



Molecular characterization of *Listeria* species isolated from frozen fish

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Key words

Listeria
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Antibiotic
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ABSTRACT:

The objective of this study was to investigate the molecular characterization of *Listeria* species isolated from frozen raw fish. A total of 219 samples consisting of 104 mackerel, 52 horse mackerel, 51 catfish and 12 herring were collected and analyzed by bacteriological, serological, antimicrobial and molecular methods. Overall, 29(56.9%) and 1(0.96%) of catfish samples and mackerel samples respectively were positive for *Listeria* spp. No *Listeria* was detected in herring and horse mackerel. In catfish, *L. welshimeri* (13.7 %) was the most commonly isolated species followed by *L. monocytogenes* (11.8 %), *L. innocua* (9.8 %), *L. grayi* subsp. *murrayi* (9.8 %), *L. grayi* subsp. *grayi* (7.8 %), and *L. ivanovii* (3.9 %). In mackerel, only *L. monocytogenes* was detected in one sample. *L. monocytogenes* isolates serotyped as type 1 and type 4 (3 isolates each) and one non-typeable. Antimicrobial resistance profiling showed all *L. monocytogenes* isolates were resistant to ampicillin and tetracycline. Two were resistant to erythromycin. However, they were susceptible to rifampicin, vancomycin, chloramphenicol and streptomycin. Four virulence-associated genes (*prfA*, *hlyA*, *actA* and *inlA*) in addition to the genus gene (*prs*) were investigated using multiplex PCR. All the isolates were positive for *prs* gene but, only *L. monocytogenes* isolates were positive for all tested virulence genes. Our study indicates that imported raw catfish can represent a significant source of *L. monocytogenes* and potential health risk for listeriosis.

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1. INTRODUCTION

The genus *Listeria* consists of Gram-positive, facultative anaerobic, motile, non-spore forming bacteria (Wong and Freitag, 2004) and comprised of eight species: *Listeria monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria grayi*, *Listeria marthii* and *Listeria rocourtiae* (Graves et al., 2010; Leclercq et al., 2010; Schmid et al., 2005). Although *L. ivanovii* and *L. monocytogenes* are mammalian pathogens, *L. ivanovii* appears to be rare and predominantly causes disease in ruminants (Guillet et al., 2010). *L. monocytogenes* is most commonly associated with disease in both animals and humans. Pregnant women, neonates,

elderly, or immunocompromised people are particularly susceptible to *Listeria* which manifests as abortion, stillbirth, septicemia, meningitis and meningoencephalitis (WHO, 2004). Almost all cases of human listeriosis are due to *L. monocytogenes*; very rare infections due to *Listeria ivanovii* and *Listeria seeligeri* have been described (Wagner and McLauchlin, 2008). *L. monocytogenes* is also the major pathogen of listeriosis in animals, although approximately 10% of septicaemia in sheep caused by *L. ivanovii* (Wagner and McLauchlin, 2008). According to FoodNet US, listeriosis was responsible for 30% of foodborne deaths from 1996 to 2005 and had a high case fatality rate of 16.9% (Barton et al., 2011). According to epidemiological data,

the minimal infectious dose for listeriosis is at least 100 CFU/g of food (Ooi and Lorber, 2005; Yang et al., 2007). Infection can also be caused by a prolonged daily consumption of food containing 10^1 - 10^5 CFU/g of *L. monocytogenes* (Maijala et al., 2001).

L. monocytogenes is psychrotrophic and halotolerant (Seeliger and Jones, 1986) and can, under otherwise optimal conditions, grow in the range of 1 to 45 °C. It can survive or even grow at pH values as low as 4.4 and at salt concentrations of up to 14%. As a consequence, it has been isolated from a variety of sources, including fish and fishery products (de Simón et al., 1992; Els et al., 2004; Gudmundsdóttir et al., 2005; Karunasagar and Karunasagar, 2000; Nakamura et al., 2004). Considerable contamination occurs during evisceration, fish handling and packing as a result of inadequate hygiene (Katzav et al., 2006). Frozen fish either the raw fish or the smoked product, particularly fatty fish such as herring, mackerel and horse mackerel, offers a good protection and that reduction of numbers of *L. monocytogenes* due to freezing is marginal. As a result, strict legislation governs both the detection limits and permissible levels of *L. monocytogenes* in ready-to-eat (RTE) foods. The U.S. Food and Drug Administration (FDA) maintains a zero-tolerance policy (Engel et al., 1990; FDA, 2003), while in Europe, legislation (no. 2073/2005) imposes a zero-tolerance policy in respect to certain foods destined for high-risk consumer groups and otherwise limits these bacteria to below 100 CFU/g (European Commission, 2005).

