage, as Listeria spp. in particular can sustain its growth rate in low salt concentration medium and at low temperatures (4°C) (Gill & Reichel, 1989). Consequently, the cheese may serve as an appropriate medium for the development and proliferation of many Listeria species, including *L. monocytogenes.* Variations in the levels of Listeria spp. contamination in dairy products may be caused by variances in the original material's characteristics as well as processing and environmental factors. According to the Egyptian Standard for Kareish cheese (1008/4/2005), *L. monocytogenes* ought to be free from it. To guarantee the microbiological safety of cheese, it is recommended to strictly adhere to appropriate manufacturing, distribution, and retail storage procedures.

One of the most popular fermented dairy products in Egypt is yogurt. In all of the yogurt samples investigated throughout this study, *L. monocytogenes* was not discovered (Table 1). This finding is consistent with previous research (Ismaiel *et al.,* 2014; Metwally & Ali, 2014; Elshinaway *et al.,* 2016; Elafify *et al.,* 2022b) that were unable to isolate *L. monocytogenes* from yogurt. The results of the present investigation were contradictory with those of earlier research conducted by EL-Malt and Abdelhameed (2009), El Marnissi *et al.* (2013), & Dapgh *et al.* (2020), which documented a reduced prevalence of *L. monocytogenes* in yoghurt samples. Dapgh & Salem (2022) identified a high incidence rate of 16% of *Listeria monocytogenes* in yogurt samples. According to Elshinaway *et al.* (2016), *L. monocytogenes* may not have been present in the yogurt samples that were examined because of the antimicrobial properties of certain lactic acid bacteria released in yogurt. Additionally, certain milk processing and heat treatment procedures may inhibit or eliminate the growth of bacteria.

There were no significant variations in the frequency of *L. monocytogenes* isolation across all samples under examination. Interestingly, it is clear that yogurt samples are of superior quality to raw milk and Kareish cheese, according to the absence of *L. monocytogenes.* Unsanitary conditions during production, processing, handling, and distribution are a major cause of *L. monocytogenes* contamination of milk and milk products. Serious infections in humans might result from Listeria spp., particularly *L. monocytogenes*, contaminating milk and its products. Human listeriosis has a 20–30% mortality rate and can be extremely dangerous or even fatal for specific groups of people, such as infants, expectant mothers, the elderly, and people with compromised immune systems. It results in both intermittent and widespread epidemics of illness (Phraephaisarn *et al.,* 2017 & Şanlıbaba *et al.,* 2018).

Antimicrobial resistance may arise from the misuse of antibiotics used in animal husbandry and cattle production (Elafify *et al.,* 2022a). The global health community views antibiotic resistance as a concern, particularly concerning foodborne pathogens that pose a direct risk to human health (Prabakusuma *et al.,* 2022). According to the study's accomplished results (Table 2), all eight (8) isolates of *L. monocytogenes* exhibited the ability to withstand at least five of the twelve antibacterial substances from nine distinct antibiotic classes with a high concentration of resistance to beta-lactam antimicrobial agents. The eight isolates of *L.*

monocytogenes have shown strong resistance to Ampicillin (AM), Amoxicillin (AX), Oxicillin (OX), Erythromycin (E), and Lincomycin (L). Trimethoprim-sulfamethoxazole (SXT), Vancomycin (VA), Ciprofloxacin (CP), Gentamycin (CN), and Doxycycline (DO) were nonetheless effective against most of the isolates. The pathogenic strain of *L. monocytogenes* was shown to have resistance to Ampicillin and Erythromycin, as well as moderate sensitivity to Ciprofloxacin (Arslan & Ozdemir, 2008). Since Ampicillin is the first antibiotic used to treat listeriosis in people, resistance to it is noteworthy (Conter *et al.*, 2009). A nearly identical conclusion was reached by Olaniyan *et al.* (2022); Faruk *et al.* (2023); Sharma *et al.* (2024), who discovered that although *L. monocytogenes* was susceptible to Gentamycin, Vancomycin, and Ciprofloxacin, it exhibited a significant degree of resistance to beta-lactam antibiotics and was resistant to Ampicillin, Cefixime, Cefalexin, and Erythromycin. As per Islam *et al.* (2016), *Listeria monocytogenes* was shown to be sensitive to Gentamycin and Vancomycin but tolerant to Ampicillin, which is consistent with Al-Nabulsi *et al.* (2015) outcomes, which also discovered that *L. monocytogenes* exhibited Erythromycin resistance. Sarker & Ahmed (2015) discovered that *Listeria monocytogenes* is Ampicillin and Erythromycin-resistant, despite the fact that approximately 70% of the isolates exhibited Oxytetracycline resistance, while Elafify *et al.* (2022b) discovered that all isolates of *L. monocytogenes* demonstrated resistance to both Erythromycin and Streptomycin. But Ampicillin, Ciprofloxacin, and Gentamicin could all be used to treat more than 70% of the recovered isolates. Moreover, El-Demerdash *et al.* (2023) revealed that isolates of *L. monocytogenes* had strong resistance to β-lactam antibiotics, like Amoxicillin and Clavulanic acid, and a high susceptibility to Vancomycin. Abdeen *et al.* (2021) observed that several food isolates of *L. monocytogenes* were resistant to various antibiotics, including Levofloxacin, Azithromycin, Oxytetracycline, Trimethoprim- Sulfamethoxazole, Amoxicillin, Ampicillin, Erythromycin, Rifampicin, and Chloramphenicol. In addition, antibiotic resistance in bacteria not only makes treatment measures ineffective but also makes it easier for other bacterial strains to transfer these genes horizontally, which is extremely dangerous for humans.

