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Microbiological study on *Listeria* species isolated from some food products of animal origin

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A B S T R A C T

The study was carried out to investigate the presence of Listeria species in some food products of animal origin. A total of 200 different samples were collected including; milk (100), kariesh cheese (25), beef meat (25), hot dog (25) and Tilapia fish (25). The obtained results showed that the highest rate of isolation of Listeria was recorded in Tilapia fish (40%), followed by beef meat (28%) then raw milk (25%), Hot dog (24%) and finally kariesh cheese (20%). Distribution of Listeria isolated from raw milk clarified the identification of L. monocytogenes (2%), L. ivanovii (7%), L. innocua (5%), L. seeligeri (6%) and L. murrayi (5%), from kariesh cheese samples, L. monocytogenes (4%), L. ivanovii (8%), L. innocua (4%), and L. seeligeri (4%), from beef meat samples, L. monocytogenes (8%), L. ivanovii (4%), L. innocua (4%), L. seeligeri (4%) and L. welshimeri (8%), from Hot dog samples, L. monocytogenes (4%), L. ivanovii (8%) and L. welshimeri (12%) and finally, from Tilapia fish samples clarified the identification of L. monocytogenes (4%), L. ivanovii (12%), L. innocua (4%), L. seeligeri (4%) and L. welshimeri (16%). Finally, PCR was applied successfully to detect LM1 gene in identification of isolates of L. monocytogenes. The data obtained in this study provides useful information for assessment of the possible risk posed to Libyan consumers and will have a significant public health impact in Libya.

Keywords: Listeria, Food products, Isolation, PCR

1. Introduction

Listeriosis is one of the most serious foodborne diseases in human. It has worldwide distribution with sporadic occurrence comparing to other food borne infections but with significant fatality rate. It is said that listeriosis is responsible for the deaths of about 400 – 500 death cases per year and over 2500 persons are reported to have falling ill (FDA, 2018). Listeriosis is caused by members of the genus Listeria which represents a groups of closely related Gram positive, non-spore forming, facultative anaerobic rod shaped bacteria. It is capable to grow at refrigeration temperature, at pH values of 5 and above, in high salt concentration (up to 10 %) and are relative resistance to freezing and drying (Arslan and Ozdemir, 2008). Also, Listeria is an Opportunistic pathogen found in soil, manure, faeces, sewage, silage, water and plant surfaces and as a result they easily enter the food chain. Among the genus Listeria, which causes the infection of listeriosis in both animal and man, Listeria monocytogenes is a major pathogenic microorganism while, L. ivanovii principally affects animals and rarely affects man, (Aygun and Pehlivanlar, 2006).

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Listeriosis is transmitted primarily through various foods as meat, meat products, fish, eggs, fruits and vegetables. In addition, Milk and other dairy products such as cheese and ice cream which are produced from unpasteurized milk are often contaminated with this pathogen and have been reported as source of listeriosis in numerous widely publicized incidents (Brooks et al., 2012). Farm animals and their environment act as an important source of food contamination and infection for humans (Jemmi and Stephen, 2006).

L. monocytogenes was found in at least 37 species of mammals, both domestic and wild, as well as up to 10% of humans may be intestinal carriers. It had been detected in 17 species of birds, some species of fish and shellfish, and is especially pathogenic to high risk populations, such as newborn, pregnant women, elderly, and immunocompromised individuals (Mugampoza et al., 2011). The ability of L. monocytogenes to persist in food-processing environments and multiply under refrigeration temperatures makes it a significant threat to public health (Jemmi and Stephan, 2006). Although L. monocytogenes is the primary human pathogen, there have been several reports of illnesses caused by L. seeligeri, L. ivanovii, L. innocua, L. welshimeri and L. gyayi in human (Guillet et al., 2010).

