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Molecular Detection of *Listeria Monocytogenes* in Milk and Some Milk Products

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ABSTRACT

Listeria monocytogenes is considered one of the major potential foodborne pathogens which often threaten human health and life. Our study assessed both existence and virulence of *L. monocytogenes* in raw milk and different milk byproducts in Damietta Governorate, Egypt. One hundred and fifty random samples of raw milk, Kariesh cheese, Domiati cheese, Roomi cheese, yogurt and cream (twenty-five samples for each) were gathered from small retails and various supermarkets at for isolation of *L. monocytogenes* as well as direct detection of the pathogen in the samples' using PCR technique that targeting 16S rRNA. Also, the study aimed to investigate the presence of virulence genes; (hly A, inl A, inl B, prf A, plc A) in *L. monocytogenes* isolates. The PCR findings for direct detection of *L. monocytogenes* declared that 41 out 150 examined samples were positive to 16S rRNA meanwhile by culture methods *L. monocytogenes* was recovered from 36 out of 150 examined samples. With regard to detection of virulence genes in isolates, 100% of the isolates (36) harbor inl A gene while 35 isolate (97.2%) harbor hly A gene. It was found that 100% of the samples (41) that were positive to 16S rRNA were also found positive to hly A and inl A genes. plcA gene was not detected in all samples whether directly or in isolates. inlB was detected in 8 out of 41 (19.5%) positive samples directly detected while it was detected in 15 out of 36 (41.6%) of isolates. prfA gene was detected in 25 out of 41 (60.9%) positive samples while it was detected in 20 out of 36 (55.5%) of isolates. Our study revealed that the considerable high isolation of *L. monocytogenes* among examined raw milk and its byproducts constitutes a potential public health hazard. Usually, PCR is seen as important technique for precise diagnosis of microorganisms rather than the classical cultural methods.

Key words: *Listeria monocytogenes*, Milk products, PCR, Raw milk, Virulence genes.

INTRODUCTION

Listeria monocytogenes is known as a Gram-positive, facultative anaerobic intracellular foodborne pathogen presents everywhere. Members of the genus *Listeria* are short rods, Gram-positive, non-spore or capsule forming, dispense individually or conform short chains, sometimes take letters V and Y shapes. In direct smear, they can be observed as coccoid and thence mistaken with streptococci (Todar 2009).

The pathogen induces a rare but dangerous illness called listeriosis in humans and animals with a death rate up to 25-30% (Karen 2015). Listeriosis is manifested in healthy individuals as noninvasive, self-limiting, or slight gastrointestinal disorders. On the other hand, the disease course becomes more severe; invasive and systemic among the immune-compromised persons; pregnant women, elder and young, leading to septicemia, encephalitis, meningitis, crossing the placenta barrier resulting in occurrence of abortion (Stavru et al. 2011).

It is frequently associated to consumption of ready-to-eat (RTE) foods; fresh produced raw milk, milk byproducts such as different types of cheese (Fagerlund et al. 2020). The bacterium has also been isolated from meat, seafood, decaying vegetables, plants, feces, soil, water, as well as asymptomatic human and animal carriers (Hain et al. 2006; Abdel et al. 2020; Umaima and Atia 2020). Addis et al. (2019) mentioned that *L. monocytogenes* can induce chronic intra-mammary infection in clinical healthy goats, which exemplifies a significant bacterial shedding in the farms and constitutes a source of milk contamination. As raw milk and RTE food products, comprising soft or semisoft cheese are primly implemented in listeriosis outbreaks (Akrami-Mohajeri et al. 2018).

The outbreaks most often take place via consumption of raw milk and dairy products because of the capability of the *Listeria* organism to multiplicate slowly in refrigerated foods (Fleming et al. 1985). Notably, *L. monocytogenes* has certain significant characteristics; the ability to grow at wide range of temperatures (1-44°C), relative resistance to freezing and drying, in high salt concentrations or at pH values of 5.0 and above (Lovett 1989).

