Open Veterinary Journal, (2021), Vol. 11(3): 517–524
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)

Original Research
DOI: *10.5455/OVJ.2021.v11.i3.25*

Submitted: 21/06/2021 Accepted: 01/09/2021 Published: 30/09/2021

Molecular characterization and in vivo pathogenicity study of *Listeria monocytogenes* isolated from fresh and frozen local and imported fish in Jordan

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Abstract
Background: *Listeria monocytogenes (L. monocytogenes)* is a serious zoonotic and food transmitted human pathogen causing meningitis and abortions. Several outbreaks of listeriosis have been associated with the consumption of ready-to-eat food products; dairy, meat, fish, and contaminated fruits and vegetables worldwide.
Aim: This study was designed to detect and characterize *L. monocytogenes* isolated from local and imported fish in Jordan.
Methods: A total of 170 fish (70 local and 100 imported), of which 140 fresh and 30 frozen samples were used in this study. *Listeria monocytogenes* was cultured and initially identified using conventional microbiological methods. For confirmation and serotyping of the *L. monocytogenes* isolates, PCR techniques were used. Using oral and intraperitoneal administration, mice were used to determine the pathogenicity and LD_{50} of the isolated *L. monocytogenes*.
Results: A total of 72 *Listeria* spp. isolates were cultured from fish. Of those, 24 were positively identified as *L. monocytogenes.* Other strains of *Listeria* spp. were *L. ivanovii* (21), *L. innocua* (11), and *L. grayi* (16). Serotyping of the *L. monocytogenes* indicated that 14 isolates belonged to the 1/2b, 3b serotypes whereas 10 isolates belonged to the 4a and 4c serotypes. All isolates were virulent to mice with an LD_{50} dose ranging from 3×10^{10} CFU/ml to $3 \times 10^{7.5}$ CFU/ml. All the virulent isolates belonged to the serotype 1/2b. Histopathologically, dead mice showed multiple necrotic lesions in the liver and spleen.
Conclusion: Results of this study showed the presence of potentially pathogenic *L. monocytogenes* in fresh and frozen, local, and imported fish in Jordan. Strict monitoring and quality control regulatory measures must be adopted to prevent future outbreaks of food poisoning associated with fish consumption.
Keywords: *L. monocytogenes*, Fish, Serotypes, Virulence, Nested PCR.

Introduction

Members of the genus *Listeria* are widely distributed in the environment and consist of eight species; *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welashimari*, *L. grayi*, *L. marthii* and *L. rocourtiae* (Skowron *et al*., 2019). *Listeria monocytogenes* survive under food preservation conditions, including pH, salinity, and refrigeration (Swaminathan and Gerner-Smidt, 2007; Buchanan *et al*., 2017; Jordan and McAuliffe, 2018; Cabal *et al*., 2019). It can also withstand the high pressure and light therapy commonly used to control the growth of organisms in food (Buchanan *et al*., 2017; Jordan and McAuliffe, 2018; Cabal *et al*., 2019).

Listeria monocytogenes is an important zoonotic and food-borne human pathogen causing listeriosis. Listeriosis is a serious disease characterized by acute meningitis and spontaneous abortions commonly affecting pregnant women, neonates, adults, and immunocompromised patients (Wang *et al*., 2015; Jensen *et al*., 2016; Moura *et al*., 2016; Skowron *et al*., 2019; Chen *et al*., 2020; Li *et al*., 2020). In recent years, several outbreaks were reported worldwide, most often associated with consuming ready-to-eat food (RTE) products (Jami *et al*., 2014; European Centre for Disease Prevention and Control and European Food Safety Authority, 2018, 2019). Recently, 22 cases of food-borne listeriosis were reported in 5 EU countries: Denmark (9 cases), Estonia (6), Finland (2), France (1), and Sweden (4) (European Centre for Disease Prevention and Control and European Food Safety Authority, 2019). In eight patients, infection was positively linked to the consumption of cold-smoked fish products (European Centre for Disease Prevention and Control and European Food Safety Authority, 2019).

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In Jordan, fresh and frozen fish are commonly consumed by people, surprisingly no outbreaks of listeriosis associated with fish consumption have been reported. However, *Listeria* exist in fish and fish products may cause significant public health concern. Therefore, this study aimed to isolate, characterize, and serotype *Listeria* species from fresh and frozen, local and imported fish in Jordan and to determine their virulence factors and *in vivo* pathogenicity in mice.