Based on the data analysis, it was shown that there were substantial statistical differences ($P<0.0001$, $\chi 2 = 63.09$) in the antimicrobial resistance of the various drugs. One possible reason for the substantial rise of *L. monocytogenes* antimicrobial resistance against the most common antibiotics for the therapy of listeriosis in humans and animals is antibiotic abuse. Because of the enzyme's poor or absent affinity during the latter stage of cell wall formation, *L. monocytogenes* has intrinsic resistance to these antibiotics (Al-Nabulsi *et al.*, 2015). The discovery of multidrug-resistant strains of Listeria from various food sources and geographical locations is important because these strains may serve as reservoirs for antimicrobial resistance genes, contributing to the emergence and spread of additional resistant strains to drugs (Chin *et al., 2018*).

The results of the present study demonstrated that all eight (8) isolates of Listeria exhibited characteristic resistance to multiple drug expression with multiple patterns on the antibiogram profile, highlighting the challenges brought about by the emergence of multidrug-resistant (MDR) species and the consequential issues that arise when treating foodborne diseases clinically. These issues include relapse and multiple drug resistance. El-Demerdash *et al.* (2023); Olaniyan *et al.* (2022), and Abdeen *et al.* (2021) yielded almost identical findings. Conversely, a current investigation by Oliveira *et al.* (2018) demonstrated that *L. monocytogenes* isolates were 100% susceptible to the majority of tested antibiotics. This underscored the importance of continuously

monitoring antimicrobial susceptibility patterns and their impact on public health. Therefore, these findings which show resistance to three or more antimicrobial classes are crucial for helping to design appropriate policies and strategies for antibiotic stewardship in animal production (Table 3). According to this research, a large proportion of multi-resistant isolates may indicate the acquisition and transmission of novel resistance genes to different strains, bacterial species, or genera. Treatment for listeriosis in people and animals is seriously threatened by multidrug resistance. Several studies have shown that infections generated by strains of *Listeria monocytogenes* that are resistant to drugs are more dangerous than infections caused by susceptible strains, as they can result in serious treatment delays or even fatal outcomes (Llor and Bjerrum, 2014). In addition, Table (3) demonstrates that the eight *L. monocytogenes* isolates had MAR index values ranging from 0.500 to 0.833. Similar findings were made by El-Demerdash *et al.* (2023) & SU *et al.* (2023), illustrating the high degree of antibiotic resistance of *L. monocytogenes* strains isolated from milk. While Mpondo *et al.* (2021) mentioned that all *L. monocytogenes* had MARI values ranging from 0.87 to 1. The obtained results were almost identical to those of Al-Mayahi and Jaber (2020), who found that 15.4% of isolates were >0.2. Antibiotic resistance was shown to be correlated with the quantity of virulence genes present in the microorganism, suggesting that virulence genes and antibiotic resistance are related. The number of virulence genes and the isolates' degree of antibiotic resistance showed a strong positive connection (Table 3).

According to the data analysis, there were variations among MARI that were highly statistically significant ($p<0.0001$, $t = 13.11$). A MARI value greater than 0.2, in general, suggests that the bacteria have been exposed and originate from a contaminated source that poses a significant danger as well as an environment where antibiotics may be misused (Khan *et al.*, 2015).