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From other sight, *L. monocytogenes* usually harbor several virulence genes; the internalins (encoded by *inl* A, *inl* B) (Liu et al. 2007), listeriolysin O (LLO encoded by *hly* A), phosphatidylinositol-phospholipase C (PI-PLC encoded by *plc* A), as well as virulence regulator factor (encoded by *prf* A). These virulence agents play essential roles in the bacterial pathogenicity and infection consequence (Vázquez-Boland et al. 2001). It has been noticed that these virulence factors act synergistically and facilitate intracellular growth and dispersal of the bacterium within the mammalian host leading to the pathogenicity augmentation of the organism (Joseph and Goebel 2007, Camejo et al. 2011).

There is mounting evidence that virulence is not a stable feature but can be influenced by environmental circumstances. For instance, it has been observed that salt and acid stress boost the expression of virulence genes and in-*vitro* pathogenicity of the organism (Olsen 2000). Furthermore, virulence prospects are also affected by the temperature, pH, osmotic stress and presence or absence of oxygen (Walecka et al. 2011). Also, there was another substantial point; spontaneous mutation which is in charge of lowering the virulence of *L. monocytogenes* strains as a result of losing one or more virulence determinants (Témoin et al. 2008).

Harb et al. (2020) reported that the mPCR targeting the 16S rRNA and *hly*A genes can efficiently disclose *L. monocytogenes* in food samples. The implementation of diplex or multiplex PCR for the disclosure of two or more virulence genes in a sole tube is coveted because it diminishes labor and cost, also will be useful in a great scale determination of virulent *Listeria* strains (Rawool et al. 2007).

It is suggested that not only, in developing countries but also among industrialized countries; raw milk, cheeses of unpasteurized milk origin and other dairy based products constitute a good medium for the growth and survival of many pathogenic organisms (Makino et al. 2005; Manfreda et al. 2005).

As the level of contamination of both milk and its products with *L. monocytogenes* constitutes serious problems for consumers, so, the current study was carried out to estimate the incidence of *L. monocytogenes* in raw milk, and local milk byproducts; Domiati, Kariesh and Roomi cheeses, yogurt and cream at Damietta Governorate, Egypt. In addition, detection of some virulence factors was also done in isolates and directly in samples by PCR technique.

MATERIALS AND METHODS

Samples

A number of one hundred and fifty random samples of raw milk, Domiati, Kariesh, Roomi cheeses, yogurt and cream (25 samples for each) were compiled from small retails and several supermarkets in Damietta Governorate, East Delta region, Egypt in sterile plastic bags, retained in ice box and conveyed with a slightest delay to the laboratory for studying the presence of *L. monocytogenes* besides its virulence genes.

Bacteriological Examinations

Isolation of *L. monocytogenes* (FDA 2011)

A 25mL portion of sample was inoculated into 225mL of buffered *Listeria* enrichment broth (BLEB) base (Himedia) and kept 4 hrs at 30±1°C for thrive. After that selective agent (Himedia) were added to the medium and the samples were incubated for a total time of 48 hrs, at 30°C. BLEB grown cultures were streaked on both PALCAM agar and Oxford agar (Oxoid) then incubated at 35±1°C for 24-48 hrs.

Confirmatory Tests

A number of tests were applied to confirm *L. monocytogenes* isolates; (Gram-staining, umbrella or tumbling motility, β-hemolysis, catalase and oxidase production, carbohydrate utilization, nitrate reduction, Anton's test and CAMP test).

Genotypic Detection of *L. monocytogenes* Isolates and Virulence Genes using PCR

DNA extraction

The extraction of DNA from enriched samples was achieved using QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications tracked the manufacturer's guides. After that the sample was thoroughly washed and centrifuged. Nucleic acid was eluted with adding of 100µL of elution buffer.

Oligonucleotide Primer

Metabion (Germany) provided the utilized specific primers which have been listed in Table 1.