Materials and Methods

Fish sample collection

A total of 170 fish samples were used in this study (40 local and 130 imported) of which 140 were fresh and 30 frozen fish. Imported fish were from Yemen, Egypt, Qatar, Saudi Arabia, and Oman. Samples belonged to various fish types, including *Nemipterus tambuloides*, *Lutjanus malabaricus*, *Lethrinus lentjan*, *Lutjanus monostigma*, and *Nemipterus nematophorus*. Whole fish were collected and placed in an ice box and transported to the laboratory for immediate processing.

Bacterial culture and identification

All processing techniques were conducted under strict aseptic conditions. Each fish sample was divided into three tissue portions: the gills, intestine, and scales/muscles (in total 510 portions). From each portion, 50 g were homogenized (Karl Kolb, Germany) in 150 ml sterile saline. Then, 0.1 ml of the homogenate was cultured on sheep blood agar (Oxoid, UK), tryptic soy agar (Oxoid, UK), Fraser broth (Conda, Spain), and *Listeria* selective chromogenic base supplemented with polymyxin B, ceftazidime, nalidixic acid, and cycloheximide (Conda, Spain). All the inoculated media were incubated aerobically at 37°C for 24–48 hours. The inoculated broth tubes were kept in the refrigerator for 2–4 weeks (cold enrichment). Suspected *L. monocytogenes* isolates were identified initially based on culture and Gram stain characteristics. Biochemical characterization was followed using the esculin hydrolysis, hemolysis assay, and Microgen *Listeria* 12L Kit (Microgen, USA) according to the manufacturer's instructions. Finally, the identified *L. monocytogenes* isolates were frozen and stored in brain-heart infusion broth (BHI, Merck) with 15% glycerol at −80°C until further analysis was performed. This study used the reference bacterial strain *L. monocytogenes* (ATCC 19116) as positive control.

Molecular identification of L. monocytogenes

The genomic DNA was extracted from isolated strains of *L. monocytogenes* using i-genomic BYF DNA Extraction Mini Kit (Boca Scientific, USA) according to the manufacture guidelines. Nested PCR was used to positively identify *L. monocytogenes* targeting the universal *hly*A gene of *L. monocytogenes* (Mojgani *et al*., 2006) using commercially primers (Intron Biotechnology, USA). Nested PCR of two rounds were performed. In the first round, the primer pair LM1 (5′-CCTAAGACGCCAATCGAA- 3′) and LM2 (5′-AAGCGCTTGCAACTGCTC-3′) derived from the listeriolysin O gene were used for PCR products target *hlyA* gene of *L. monocytogenes*. The amplification conditions were initial denaturation at 95°C for 3 minutes, followed by 30 cycles denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, DNA extension at 72°C for 1 minute. A final extension completed the reaction at 72°C for 5 minutes, and the PCR product of 702 bp fragment of the *hlyA* gene was cooled at 4°C. The second round of nested PCR was conducted using the nested primers: LL5 (5′-AACCTATCCAGGTGCTC-3′) and LL6 (5′- CTGTAAGCAATTTCGTC-3′) targeting the *hlyA* gene. The amplification conditions were described in round one, except primer annealing was at 55°C for 1 minute. The PCR products were viewed under UV light after being run in agarose gel electrophoresis stained with ethidium bromide. Real time PCR was used to amplify *L. monocytogenes* specific gene *prfA* using the PowerChek Kit (Kogene Biotech, South Korea). The PCR products were viewed under UV light after being run in agarose gel electrophoresis stained with ethidium bromide.

Serotyping of L. monocytogenes

All *L. monocytogenes* were subjected to serotyping using a PCR (Bio-Rad, USA). The *D1*, *D2*, *GLT*, *FlaA*, and *MAMA* primers (Table 1) have been used at different PCR conditions (Table 2). The serotyping procedure was performed according to the study reported by Borucki and Call (2003).