Recently, media have been described which distinguish L. monocytogenes from other Listeria spp., based on haemolysis or on a chromogenic substrate (ISO, 1996). Using chromogenic agars, the presumptive identification of L. monocytogenes is possible after 24 h, compared with 3-4 days using Oxford and other conventional agars (Greenwood et al., 2005). DNA-based methods such as conventional PCR have been developed as safe, useful, sensitive, and accurate methods for the detection of L. monocytogenes in clinical specimens (O' Grady et al., 2010). The expansion of PCR-based serotyping procedures has provided further benefits for the identification and grouping of L. monocytogenes (Doumith et al., 2004). Control of listeriosis is achieved by avoid consumption of unpasteurized milk and milk products. Pregnant women and immunosuppressive individuals should take stringent precautions to avoid infection by listeriosis and other foodborne pathogens. So, the present work was carried out to determine the occurrence of Listeria spp. in different food samples (milk, cheese, meat products and fish) beside application of PCR for identification of Listeria monocytogenes virulence genes.

2. Material and methods

2.1. Collection of samples:

A grand total of 200 different food samples were collected from local markets including; milk samples (100), kariesh cheese (25), beef meat Hot dog (heat treated meat product) (25) and Tilapia fish (25). Samples were kept in separate sterile plastic bags and transferred in an ice box as soon as to the laboratory.

2.2. Preparation of samples:

For milk and kariesh cheese; 25 ml or 25 g of each sample was aseptically taken and homogenized in 225 ml of Listeria enrichment broth (Oxoid) supplemented with Listeria selective enrichment supplement (Oxoid) using a Lab-blender 400 stomacher (Interscience, France) for 2 - 4 minutes and incubated at 30 °C for 48hours. For meat products and fish; 25g of each sample was homogenized in 225 of half Fraser broth using a

Lab-blender 400 stomacher (Interscience, France) for 2 - 4 minutes and incubated at 30 °C for 48 hours.

2. 3. Isolation procedures

It was performed according to the technique suggested by ISO 11290-1 (2017). Accurately, 1 ml of primary enrichment was transferred to 10 ml of Fraser broth and incubated at 30 °C for 48 hours. A loopful of the incubated broth was streaked onto Oxford agar and PALCAM agar (Oxoid) and incubated at 30 °C for 48 hours. All colonies (2-3 mm diameter with a sunken centre) surrounded by a brownish green and/or black halo were taken as possible Listeria spp. Five typical suspected Listeria colonies from each plate were subcultured onto tryptic soy agar supplemented with 0.6 % of yeast extract (Oxoid) and incubated at 30 °C for 24 hours. All of the isolates were subjected to standard biochemical tests, including Gram staining, catalase test, motility test at 25 and 37 °C, nitrate reduction, MR/VP test, β-haemolysis activity, CAMP test and acid production from glucose, mannitol, rhamnose and xylose.

2.4. Molecular identification of Listeria monocytoens1 gene specific for Listeria monocytoens

Biochemically positive L. monocytogenes isolates were examined for the presence of LM1 gene specific for L. monocytogenes as will discussed in the section of molecular identification.

2.4.1. DNA extraction technique

Into the bottom of a 1.5 ml microcentrifuge tube, 20 µl protease was pipetted. To this 1.5 ml microcentrifuged tube, 200 µl of the sample were added followed by addition of 200 µl AL buffer. The mixture was well mixed by pulse vortexing for 15 seconds and then incubated at 56˚C for 10 minutes. The 1.5 ml microcentrifuged tube was then centrifuged to remove drops from the inside of the lid. About 200 ml ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated to remove drops from the inside of the lid. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. 8- The QIAamp mini spin column was carefully opened and 500 µl AW1 buffer was added without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1 minutes and then placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. AW2 buffer (500µl) was added to the minispin column without wetting the rim. The cap was closed, and centrifuged at full speed for 3 minutes and then was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1minutes was done. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. All buffer (100 µl) was added carefully to the minispin columns, incubated at room temperature (15-25 C) for 1 minutes and then centrifuged at 8000 rpm for 1 minute.