Given that not all *L. monocytogenes* strains have an equal capacity to cause disease, the application of stringent zero-tolerance policies to all *L. monocytogenes* strains is questionable (Clayton et al., 2011). Serotyping has been used to

characterize *L. monocytogenes* that differs in their virulence and pathogenicity (Doumith et al., 2004b; Liu, 2006). Currently, 13 serotypes of *L. monocytogenes* have been described; three of them (1/2a, 1/2b and 4b) have been isolated in more than 90% of the cases from human and animal listeriosis (Gianfranceschi et al., 2009; Orsi et al., 2011). *L. monocytogenes* consists of at least four evolutionary lineages (I, II, III, and IV) with different but overlapping ecological niches. Most *L. monocytogenes* isolates belong to lineages I and II, which include the serotypes most commonly associated with human clinical cases, such as serotypes 1/2a (lineage II), and 1/2b and 4b (lineage I). Lineage II strains are common in foods and widespread in the natural and farm environments, and are often isolated from animal listeriosis cases and sporadic human cases. However, most human listeriosis outbreaks are associated with lineage I isolates. Lineage III and IV strains are rare and predominantly isolated from animal sources (Doumith et al., 2004b; Orsi et al., 2011; Ragon et al., 2008; Shen et al., 2013).

L. monocytogenes is varied in its susceptibility to antimicrobial drugs and influenced by antimicrobial use to humans and animals, as well as the geographical differences (Carpentier and Courvalin, 1999; Nwachukwu et al., 2010; Soni et al., 2013). Drug-sensitive strains of *L. monocytogenes* were isolated from clinical and food samples by Dhanashree et al. (2003). While, Sharma et al. (2012) reported multidrug-resistance strains from milk samples. The antimicrobial resistance of *L. monocytogenes* may be associated with the presence of a plasmid or may be determined by genes that are transferred by conjugation and mutational events in chromosomal genes (Harakeh et al., 2009; Poros-Gluchowska and Markiewicz, 2003). The widespread distribution of epidemiologically important

serotypes of *L. monocytogenes* and their resistance to commonly used antibiotics indicate a potential public health risk. The situation assumed a monitoring of antimicrobial resistance (AMR) is extremely important to decide the proper treatment of listeriosis.

Isolation, identification and characterization of *L. monocytogenes* rely on traditional culture methods and several polymerase chain reaction (PCR)-based techniques (Adzitey and Huda, 2010). Reliable detection of pathogenic *L. monocytogenes* has been suggested to be ideally based upon the detection of virulence markers such as internalins (*inlA*, *inlC*, *inlJ*), listeriolysin O (*hlyA*), actin polymerization protein (*actA*), phosphatidyl-inositol-phospholipase C (*plcA*), invasive associated protein (*iap*) and virulence regulator (*prfA*) which have been reported for their importance in *L. monocytogenes* infection and its pathogenesis (Liu et al., 2007; Vazquez-Boland et al., 2001). Therefore, detection of just one virulence-associated gene by PCR is not always sufficient to identify *L. monocytogenes* (Di Ciccio et al., 2012)). In addition, some *L. monocytogenes* strain may lack one or more virulence determinants because of spontaneous mutations (Clayton et al., 2011; Cotter et al., 2008; Shen et al., 2013; Soni et al., 2013). So, simultaneous detection of virulent genes in a single step reduces time and shall be useful in a large scale survey for detecting virulent strain of *Listeria*. Multiplex PCR (MPCR) involving detection of different foodborne pathogens has evolved as an area of great potential and could become a viable alternative to the conventional methods for detection of *L. monocytogenes* (Chen and Knabel, 2007; Jadhav et al., 2012; Kerouanton et al., 2010; Kim et al., 2007 and Touron et al., 2005). Egypt is a net importer of fish and fishery products in spite of the rise of fish production for the last few years. However, to the best of our knowledge, no published information

regarding the presence of *L. monocytogenes* in imported fish to Egypt is available. Due to the lack of information about contamination rates of *Listeria* in mackerel, horse mackerel, catfish and herring fish in Egypt and the risk of getting listeriosis from consuming these types, we run this investigation to determine the incidence of *L. monocytogenes* and to how extent listeriosis can occur. To achieve these objectives, the study of serotyping, antimicrobial resistance and virulence associated genes (*prfA*, *hlyA*, *actA* and *inlA*) of *L. monocytogenes* isolates from imported frozen fish was carried out.