Mouse lethality assay

To determine the lethality and discriminate between virulent and avirulent isolates, 0.5 ml of 3×10^9 CFU/ml of each isolate was injected intraperitoneally into mice (Swiss Albino, strain BALB/C), reared, and reproduced at the Animal House unit at the Faculty of Veterinary Medicine/JUST. The *L. monocytogenes* (ATCC 19116) reference strain was used as a positive control. Monitoring of mice was extended for 2 weeks (Liu, 2004).

In vivo determination of LD_{50} was performed using 5 mice groups ($n = 8$). Mice in each group were inoculated intraperitoneally with 0.5 ml of different dilutions of 24 hours old culture of *L. monocytogenes* (10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). Mice were monitored daily, and after 2 weeks, all mice have died. The death time and the mortalities were recorded and compared with the number and time of deaths induced by the reference strain. All dead mice were subjected to necropsy and re-isolation of *L. monocytogenes* (Liu, 2004).

In vivo pathogenicity study of fish tissue contaminated with L. monocytogenes

Twelve mice were used in this experiment. Two mice were fed 100 g (50 g each) of sterile fish tissue (that did not show any bacterial growth when inoculated on different bacteriological media) after being fasted for 12 hours. Another two mice were injected intraperitoneally with 0.5 ml of sterile fish tissue homogenate (which

Table 1. Primers used to serotype *L. monocytogenes* isolated from fish samples in Jordan.

Primer set	Primer pairs	Product size (bp)
D1	CGATATTTTATCTACTTTGTCA (F) TTGCTCCAAAGCAGGGCAT (R)	214
D2	GCGGAGAAAGCTATCGCA (F) TTGTTCAAACATAGGGCTA (R)	140
FlaA	TTACTAGATCAAACTGCTCC (F) AAGAAAAGCCCCTCGTCC (R)	538
Glt	AAAGTGAGTTCTTACGAGATTT (F) AATTAGGAAATCGACCTTCT (R)	483
MAMA	CAGTTGCAAGCGCTTGGAGT (F) GTAAGTCTCCGAGGTTGCAA (R)	268

Table 2. Primers and PCR conditions used to serotype *L. monocytogenes* isolated from fish samples in Jordan.

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
D1, D2, Glt	95°C/3 m/1 c	95°C/30 s/25 cs	45–59°C/30 s/25 cs	72°C/1 m/25 cs	72°C/10 m/1 c
FlaA	95°C/3 m/1 c	95°C/30 s/30 cs	45–59°C/30 s/30 cs	72°C/1 m/30 cs	72°C/10 m/1 c
MAMA	95°C/10 m/1 c	95°C/30 s/40 cs	55°C/1 m/40 cs	72°C/1 m/40 cs	72°C/10 m/1 c

(m): minutes; (c): cycle; (s): second; (cs): cycles.

was prepared by homogenizing 50 g fish tissue (Karl Kolb homogenizer, Germany) in 150 ml sterile normal saline). The two mice fed orally with sterile fish tissue, and the two mice injected intraperitoneally with sterile tissue homogenate were considered as control groups. The same protocols were repeated using contaminated fish tissues with 3×10^9 CFU/ml of *L. monocytogenes* (4 mice were fed 50 g each, and 2 mice were injected intraperitoneally with 0.5 ml of contaminated fish tissue homogenate). The last group (two mice) was injected intraperitoneally with 3×10^9 CFU/ml of the *L. monocytogenes*. All of the mice were monitored daily for 2 weeks.

Histopathology

All dead mice were subjected to a thorough necropsy procedure. Tissue samples from the liver, spleen, brain, and heart were collected in 10% buffered formalin, dehydrated in alcohol, embedded in paraffin wax, cut at 5 microns (Microtome, Thermo Scientific, USA), and stained using hematoxylin and eosin stain. The stained sections were examined under a light microscope.

Ethical approval

Treatment and the maintenance of the animals were in accordance with the Jordan University of Science and Technology Animal Care and Use Committee which follows the international animal care and use guidelines (Institute of Laboratory Animal Resources (ILAR), 1996).

Results

The prevalence of *Listeria* spp. in fresh and frozen fish in Jordan are presented in Tables 3 and 4. A total of 72 *Listeria* isolates were obtained from 170 examined fish. Eighteen *L. monocytogenes* isolates were positively identified from fresh fish and six isolates from frozen fish. Additionally, 16, 9, and 10 *Listeria* species isolated from fresh fish, and other 5, 2, and 6 isolates from the frozen fish were diagnosed as *L. ivanovii*, *L. innocua*, and *L. grayi*, respectively.