2.4.2. Polymerase chain reaction technique

Oligonucleotide primers used for detection of hylA virulent gene by PCR according to Swetha et al., (2012). The forward primer sequence (CCTAAGACGCCAATCGAA) and reverse primer sequence (AAGCGCTTAACTGCTC) were used for detection of L. monocytogenes at 702 bp region. Purified DNA of L. monocytogenes isolates was subjected to conventional PCR for the tested gene using the cycling condition listed in the following table;

Gene	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
LM1	95˚C 3 min.	94˚C 30 sec.	53˚C 30 sec.	72˚C 1 min.	35	72˚C 10 min then hold 4 sec.

2.4. Statistical analysis

The statistical analysis was carried-out using Chi2-test for study the prevalence of certain parameters among different studied tests according to SAS, (2014).

4. Results

Table (1): Rate of isolation of Listeria spp. from different food samples

Food samples	No. of examined samples	Positive	
		No.	%
Raw milk	100	25	25.0
kariesh cheese	25	5	20.0
Beef meat	25	7	28.0
Hot dog	25	6	24.0
Tilapia fish	25	10	40.0
Total	200	53	26.5
Chi^2 value	8.55*		

* = Significant at (P < 0.05)

Table (2): Distribution of Listeria spp. isolated from samples of raw milk and kariesh

Listeria spp.	Raw milk (n=100)		kariesh cheese (n=25)		Total (n=125)	
	Frequency	%	Frequency	%	Frequency	%
L. monocytogenes	2	2.0	1	4.0	3	2.4
L. ivanovii	7	7.0	2	8.0	9	7.2
L. innocua	5	5.0	1	4.0	6	4.8
L. seeligeri	6	6.0	1	4.0	7	5.6
L. murrayi	5	5.0	0	0.0	5	4.0
Total	25	25.0	5	20.0	30	24.0
Chi^2	3.25 NS		8.99*		11.45**	
Total Chi^2-value	9.78**					

Table (3): Distribution of Listeria spp. isolated from samples of beef meat and Hot dog

Listeria spp.	Beef meat (n=25)		Hot dog (n=25)		Total (n=50)	
	Frequency	%	Frequency	%	Frequency	%
L. monocytogenes	2	8.0	1	4.0	3	6.0
L. ivanovii	1	4.0	2	8.0	3	6.0
L. innocua	1	4.0	0	0.0	1	2.0
L. seeligeri	1	4.0	0	0.0	1	2.0
L. welshimeri	2	8.0	3	12.0	5	10.0
Total	7	28.0	6	24.0	13	26.0

Chi^2	4.27*	9.55**	6.55**
Total Chi^2-value	19.56**		

* = Significant at (P < 0.05)
** = Significant at (P < 0.01)

Table (4): Distribution of Listeria spp. isolated from samples of Tilapia fish

Listeria spp.	Tilapia fish (n=25)	
	Frequency	%
L. monocytogenes	1	4.0
L. ivanovii	3	12.0
L. innocua	1	4.0
L. grayi	1	4.0
L. welshimeri	4	16.0
Total	10	40.0
Chi^2	6.55**	

Table (5): Prevalence of L. monocytogenes in food products based on molecular identification of LM1 gene of isolated of L. monocytogenes strains

Sample type	No. of examined isolates	No. of isolates positive for LM1 gene
Milk	1	1
Meat	1	1
Fish	1	1
Hot dog	1	0
Cheese	1	0
Total	5	(3/5) 60%