2. MATERIALS AND METHODS

2.1. Collection of fish samples

A total of 219 imported frozen raw fish samples consisting of 104 mackerel, 52 horse mackerel, 51 catfish and 12 herring were used in this study. All samples were dissected and put in sterile stomacher bags and assigned an identification code in order to maintain a database of the isolates then, taken immediately in ice boxes to the laboratory of microbiology department, Faculty of Veterinary Medicine, Alexandria University and preserved at -20°C until further analysis. Fish gills, skin and flesh were the principal tissues of investigation. However, care was taken to include other tissues or whole organisms consistent with Egyptian dietary habits.

2.2. Isolation and identification of *Listeria* spp.

Two-step method for enrichment of *L. monocytogenes* and other *Listeria* spp. was performed in accordance to the EN ISO 11290-1/A1:2004 standard. Briefly, samples were incubated in half-Fraser broth (Oxoid) at 30°C for 24 h. Subsequently, 0.1 ml was transferred to a new tube containing 10 ml of full-strength Fraser broth and incubated at 37°C for 48 h. Positive enrichment cultures (broth

darkening) were streaked onto Oxford (Oxoid) or PALCAM (Oxoid) agar. The plates were incubated under microaerophilic conditions at 37°C for 24-48 h. 2 to 3 presumptive colonies on Oxford and/or PALCAM agar (typical gray greenish colonies with black sunken centers as shown in Fig. 1A and 1B) were isolated and purified by re-streaking on ALOA agar (Agosti & Ottaviani *Listeria* Agar; LabM, Lancashire, UK), followed by submitting to Gram staining, Catalase test, CAMP test with *Staphylococcus aureus*, motility test at 20–25°C, carbohydrate utilization. Positive colonies

on ALOA (typical green – blue colored colonies with and without a distinctive opaque halo as shown in Fig. 1C and 1D) and other tests were further confirmed using the Microbact 12L kit carried out according to the manufacturer recommendations (MB1128A, Oxoid). Single pure isolated colonies were grown overnight in tryptic soy broth (TSB) and stored in cryovials containing 25% glycerol at –20°C. As needed, stocks were thawed and subcultured on tryptone soya yeast extract agar ((TSA-YE; Oxoid) before use.

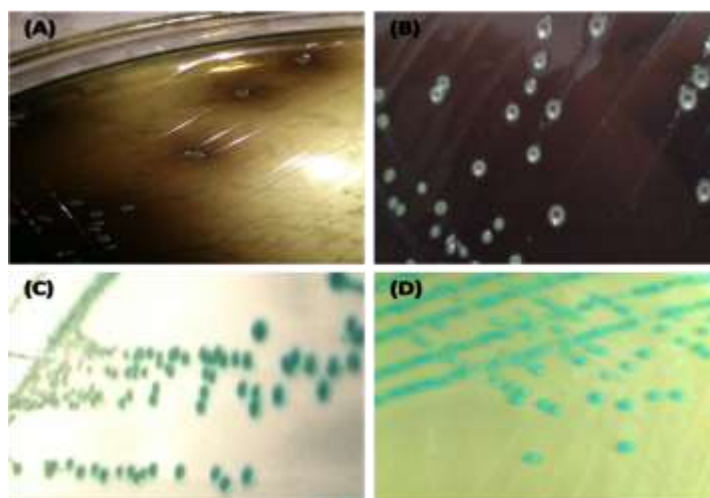


Figure 1. *Listeria* spp. cultured on different selective agar media. A: Colonies of suspected *Listeria* spp. on Oxford agar media, B: Colonies of suspected *Listeria* spp. on PALCAM agar media, C: Colonies of suspected *L. monocytogenes* or *L. ivanovii* on ALOA agar, D: Colonies of suspected *Listeria* spp. other than *L. monocytogenes* and *L. ivanovii* on ALOA agar.

2.3. Serology of *L. monocytogenes*

Listeria O antiserum types 1 and 4 (Difco Laboratories) were used for serological identification of *L. monocytogenes* by using the slide agglutination test according to the manufacturer's instructions.