Listeria monocytogenes were isolated and confirmed using PCR. Figure 1 shows bands corresponding to the 454 bp target *prfA* gene specific for the *L. monocytogenes*. Lanes representing *L. grayi*, *L. innocua*, and *L. ivanovii* were negative for this gene. In Figure 2, lanes 1–27 lanes (except lanes 5, 14, and 26) represent *L. monocytogenes* isolates with bands of 267 bp of the target *hlyA* gene. Lanes 5, 14, and 26 represent *L. grayi*, *L. innocua*, and *L. ivanovii*, respectively, which were negative for this gene.

Results of the serotyping of *L. monocytogenes* are presented in Figure 3 and Table 5. Fourteen isolates (58%) revealed bands of 483 bp that match the *Glt* primer band, which indicates that these isolates fall within the spectrum of 1/2b, 3b serotyping. The rest 10 (42%) isolates that were found negative with the *Glt* primer were run using the 268 bp *MAMA* primer. They all showed bands corresponding to that of the *MAMA* primer, indicating that these isolates fall within the 4a and 4c serotypes. Serotyping of the *L. monocytogenes* indicated that 14 isolates belonged to the 1/2b, 3b serotypes whereas 10 isolates belonged to the 4a and 4c serotypes.

Table 3. Number of *Listeria spp.* isolates from different portions of fresh and frozen fish in Jordan.

Fish status	Number samples	Fish portion	Number of isolates	Percentage
Fresh	140	Gills	51	37
		Intestines	0	0
		Scales and muscles	13	9
Frozen	30	Gills	6	20
		Intestines	0	0
		Scales and muscles	2	7
Total	170		72/510	14.1

Table 4. Number and percentages of *Listeria* species isolated from fresh and frozen fish in Jordan.

Fish status	Number of fish portions	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. grayi*
Fresh	420	18 (4.28%)	16 (3.8%)	9 (2.1%)	10 (2.4%)
Frozen	90	6 (6.6%)	5 (5.5%)	2 (2.2%)	6 (6.6%)
Total	510	24 (4.70%)	21 (4.1%)	11 (2.2%)	16 (3.1)%