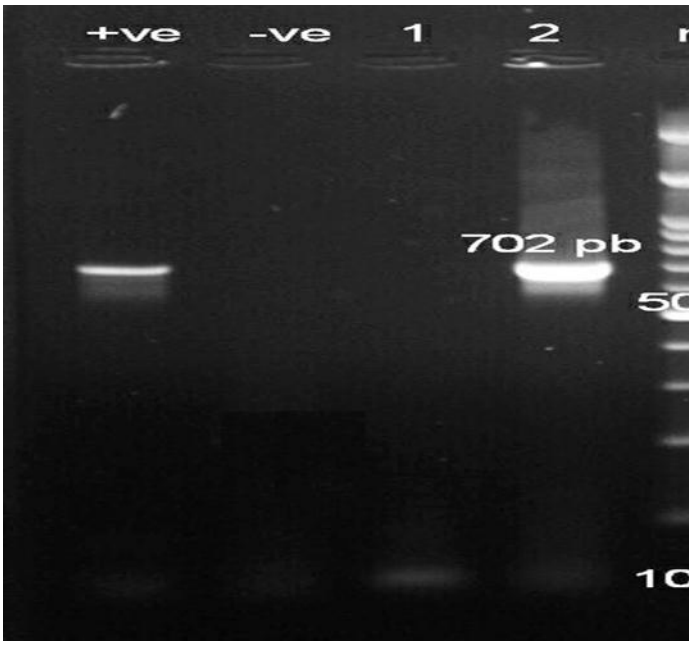

Photo: Sampler of agarose gel electrophoresis of molecular identification of hylA gene of isolated of L. monocytogenes strains
Lane M: 100 bp ladder as molecular size DNA marker
Lane C+: Control positive
Lane C-: Control negative
Lanes 2, 3 and 4: Positive L. monocytogenes for hylA gene

4. Discussion

Listeriosis is one of the most serious foodborne diseases in human. It frequently occurs as a result of incidental contamination of ready-to- eat and raw food products. Outbreaks and sporadic cases of Listeriosis have been related with consumption of contaminated milk, soft cheese, undercooked meat and unwashed raw vegetables (Rahimi et al. 2010).

Listeriosis is also considered as a serious disease for public health. Hence food industry and food laboratories should direct a special attention to such disease. The eating habits of Libyan people are different from those in western countries. The majority of people prefer to consume traditionally produced foods. Traditional dairy products in Libya are produced in small productive centers mostly located in urban areas and distributed unpacked. These products may be produced from unpasteurized milk. There have not been comprehensive studies performed on food contamination by Listeria and listeriosis in most developing countries.

As shown in Table (1), isolation of Listeria spp. from different food samples revealed that the highest rate of isolation was recorded in the examined samples of Tilapia fish (40%), followed by beef meat (28%) then raw milk (25%), Hot dog (24%) and finally kariesh cheese (20%) with statistically significant association between these rates of isolation. Concerning milk samples, the recorded result was higher than that recorded by Seyom et al. (2015))18.9%,(Girma and Abebe (2018) (20.88%)and Haggag et al., (2019) (8.4%) while it was lower than that recorded by El-Malt and Abdel-Hameed (2009) who detected Listeria species in 15 out of 50 raw milk samples (30%). Concerning kariesh cheese, the recorded result was lower than recorded by Arslan and Özdemir (2008) who found that the overall incidence of Listeria species was 33.2% in homemade cheese in turkey. On contrary, it was higher than that recorded by El-Shenawy et al., (2017) who examined 40 samples of kariesh cheese collected randomly from Beni-Suef Governorate, Egypt and found that the occurrence of Listeria spp. in kariesh cheese was 12.5%. Concerning beef meat, the recorded result was higher that recorded by Ismaiel et al. (2014) who investigated sixty beef meat samples for Listeria isolation and found that the incidence rate was 10%. Concerning Hot dog, the obtained result was nearly similar with that of Telles, et al. (2006) who examined 394 samples of hot dog and found that 101(25.6%) samples were contaminated with Listeria species. Concerning fish, the obtained result was higher than that recorded by Rahimi et al. (2012) who examined a total of 140 fish samples collected from retail stores for the presence of Listeria species and they could isolate 8 Listeria isolates (5.7%).