PCR Amplification

Primers were amplified in a 50µL PCR reaction tube containing 25µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 2µL of each primer of 20 pmol concentrations, 9µL of distilled water, and 12µL of DNA template. The reaction was carried out in 35 cycles illustrated in Table 1 using Applied biosystem 2720 thermal cycler.

Checking of the PCR Products

Using 1.5% agarose gel obtained from Applichem GmbH, Germany) for electrophoresis of the amplified PCR products in 1x TBE buffer at room temperature through propensity of 5V/cm. 20µL of each product was put in each gel slot for gel analysis. For fragment sizes' determination, two types of Ladders were used; Gelpilot 100bp plus (Qiagen GmbH, Germany) and generuler 100bp (Fermentas, Thermo, Germany). The (Alpha Innotech, Biometra) gel documentation system was used for photographing and analysis of the electrophoresed gel via computer software.

RESULTS

The bacteriological examinations exposed that *L. monocytogenes* was isolated from 36 out of 150 examined samples (24%) as illustrated in Table 2. The well grown isolates exhibited on Oxford agar a picture of black colonies with dimpled centers while they showed on PALCAM agar; green -gray colonies with black frustrated button center and surrounded by black zones. The presumptive isolates were Gram + bacilli or coccobacilli, demonstrating tumbling or pattern umbrella motility. Biochemical reactions displayed that all isolates were oxidase (-) and catalase (+), also produce acid with L- rhamnose and dextrose, but not with sucrose mannitol or D- xylose.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions used for identification of *Listeria monocytogenes* and its virulence genes in samples

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16S rRNA	GGACCGGGG CTA ATA CCG AAT GAT AA TTCATGTAGGCGAGTTGCAGC 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)
plcA	ACA AGC TGC ACC TGT TGC AG TGA CAG CGT GTG TAG TAG CA	1484	94˚C 5 min.	94˚C 30 sec.	60˚C 50 sec.	72˚C 1 min.	72˚C 12 min.	Soni et al. (2014)
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)

Table 2: Comparison between culture methods and PCR technique for the detection of *L. monocytogenes* in collected raw milk and some milk byproducts samples

Sample	Number of examined samples	Positive samples by culture method		Positive samples by PCR	
		Number	Percentage	Number	Percentage
Raw milk	25	11	44	11	44
Domiati cheese	25	6	24	7	28
Kariesh cheese	25	6	24	6	24
Roomi cheese	25	2	8	5	20
Yogurt	25	4	16	5	20
Cream	25	7	28	7	28
Total	150	36	24	41	27.3

The obtained data of virulence assessment exhibited that, all *L. monocytogenes* isolates displayed narrow area of β-hemolysis on 5% sheep blood agar, positive CAMP test with β-hemolysis zone at the intersection of examined isolates and *S. aureus* strain. Concerning animal pathogenicity (Anton's test); all isolates induced positive reaction manifested by suppurative conjunctivitis within 1-2 days pursued by keratitis in all tested rabbits.

The PCR results for direct detection of *L. monocytogenes* showed that 41 out of 150 examined samples were positive to 16S rRNA (27.3%), (Fig. 1). In respect to detection of virulence genes; 35out of 36 isolates (97.2%) harbor *hly* A gene while 100% of the direct samples carry the mentioned gene (Fig. 2). All isolates (36) and direct samples (41) were found to harbor *inl*A gene (Fig. 3). *inl*B gene was detected in 15 out of 36 (41.6%) of isolates while 8 out of 41 (19.5%) directly detected samples were positive (Fig. 4). On the other hand, *prf*A gene was detected in 25 out of 41 (60.9%) direct samples while it was detected in 20 out of 36 (55.5%) of isolates (Fig. 5). Finally, *plc*A gene was not detected in all samples whether isolates or directly detected samples (Fig. 6).