The obtained result (Table 4 & photo 1) revealed that the 16S rRNA gene was present within each isolate that was studied. Comparable findings were achieved via Abdeen *et al.* (2021) & El-Demerdash *et al.* (2023). All of the isolates of *L. monocytogenes* examined in this study were positive for four virulence-associated genes, namely the genes encoding particular virulence factors, namely internalins (*inlA*, *inlB*), hemolysin, and Listeriolysin O encoded by the *hylA* gene, which is a major virulence factor in Listeria and is believed to be crucial for identifying *L. monocytogenes* (*hlyA*) and (*prfA*), of which seven isolates carried both *inlA* and *inlB*, six isolates carried *hlyA*, and five isolates carried the *prfA* gene, implying consumers of dairy products may be susceptible to food-borne listeriosis (Table 4& photos 2, 3, 4, 5). The same outcomes have been achieved by Matle *et al*. (2019), Abdeen *et al.* (2021), and Gana *et al.* (2024). The registered results are consistent with a previous Egyptian investigation that identified four virulence genes (*inlA, actA, prfA, hlyA*) in isolates of *L. monocytogenes* from animal food items (El-Demerdash & Raslan, 2019; El-Demerdash *et al.*, 2023). The research has provided ample evidence of the roles these virulence genes play in the pathophysiology of clinical listeriosis that results from consuming products contaminated with Listeria (Loo *et al., 2020*). The multimodality of listeriosis-causing mechanisms exhibited by *L. monocytogenes* is widely recognized. The six virulence genes, *actA, mpl, plcA, hly, plcB,* and *prfA*, are its main culprits. These genes are members of the pathogenic *PrfA*-dependent gene cluster called *LIPI*-1 (Raschle *et al.*, 2021). Furthermore, Listeria virulence islands, internalin (*inl*) genes, and genomic islands *LIPI-1, LIPI-2, LIPI-3,* and *LIPI-4* have been found (Wagner *et al.*, 2022). The presence of *Listeria monocytogenes* in milk and other dairy products makes it difficult to treat the

bacteria therapeutically; thus, it is crucial to track the pathogen's spread and resistance mechanisms along its many food chains. Preserving safe time and temperature control (TCS), putting good manufacturing procedures (GMP) and HACCP into operation from the farm to the outside of the farm, utilizing metabolomics techniques for molecular epidemiological assessment, and implementing good hygiene practices (GHP) are some of the monitoring and controlling actions (Li *et al.,* 2021; Prabakusuma *et al.,* 2022).

Concerning Table (5), milk that was pasteurized for 15 seconds at 85 and 95 °C is feasible as the *listeria monocytogene* count was zero. This complies with the Egyptian Regulations, which demand that *Listeria monocytogenes* be completely absent. A noteworthy association was discovered between the frequency of *L. monocytogenes* carrying virulence genes immediately following inoculation and following a two-week storage period at 4°C. This is in accordance with the opinion of Malaka *et al.* (2014), who discovered that fresh milk and HTST pasteurized milk were contaminated with *L. monocytogenes* during refrigerator storage because of the bacteria's ability to thrive at low temperatures.

However, Malaka *et al.* (2019) found that after a day of storage, pasteurization at 95 °C did not reveal the presence of *L. monocytogenes*. But, after a week or two, there was a suspected level of the bacteria, suggesting that pasteurization only causes the bacteria to go dormant. Additionally, the presence of polymorph nuclear leucocytes (PMNL) in milk may influence the bacterium and contribute to cross-contamination after pasteurization. It was confirmed by Elafify *et al.* (2022b) that lowering the refrigerator temperature to 10°C resulted in a substantial ($P < 0.05$) decrease in the number of *L. monocytogenes.*

It is important to emphasize that, in the current study's conditions, the anaerobic techniques used to grow *L. monocytogenes* at 43°C produced higher values for the positive control and HTST 75°C milk samples than the results of previous studies that used aerobic plating after one and two weeks of growth at 37°C. However, results showed no significant increase in CFUs between them directly after inoculation. This was by Knabel *et al.* (1990), who stated that the theory that the recovery of severely heat-injured *L. monocytogenes* was caused by the absence of 02 was accurate. This led to the accumulation of toxic levels of 02 products, which would have caused *L. monocytogenes* to become an indispensable anaerobe.

The highly significant factor ($P < 0.0002$) was observed between either the control, HTST of 75 °C samples and both treatment two and three of aerobic and anaerobic incubation, while the sample of treatment one that aerobically grew was significant only for the positive control in aerobic and anaerobic recovery. These bacteria are considered hazardous owing to their ability to adapt to a variety of environmental stresses, including heat, cold, and osmotic pressure (Guenther *et al.,* 2009). A crucial aspect for the wellness of humans is the survival of *Listeria monocytogenes* in HTST milk that has been pasteurized.