2.4. Antimicrobial resistance test of *L. monocytogenes*

The sensitivities of *L. monocytogenes* isolates and resistant variants to a variety of antibiotics were assayed by standard

disc-diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009). The filter discs (Bioanalyse) with the following concentrations were used: ampicillin (10 ug), chloramphenicol (30 ug), erythromycin (15 ug), gentamicin (10 ug), rifampicin (5 ug), streptomycin (10 ug) and tetracycline (30 ug). These antibiotics were selected to include antimicrobials used in the veterinary and human therapy. All the isolates of *L. monocytogenes* were revived in TSA-YE agar for 24 h at 37°C (Yücel et al., 2005).

3 to 5 colonies from TSA plate were transferred into 5-ml Mueller-Hinton Broth (MHB; Oxoid) until its turbidity was equivalent to the turbidity of the 0.5 McFarland standards. The suspension was streaked uniformly onto Mueller Hinton Agar (MHA; Oxoid) with sterile swabs. The filter discs were then dispensed onto the agar using the antibiotic disc dispenser. For each antibiotic, at least three independent disk diffusion assays were performed. The plates were incubated at 37°C for 24 h. To maintain quality control of performance and reliability to the results, the standard strain of *Staphylococcus aureus* ATCC 25923 was used. The diameter of growth inhibitory zone was measured and interpreted according to the CLSI (2009).

2.5. DNA extraction of *L. monocytogenes*

DNA extraction was performed using the boiled-cell method (Chai et al., 2007), that modified by (Marian et al., 2012). Briefly, presumptive colonies were cultured in 6 ml tubes containing *Listeria* enrichment broth and incubated at 37 °C for 24-48 h. The tubes were centrifuged at 10,000 rpm for 2 min to pellet the cells. Harvested cells were washed with 500 ul of sterile distilled water and vortexed to re-suspend the pellets. Tubes were boiled at 100°C for 10 min and allowed to cool at - 20 °C for 5 min, followed by a centrifugation at 12,000 rpm for another 5 min. The clear supernatant was ready to be used as template DNA in PCR assay.

2.6. Detection of virulence genes of *L. monocytogenes* by MPCR

The presence of four virulence associated genes of pathogenic *L. monocytogenes* (*prfA*, *hlyA*, *actA* and *inlA*) was investigated using MPCR. The *prs* gene, specific for strains of the genus *Listeria* (Doumith et al. 2004a) was included to confirm the genus in all tested isolates.

The target genes, oligonucleotide primers (Bioneer, Korea) and expected product sizes are listed in Table 1. *L. monocytogenes* ATCC 7644 was used as a positive control. *L. monocytogenes* (seven isolates) and other *Listeria* spp. (one isolate randomly selected from each *L. welshimeri*, *L. ivanovii*, *L. innocua*, *L. grayi* subsp. *grayi* and *L. grayi* subsp. *murrayi* isolates) were examined for the presence of genus and virulence associated genes. A PCR mixture with primers, but with no added DNA, was used as a negative control. Two sets of MPCR with the same temperature cycling parameters were conducted for *L. monocytogenes* and other *Listeria* spp. respectively. The amplification was carried out in a 50 µL reaction mixture containing 10 µL of 5× PCR buffer (50 mM NaCl, 50 mM Tris-HCl, pH 9.0); 250 µM each deoxynucleotide triphosphate; 2 mM MgCl₂; and 1 U of GoTaq Flexi DNA polymerase (Promega, U.S.). Each primer was added at the final concentrations of 15 pmol. 5 µL of template DNA was added to each reaction mixture. The final volume of the reaction mixture was adjusted to 50 µL with sterile deionized distilled water. Amplification of DNA segment was performed in a thermal cycler (Applied Biosystems, U.S.) with the following temperature cycling parameters: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 2 min, primer extension at 72 °C for 1 min and a final extension at 72 °C for 15 min. 10 µL of each amplified product was loaded in 1.3% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089M Trisbase, 0.089M boric acid, and 0.002 M EDTA (pH 8.0)] at 100 volts for 40 min. After electrophoresis, amplification products were detected by visualization of the bands under UV transilluminator (Bio-Rad, Hercules, CA, U.S.) after staining with ethidium bromide. A 100-DNA ladder (Bioron, Ludwigshafen, Germany) was

used as a molecular marker to indicate the size of the amplicons.