As shown in Table (2), distribution of Listeria spp. isolated from raw milk samples clarified the identification of L. monocytogenes (2%), L. ivanovii (7%), L. innocua (5%), L. seeligeri (6%) and L. murrayi (5%) with statistically non-significant association between these rates of isolation. Several studies have shown contamination of raw milk with Listeria spp.; El-Malt and Abdel-Hameed (2009) identified L. welshimeri in one sample (2%), L. inoccu present in 2 samples (4%) and L. grayi was detected in 5 samples (10%). Suspected L. monocytogenes has been recovered in 3 samples (6%) of raw milk, Meshref et al., (2015) examined 51 raw milk samples randomly collected from retail markets and grocery stores in Beni-Suef governorate, Egypt and found that the rate of isolation of Listeria spp. was 27.45% and the most recovered species was L. innocua (22.22%) followed by L. ivanovii and L. seeligeri (18.52% for each) then L. grayi (14.815%), L. welshimeri (14.815%) and lastly, L. monocytogenes (11.11%), Seyom et al. (2015) found that the prevalence rate of L. monocytogenes in milk was 2.04%, Hesham et al., (2017) who studied the prevalence of Listeria spp. in retail raw animal food products covering most Tripoli city in Libya. They tested 180 samples of dairy, meat and their products and found that 79 (43.8%) tested positive for Listeria spp., 32 (40%) samples were positive from different dairy products (7 laben, 9 raw cow's milk, 8 Ricotta cheese, 8 Maassora cheese). Listeria spp. were isolated as follows: 17 (10.8%) from raw milk, 10 (6.3%) Laben, 12 (7.6%) Massora cheese, 25 (15.8%) chicken meat, 41 (25.9%) raw beef, 11 (7%) beef burger. While 14 (8.7%) was isolated from Ricotta cheese, chicken burger and beef sausage, El-Gohary et al. (2018) found that 3 (12.5%) samples were positive for L. monocytogenes. and Haggag et al., (2019) who clarified the presence of L. monocytogenes (3 isolates) at a percentage of 2%, L. ivanovii (2 isolates) at a percentage

of 1.33%, L. innocua (2 isolates) at a percentage of 1.33% and L. grayi (4 isolates) at a percentage of 2.67%.

These results indicated the role of raw milk as a vehicle of listeriosis infection to human as it is clearly a food borne zoonoses. The sources of Listeria spp. in raw milk have been documented to be faecal and environmental contamination during milking, storage and transport, infected dairy animals and silage quality (Bemrah et al., 1998) so, strict hygienic measures including hygienic disposal of animal manure with cleaning and disinfection and avoidance of using of animal manure especially sheep manure as agricultural fertilizer.

In addition, data presented in Table (2) showed that the distribution of Listeria spp. isolated from kariesh cheese samples clarified the identification of L. monocytogenes (4%), L. ivanovii (8%), L. innocua (4%), and L. seeligeri (4%) with statistically significant association between these rates of isolation. This finding was supported by that of Arslan and Özdemir (2008) who differentiated various Listeria species with percentages of 9.2% (13/142) for L. monocytogenes, 5.6% for L. innocua, 4.9% for L. grayi and 2.1% for each of L. ivanovii and L. welshimeri.

It was interesting that the milk and kariesh cheese samples were contaminated with Listeria spp. this may explain the utmost zoonotic role of milk in spread and disseminating this zoonotic agent to human consumers.

Data presented in Table (3) showed that the distribution of Listeria spp. isolated from beef meat samples clarified the identification of L. monocytogenes (8%), L. ivanovii (4%), L. innocua (4%), L. seeligeri (4%) and L. welshimeri (8%) with statistically significant association between these rates of isolation. The recorded result was lower than that recorded by Indrawattana et al., (2011) who found that the prevalence of L. monocytogenes in raw meats marketed in Bangkok was 15.4%. This finding was supported by that of Ismaiel et al. (2014) who isolated 6 isolates of Listeria which comprised 1.66% (1/60) for each of L. monocytogenes, L. welshimeri and L. innocua and 5% (3/60) for L. ivanovii and Hesham et al., (2017) who studied the prevalence of Listeria spp. in retail raw animal food products covering most Tripoli city in Libya. They tested 180 samples of dairy, meat and their products and found that 47 (47%) samples from various meat and its products (9 chicken meat, 12 chicken burger, 3 raw beef, 12 beef burger, 11 beef sausage).