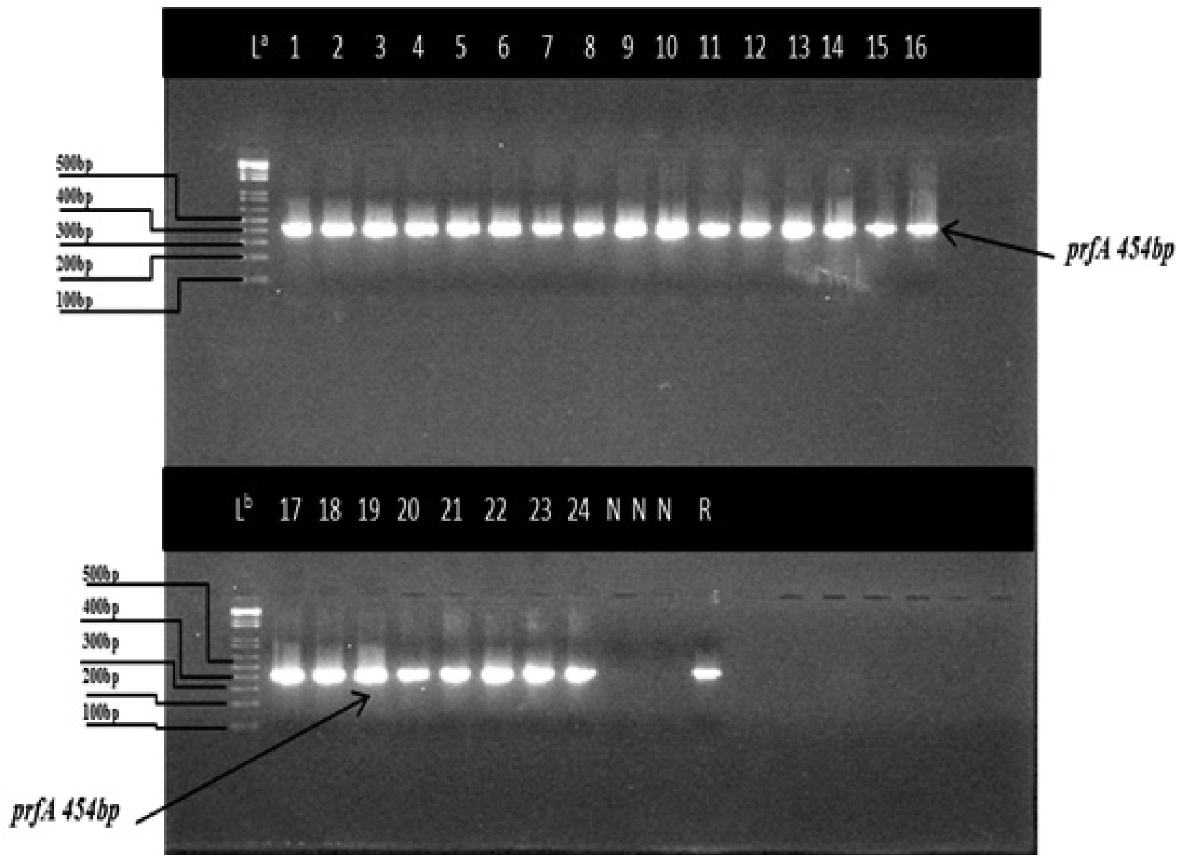

Fig. 1. Agarose gel electrophoresis (2%) image representing the *prfA* gene of *L. monocytogenes* isolates from fresh and frozen fish. Lanes: L^a and L^b: 100 bp molecular weight marker, Lanes: 1–24 prfA 454 bp specific for *L. monocytogenes*, Lanes N: no bands, negative for (*L. grayi*, *L. innocua*, and *L. ivanovii*), Lanes (R) positive reference strain (ATCC 1932).

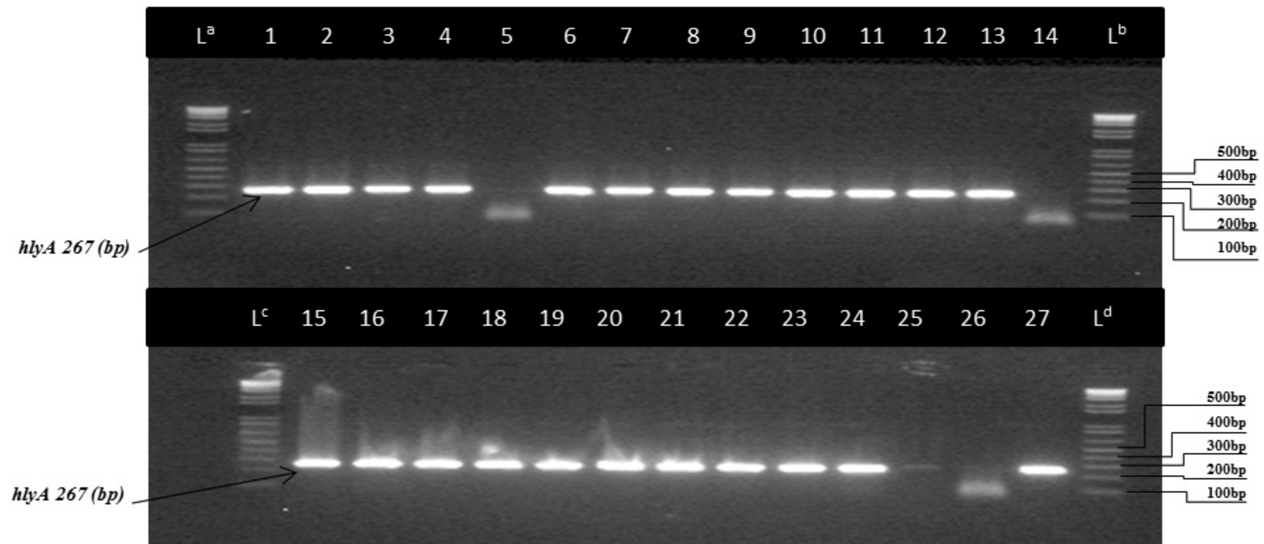

Fig. 2. Image of the nested PCR product on agarose gel electrophoresis (2%) targeting the *hlyA* gene of *L. monocytogenes* isolates from fresh and frozen fish. Lanes: (L[a], L[b], L[c], and L[d]): 100 bp molecular weight marker. Lanes:1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, and 27 represent *L. monocytogenes* while lanes 5, 14, and 26 represent *L. grayi, L. innocua*, and *L. ivanovii*, respectively.