CONCLUSION

The outcome of this investigation revealed that multidrug-resistant *L. monocytogenes* was isolated from dairy products sold in Assiut, Egypt, and gave a thorough picture of the virulence factors, antibiotic resistance, and prevalence of *L. monocytogenes* divorced from certain dairy products and milk. The presence of *L. monocytogenes* in some dairy products is most likely caused by unhygienic production practices, inadequate pasteurization temperatures, and environmental pollution from animal feces,

which may contribute to the contamination. These bacteria are considered harmful because of their adaptability to a variety of external stresses, including heat, cold, and osmotic pressure. Consumption of raw milk and its products, particularly kareish cheese made without proper control procedures and with insufficient heat treatment, can lead to major health issues and might cause serious health problems. The state of *L. monocytogenes* resistance to antibiotics in raw milk and its derivatives presents a potential risk, particularly in the absence of preventive measures and strict hygienic practices.

Declaration of Competing Interest:

The authors declare that they don't have any competing interests.

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الكشف الجزيئي لجينات الضراوة لميكروب الليستريا مونوسيتوجينز المعزولة من اللبن الخام وبعض منتجات الألبان المصنعة محلياً

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الالبان ومنتجاتها من أهم الأغذية التى يحتاج إليها الانسان حيث أنها تتميز بقيمتها الغذائية العالية وتحتوى على العديد من العناصر الغذائية الهامة بالاضافة الى سهولة هضمها. ونظرا لقيمتها الغذائية العالية تعتبر الالبان ومنتجاتها عرضة للتلوث اثناء صناعتها وتداولها بالعديد من الميكروبات التى تمثل خطرا على صحة المستهلك بالاضافة الى ما قد ينجم من عيوب على المظهر الخارجى للمنتج مما قد يؤثر على جودته. يعتبر ميكروب الليستريا مونوسيتوجينز أحد هذه الميكروبات لذلك تم عمل هذه الدراسة لمعرفة مدى تلوث اللبن وبعض منتجاته بهذا الميكروب. تم جمع ١٢٠ عينة من اللبن الخام والجبن القريش والزبادى البلدى (٤٠ عينة من كل نوع) من محلات الالبان والسوبرماركت والباعة فى اسواق مدينة أسيوط وقد تم فحص العينات بكتريولوجيا لوجود ميكروب الليستريا مونوسيتوجينز. كانت نسب وجود ميكروب الليستريا مونوسيتوجينز ٦,٧% من العدد الكلى حيث تم عزل ٨ عترة من ١٢٠ عينة وكانت نسبة العزل من اللبن الخام ٥ (١٢,٥ %) والجبنة القريش ٣ (٧,٥ %) ولكن عينات الزبادى كانت خالية من الميكروب. أظهرت نتائج اختبار الحساسية للعترات المعزولة لعدد اثنى عشرمن المضادات الحيوية من مجموعات مختلفة اكثر حساسية لكل من السيبروفلوكساسين ،جنتاميسين ، ڤانكوميسين، الدوكسى سيكلين وسلفاترای میثوبریم بنسب ١٠٠ ، ٨٧,٥ ، ٨٧,٥ ، ٨٧,٥ و ٦٢,٥ % على التوالى بينما كانت اكثر مقاومة لكل من الامبسيلين ، اموكسيسيلين ، الاوكسيلين ، الارثروميسن ، اللنكوميسين وسيفترایاكسون بنسب ١٠٠ ، ١٠٠ ،١٠٠ ، ١٠٠ ، ١٠٠ و ٧٥ % على التوالى وتبين كل العترات لها مقاومة متعددة للمضادات الحيوية بمعدل ١٠٠ %. تم فحص العزلات باستخدام تفاعل البلمرة التسلسلى لوجود جين ***16S rRNA*** الخاص بميكروب الليستريا مونوسيتوجينز وبعض جينات الضراوة ***inlA,inlB,hlyA*** **and** ***prfA*** .حيث تبين ان كل العترات ايجابية ***16S rRNA*** الخاص بميكروب الليستريا مونوسيتوجينز وعدد سبعة عترات حاملة لجيني ***inlA,inlB*** وستة عترات حاملة لجين ***hlyA*** وخمسة عترات حاملة لجين ***prfA*** كذلك أوضحت الدراسة ان درجة حرارة بسترة اللبن يجب الا تقل عن ٨٥ درجة مئوية لمدة دقيقة للقضاء على ميكروب الليستريا وان يتم تخزين الالبان ومنتجاتها فى اماكن جيدة التهوية وكذلك تم مناقشة المخاطر الصحية للميكروب وضرورة تطبيق جميع الاشتراطات الصحية اثناء تصنيع وتخزين وتسويق هذه المنتجات.