DISCUSSION

To secure food safety and quality, disclosure of pathogenic microbes should be a substantial target. *L. monocytogenes* has been implemented in numerous outbreaks and solitary cases of listeriosis correlated to the uptake of unpasteurized milk and other dairy products (Van Kessel et al. 2004). The implementation of molecular way has promoted the determination and characterization of leading virulence-linked genes in *L. monocytogenes* (Liu 2006). Therefore, this study was performed to estimate the scope of *L. monocytogenes* among raw milk, Domiati cheese, Kariesh cheese and Roomi cheese, yogurt and cream at Damietta Governorate, in addition, detection of some virulence factors was also done in isolates and directly in samples by PRC technique.

As shown in Table 2, the total positive samples for *L. monocytogenes* using culture method were 36 out of 150 examined samples. *L. monocytogenes* was isolated from 11 (44%), 6(24%), 6(24%), 2(8%), 4(16%) and 7(28%) of examined raw milk, Domiati cheese, Kariesh cheese, Roomi cheese, yogurt and cream, respectively. These results disagreed with the results as reported in previous studies (EL-Malt et al. 2009; El-Marnissi et al. 2013; Dapgh et al. 2020) who recorded lower incidence of *L. monocytogenes*. On the other hand, the PCR findings for direct detection of *L. monocytogenes* declared that 41 out of 150 examined samples were positive to 16SrRNA and this may be due to PCR can detect both live and died *L. monocytogenes*, some processing and heat treatment of milk may kill or suppress the growth of bacteria. These results came in accordance with those notated by Holko et al. (2002), Michael et al. (2005), Swetha et al. (2012) and Ciolacu et al. (2015). The previous authors mentioned that PCR minimized diagnosis' time hence permitting a quick recognition of *L. monocytogenes* with high degrees of specificity and sensitivity. By direct detection of *L. monocytogenes* using the primer of 16SrRNA, *L. monocytogenes* was detected in 11(44%), 7(28%), 6(24%), 5(20%), 5(20%) and 7(28%) of examined raw milk, Domiati cheese, Kariesh cheese and Roomi cheese, yogurt and cream, respectively. Lower incidence was detected by Abdeen et al. (2021) who detected *L. monocytogenes* in 6% of examined raw milk.

The pathogenicity of *Listeria* spp. is closely associated with their hemolytic activities (Gedde et al. 2000; Maarouf et al. 2007). The findings of virulence tests for isolated *Listeria* appeared that, all *L. monocytogenes* were positive to CAMP test and displayed β-hemolysis narrow zone on

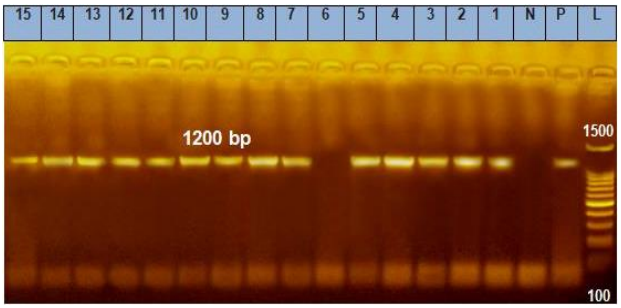

Fig. 1: PCR amplification of (16S rRNA) gene universal gene for identification of *L. monocytogenes*; lane L: 100b DNA ladder, lanes 1-5, 7-15 represent positive samples at 1200bp, Lane P is positive control while Lane N is negative control.

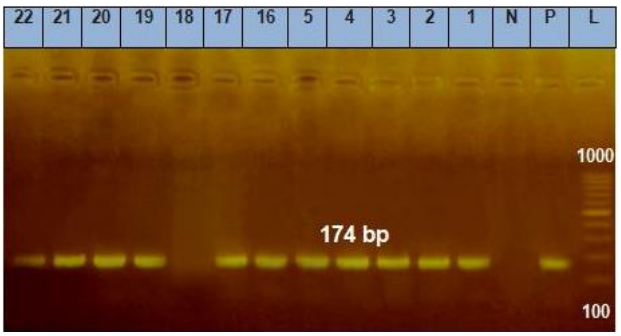

Fig. 2: PCR amplification of (*hly* A) gene virulence gene in *L. monocytogenes*; lane L: 100b DNA ladder, lanes 1-17, 19-22 represent positive samples at 174bp, Lane P is positive control while Lane N is negative control.