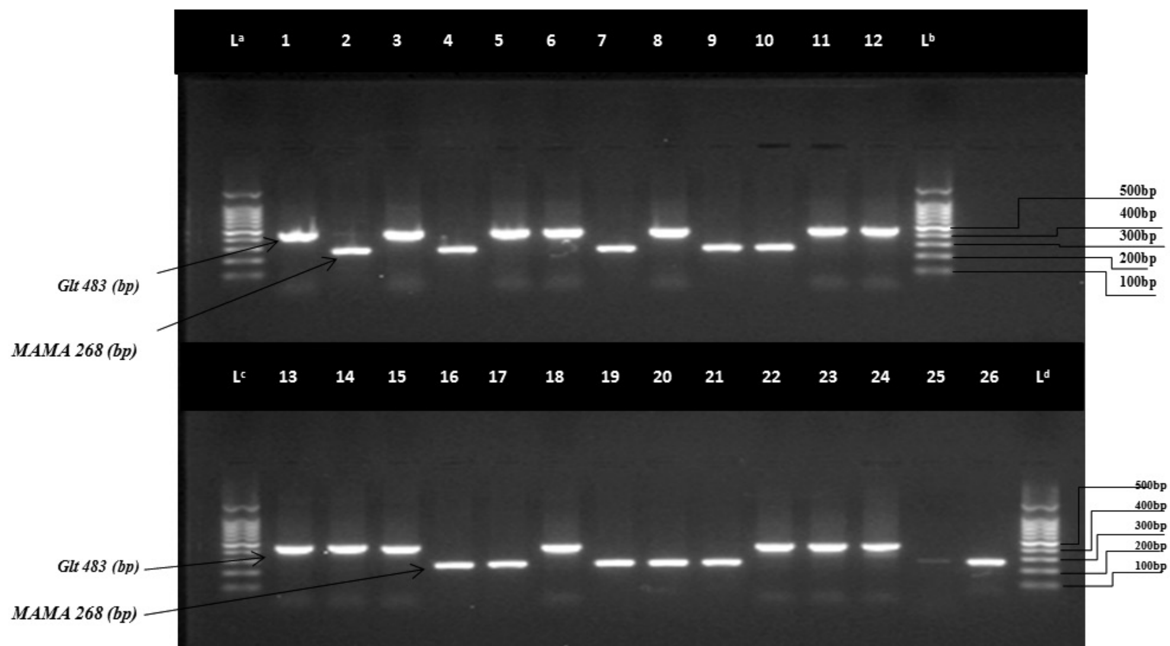

Fig. 3. PCR results of serotyping by use of *Glt* and *MAMA* primers. Agarose gel electrophoresis (1.5%) of *L. monocytogenes* isolated from fresh and frozen fish. Lanes: (L[a], L[b], L[c], and L[d]) 100 bp molecular weight markers. Lanes: 1, 3, 5, 6, 8, 11, 12, 13, 14, 15, 18, 22, 23, and 24 (+) product amplified by the primer sets of the amplicon size product 483 (bp) *Glt* gene for all *L. monocytogenes* isolates. Lanes: 2, 4, 7, 9, 10, 16, 17, 19, 20, 21, and 25 (+) product amplified by the primers sets of the amplicon size product 268 (bp) *MAMA* gene for all *L. monocytogenes* isolates. Lane 26: positive control for the (ATCC 19116) *MAMA* gene.

All *Listeria* isolates were used in the mouse lethality assay. Only 8 out of 24 *L. monocytogenes* isolates were lethal to mice within 3–6 days. Mice inoculated with other *Listeria* species (21 *L. ivanovii*, 11 *L. grayi*, and 16 *L. innocua*) survived the entire length of the experiment (14 days).

Eight isolates of *L. monocytogenes* were used to determine the LD_{50} in mice. The results showed that 0.5

Table 5. PCR serotyping of *L. monocytogenes* isolates (n = 24) from fresh and frozen fish in Jordan.