Also, data presented in Table (3) showed that the distribution of Listeria spp. isolated from Hot dog samples clarified the identification of L. monocytogenes (4%), L. ivanovii (8%) and L. welshimeri (12%) with statistically significant association between these rates of isolation. This finding was in harmony with that of Navratilova et al. (2004) who found 56 (55.4%) isolates from positive Listeria isolates from Hot dog were identified as L. monocytogenes.

The presented data in Table (4) showed that the distribution of Listeria spp. isolated from Tilapia fish samples clarified the identification of L. monocytogenes (4%), L. ivanovii (12%), L. innocua (4%), L. seeligeri (4%) and L. welshimeri (16%) with statistically significant association between these rates of isolation. This finding was in harmony with that of Rahimi et al. (2012) who recorded that L. innocua was identified in 6 samples (4.2%) while L. monocytogenes was detected in 2 samples only (1.42%) and Edris et al. (2014) who studied the incidence of L. monocytogenes in 100 fresh Tilapia fish samples in Egypt and the organism was obtained from 7% (7/100) of the examined samples and Wu et al (2015) who isolated 10 L. monocytogenes isolates which represented 6.9% of the total samples.

The variation in prevalence of L. monocytogenes in samples might be due to the differences in holding time, processing ways of the food before sale, as well as this microorganism is able to survive in low temperature (Walker et al., 1990) and tolerate cold stress (Schmid et al., 2009)

Molecular methods including; PCR were diagnostic tools for epidemiological investigation. In the present study, PCR was employed for detection of hylA gene for characterization of L. monocytogenes isolated from various isolates. PCR was applied successfully to detect hylA gene in identification of isolates of L. monocytogenes (Photo).

Several studies study the molecular detection of L. monocytogenes in food products including; Edris et al. (2014) examined 7 L. monocytogenes isolates recovered from 100 fish samples by PCR technique and found that 4 isolates only (4%) were confirmed as L. monocytogenes, Eslami et al. (2014) identified L. monocytogenes virulence factors in women with abortion by polymerase chain reaction in Iran at which 96 vaginal swab samples were collected from 96 women with abortion and subjected to molecular examination.

It was found that 16 (16.6%) isolates were identified by PCR as L. monocytogenes by detection of hylA virulent gene, Ahmed et al. (2016) identified L. monocytogenes in minced meat and Cheese in Duhok Province, Kurdistan using PCR. Fourteen suspected Listeria species isolated from 100 meat and cheese samples (50, each) were examined by PCR which yielded that 7 out of 11 (14%) isolates from meat and 1 isolate out of 3 (2%) ones from cheeses were confirmed as L. monocytogenes, El-Gohary et al. (2018) studied the zoonotic and molecular aspects of Listeria species in aborted women in Egypt. Twelve vaginal swab samples were collected from aborted women admitted for Mansoura University hospitals and some private clinical laboratories. The findings were that two (16.6%) samples revealed showed the presence of Listeria species and Owusu-Kwarteng et al (2018) characterized the prevalence of L. monocytogenes virulence gene in raw milk in Ghana. They screened the prevalence of hylA virulent gene in ten L. monocytogenes isolates obtained from raw milk by PCR. The results showed that all isolates harbored such gene.

5. Conclusion

The data obtained in this study provides useful information for assessment of the possible risk posed to Libyan consumers and will have a significant public health impact in Libya. The recorded results in the current study throw the light upon the public health hazard of Listeria where investigated samples were found to be positive for presence of Listeria with different rates that could ring the hazard bell about the significant role of different food products in transmitting these organisms to human beings. Moreover, PCR is a useful diagnostic tool for detection of L. monocytogenes because it sensitive and specific so this technique should be adopted as a complementary tool to conventional tests.

Conflict of interest statement

No conflicts of interest.

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