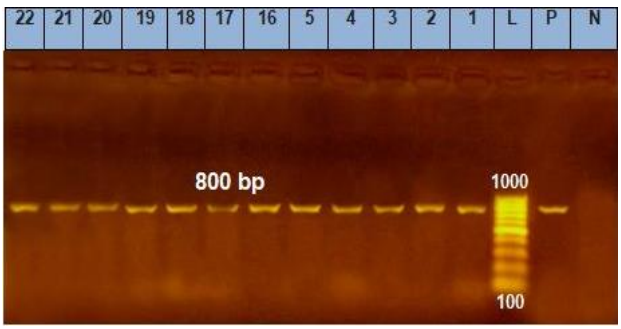

Fig. 3: PCR amplification of (*inl* A) virulence gene in *L. monocytogenes*; lane L: 100b DNA ladder, lanes 1-5, 16-22 represent positive samples at 800bp, Lane P is positive control while Lane N is negative control.

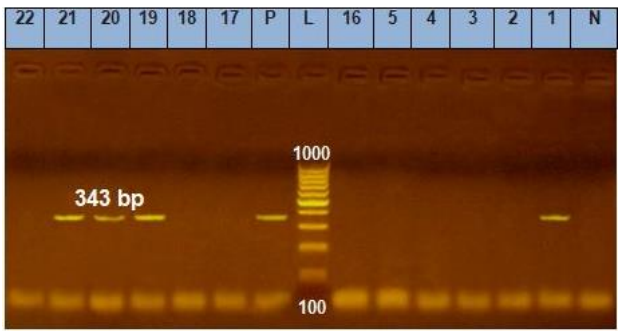

Fig. 4: PCR amplification of (*inl* B) virulence gene in *L. monocytogenes*; lane L: 100b DNA ladder, lanes 1, 19-21 represent positive samples at 343bp, Lane P is positive control while Lane N is negative control.

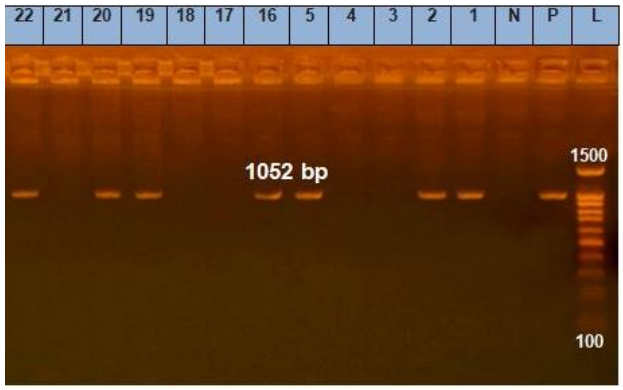

Fig. 5: PCR amplification of (*prf*A) virulence gene in *L. monocytogenes*; lane L: 100b DNA ladder, lanes 1, 2, 5, 16, 19, 20, 22 represent positive samples at 1052bp, Lane P is positive control, Lane N is negative control.