Primers	Lineages and serotypes	Number (%) of isolates
D1	DI/DIII (1/2b, 3b, 4b, 4d, 4e, 4a, 4c) (Lineage I and III)	24 (100%)
D2	DII (1/2a, 1/2c, 3a, 3c) (Lineage II)	0 (0%)
GLT	(+) (1/2b, 3b) (Lineage I)	14 (58.3%)
FlaA	(+) (1/2a, 3a) (Lineage II) (−) (1/2c, 3c) (Lineage II)	0 (0%)
MAMA	(+) (4a, 4c) (Lineage III) (−) (4b, 4d, 4e) (Lineage I)	10 (41.7%)

ml of 3×10^{10} CFU/ml of the reference strain resulted in 100% mortality while 3×10^{9} CFU/ml of the bacteria resulted in 50% mortality. The LD_{50} of the eight isolates were 3×10^{10}, $3 \times 10^{9.5}$, $3 \times 10^{7.5}$, 3×10^{9}, $3 \times 10^{9.5}$, $3 \times 10^{9.5}$, $3 \times 10^{7.5}$, and $3 \times 10^{8.5}$ CFU/ml.

In the *in vivo* pathogenicity assay, one isolate was found lethal after intraperitoneal inoculation without using the fish tissue as a vector. None of the two mice fed with sterile fish tissue homogenate had died whereas one out of the four mice fed with contaminated fish tissue had died. On the other side, the two mice that were inoculated intraperitoneally with sterile fish tissue survived over the entire period of the experiment, whereas the two mice that were inoculated intraperitoneally with contaminated fish tissue had died. All the virulent isolates were belonging to the serotype 1/2b.

Histopathologically, mice infected with LD_{50} of *L. monocytogenes* showed multiple areas of necrotic hepatocytes that are replaced with a large number of inflammatory cells as compared with normal liver. The spleen showed depletion and necrosis of lymphoid follicles as compared with the normal spleen. No lesions were seen in the brain.

Discussion

Jordan imports large quantities of fresh and frozen fish products (3,080.588 tons) for human consumption (Jordan Ministry of Agriculture (MOA) Annual Reports, 2008, 2010, 2013). Domestic fish production reached about 1,040 tons in 2010. About half of this production comes from farms in Jordan Valley. In 2008, about 160 tons were caught from the Gulf of Aqaba (Jordan Ministry of Agriculture (MOA) Annual Reports, 2008, 2010). In the last 3 years, fish consumption has increased as a reflection of a remarkable jump in the price of red meat (Jordan Ministry of Agriculture (MOA) Annual Reports, 2013). Although no outbreaks of listeriosis associated with fish consumption have been reported in Jordan, *Listeria spp*. exist in fish and fish products which may cause significant public health concerns. In fact, *L. monocytogenes* have previously been recovered from cheese, beef, and chickens in Jordan (El-Sukhon, 1993; Awaisheh, 2010). In this study, several species of *Listeria,* other than *L. monocytogenes* were recovered from fresh and frozen fish in Jordan for the first time that may need pathogenicity study.

In this study, 17% of sea fish samples collected from the fish selling markets in different locations were contaminated with *Listeria* 12–24 hours post fishing. This may reflect the unhygienic environment and the high climatic temperatures of the study locations. However, fresh fish samples originated from rivers and dams in Jordan and collected from fish selling markets showed a very high (77.5%) *Listeria* contamination. This may reflect water contamination of rivers and dams in Jordan (Fandi *et al*., 2009; Nwachukwu *et al*., 2010). In addition to transport factor and storing conditions of fish in dirty polyester boxes commonly used to store and display fish on the ground in the public markets, which increases the vulnerability to microbial contamination of fish. Circumstantial factors, such as handling, selling environment, and using unclean utensils and surfaces may influence the hygienic status of fresh fish and cross contamination after being in contact with raw foods that may increase *Listeria* contamination. These findings are similar to other studies where a high (81%) incidence of *Listeria* was recovered from freshwater fish than that (30%) in seawater fish (Nwachukwu *et al*., 2010).

In this study, *Listeria* contamination in the imported fresh sea fish samples was 22.5% and in the frozen fish was 26.6%. *Listeria monocytogenes* may naturally be present in the soil adjacent to the riverbanks as *Listeria* is frequently found in soil and water environments (Schaffter and Parriaux, 2002). The widespread of *L. monocytogenes* in soil has often been attributed to contamination from decaying plant and fecal material, with damp surface soil providing a cool, moist protective environment and the decaying material that act as substrate, which together enables the survival of *L. monocytogenes* from season to season (Gahan and Hill, 2014).

In this study, the incidence of *L. monocytogenes* was relatively high (22.5%) and (26.6%) in fresh and frozen fish, respectively. In Northern Spain, 25% of RTE smoked fish were contaminated with *L. monocytogenes* (Garrid *et al*., 2009). Variations in the rate of *Listeria* recovery from fish and fish products in different parts of the world are expected due to different methods used to collect samples, different standards in the handling

and hygiene of fish during storage, transportation, and market displaying conditions.

No previous studies to characterize and determine the pathogenicity of *L. monocytogenes* isolated from fish could be found in Jordan. The virulent strains of *L. monocytogenes* determined in this study were belonging to the 1/2b serotype. However, most virulent isolates from beef and poultry in Jordan belonged to the 1/2a, 1/2b, 1/2c, and 4b serotypes (Awaisheh, 2010). It was reported that serotype 1/2b is responsible for clinical listeriosis in humans (Heymann, 2008).

In the mouse lethality assay, seven *L. monocytogenes* isolates were lethal to mice with LD_{50} ranging between 3×10^{10} and $3 \times 10^{7.5}$ CFU/ml. Such diversity in LD_{50} values has been reported by others (Liu, 2004). The virulence and lethality assay results are in accordance with recorded serotyping results where the seven virulent isolates belonged to the serotype 1/2b. The serotypes 1/2b along with the 1/2a, and 4b serotypes are responsible for more than 96% of human listeriosis (Heymann, 2008). All the 24 *L. monocytogenes* isolates were found to belong to lineage I and III. Of those, 14 belonged to serotypes 1/2b, 3b, 7 of them were lethal to mice. These data raise an important alarm indicating potential human health hazard.

In the pathogenicity assay, none of the orally fed mice or intraperitoneally injected with sterile fish tissue or sterile fish tissue homogenate were died. While two of two mice injected intraperitoneally with fish tissue homogenate contaminated with 3×10^9 CFU/ml *L. monocytogenes* were died. Also, one of two mice injected intraperitoneally with 3×10^9 CFU/ml *L. monocytogenes* without fish tissue vector were died, and one of four mice orally fed with contaminated fish tissue was died. All these results confirm that *L. monocytogenes* are virulent and lethal to mice. This pathogenicity assay demonstrates the presence of virulent *L. monocytogenes* strains in fish in Jordanian markets. In Jordan, no standards on the allowable numbers of *L. monocytogenes* in seafood products are available. Therefore, the conduction of further studies becomes crucial to determine *L. monocytogenes* impact on public health, depending on international standards.

To control and reduce *L. monocytogenes* hazard in Jordan, strict implementation of the Hazard Analysis and Critical Control Point system and the Sanitation Standard Operation Procedure should be adopted at the retail level, fish markets, and storage conditions (Holah *et al.*, 2004). On the other hand, educating consumers and fish market dealers minimizes the risk of listeria infection (Buchanan *et al*., 2017; Kosa *et al*., 2007).

Conclusion

Virulent strains of *L. monocytogenes* and other strains of *Listeria* spp. have been recovered from fresh and frozen, local and imported fish in Jordan. Strict monitoring and quality control regulatory measures must be adopted to prevent future outbreaks of food poisoning associated with fish consumption.

Acknowledgements

The authors would like to thank the Deanship of Research at Jordan University of Science and Technology for their support.

Author contributions

YHT and SE conceived, designed, and supervised the project, performed analysis and/or interpretation of data. AA collected laboratory samples, conducted the practical part of the study, conducted biochemical analyses, and drafted the manuscript. ZBI carried out final writing and critical review/revision of the manuscript. YHT and SE had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

The authors declare that they have no conflicts of interests.

Funding

This work was supported by a grant from Deanship of Research at Jordan University of Science and Technology, Jordan (grant number 15/2012). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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