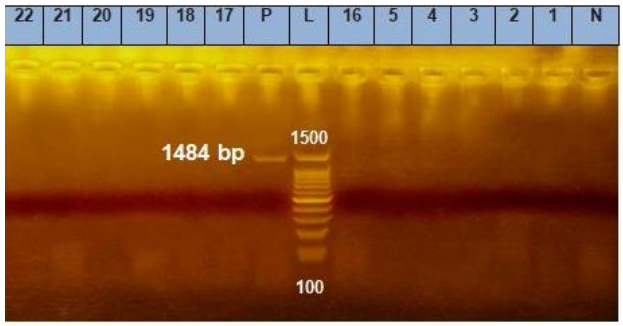

Fig. 6: PCR amplification of (*plc* A) virulence gene in *L. monocytogenes*; lane L: 100b DNA ladder, no positive samples at 1484bp, Lane P is positive control while Lane N is negative control.

blood agar. The same results were recorded by Portnoy et al. (1992) and McKellar (1994) who mentioned that all virulent *L. monocytogenes* secrete a form of hemolysin (listeriolysin O) which lyses the host vacuole, so the organism can survive in the host cytoplasm. Concerning CAMP test, it is proposed that RBC membrane was sensitized by sphingomyelinase produced by *S. aureus* prior to entire lyses with *L. monocytogenes* (McKellar 1994). Also, all isolates showed positivity for Anton's test and this result was in harmony with Shafie and Amer (2002) and Maarouf et al. (2007).

Regarding the prevalence of virulence-associated markers, the different specific primers for the following virulence genes (*hly* A, *inl* A, *inl* B, *prf*A and *plc*A) were used in PCR. 100% of the isolates (36) harbor inl A gene while 35 isolate (97.2%) harbor *hly* A gene. 100% of the samples (41) that were positive to 16S rRNA were also positive to *hly* A and *inl* A genes. *plc*A gene was not detected in all samples whether directly or in isolates. *inl*B was detected in 8 out of 41(19.5%) positive samples directly detected while it was detected in 15 out of 36(41.6%) of isolates. *prf*A gene was detected in 25 out of 41(60.9%) positive samples directly detected while it was detected in 20 out of 36(55.5%) of isolates. Parallel findings have also been reported previously (Almeida and Almeida 2000; Jaradat et al. 2002; Joseph and Goebel 2007; Liu et al. 2007; Ciolacu et al. 2015).

The data of PCR for *inl*A gene (encoding internalin A) (Fig. 3); displayed product of 800bp amplification in all

isolates (36). Almeida and Almeida (2000) and Jaradat et al. (2002) reported homologous results. Also, the data was harmonized with that mentioned by Joseph and Goebel (2007); Liu et al. (2007) as well as Shen et al. (2013) and Ciolacu et al. (2015). The data of PCR for *inl*B gene (encoding internalin B) (Fig. 4) showed product of 343bp amplification in 15(41.6%) isolates. Jaradat et al. (2002), Joseph and Goebel (2007) also Gelbicova and Karpiskova (2012) also reported nearly similar data.

The findings of PCR for *prf*A gene (Fig. 5) which positively regulates all virulence factors in *L. monocytogenes* (Ciolacu et al. 2015) demonstrated product of 1052 bp amplification in 20(55.5%) isolates. The Higher prevalence were indicated also by other previous studies Holko et al. (2002) and Jaradat et al. (2002). Also, Begley et al. (2005), Liu et al. (2007) as well as Joseph and Goebel (2007) and Shen et al. 2013 reported the same picture.

The data obtained from Fig. 6 showed that the *plc*A gene giving product of 343 bp. in positive control and has not been detected in any sample. Our findings differed from that obtained by Simranpreet et al. (2017) who detected the *plc*A gene in all samples.

Conclusion

The results of this study showed that, the higher prevalence of *L. monocytogenes* was present in raw milk followed by the cream, and this may be attributed to that these products were not treated by heat or any starter. PCR was rapid, accurate and sensitive in detection of *L. monocytogenes* than the culture method. There is no great difference between ratios of virulence genes' PCR detection either in isolates or directly in samples.

Authors Contribution

Rania, L. Salem collected the samples, performed the bacteriological isolation and biochemical tests, assisted in PCR analysis of virulence genes, collection of data and writing. Amany, N. Dapgh carried out Anton's test, conducted the PCR analysis, assisted in writing and revision of the manuscript.

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