Table 1: List of the primers employed in this study

Primer name	Sequence (5'→3')	Target gene amplicon (bp)	Ref.
PrsF	GCT GAA GAG ATT GCG AAA GAA G	Prs	370
PrsR	CAA AGA AAC CTT GGA TTT GCG G		Doumith et al., 2004
hlyAF	CCT AAG ACG CCA ATC GAA	hlyA	702
hlyAR	AAG CGC TTG CAA CTG CTC		Ciccio et al., 2012
actAF	GAC GAA AAT CCC GAA GTG AA	actA	268
actAR	CTA GCG AAG GTG CTG TTT CC		Ciccio et al., 2012
prfAF	CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG	prfA	1060
prfAR	AGC AAC CTC GGA ACC ATA TAC TAA CTC		Ciccio et al., 2012
inlAF	CCT AGC AGG TCT AAC CGC AC	inlA	255
inlAR	TCG CTA ATT TGG TTA TGC CC		Ciccio et al., 2012

2.7. Statistical analysis

The Chi-squared (χ^2) was used to determine significant differences among the different variables. A probability value of less than 0.0001 was considered statistically significant.

3. RESULT

3.1. Incidence of *Listeria spp.* in frozen imported fish

Overall, 29(56.9%) and 1(0.96%) of catfish samples and mackerel samples respectively were positive for *Listeria spp.* (Fig. 2). No *Listeria* was detected in herring and horse mackerel. In catfish, *L. welshimeri* (13.7 %) was the most commonly isolated species followed by *L. monocytogenes* (11.8 %), *L. innocua* (9.8 %), *L. grayi subsp. murrayi* (9.8 %), *L. grayi subsp. grayi* (7.8 %), and *L. ivanovii* (3.9 %). However, in mackerel, only *L. monocytogenes* was detected in one sample.

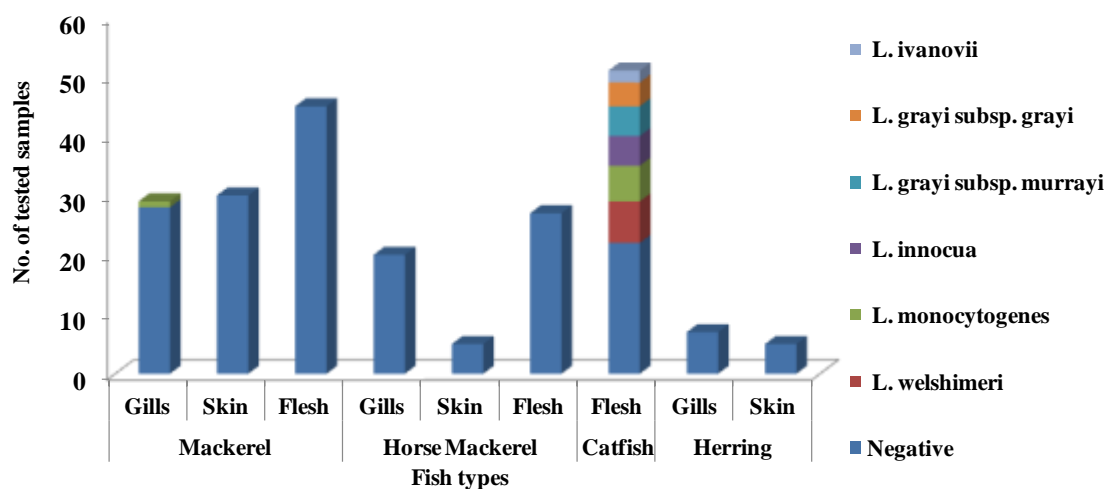


Figure 2. Incidence of *Listeria spp.* in imported frozen Fish.

3.2. *L. monocytogenes* Serotyping

The seven *L. monocytogenes* isolates were serologically typed as follows: 3 isolates type 1; 3 isolates type 4 and 1 isolate was non-typeable. The 6 serotypes were isolated from catfish, but the non-typeable one was isolated from mackerel.

3.3. Antimicrobial resistance profile

AMR profiling (Fig. 3) showed all *L. Monocytogenes* isolates were resistant to ampicillin and tetracycline. Two isolates were resistant to erythromycin. However, they were susceptible to rifampicin, vancomycin, chloramphenicol and streptomycin.

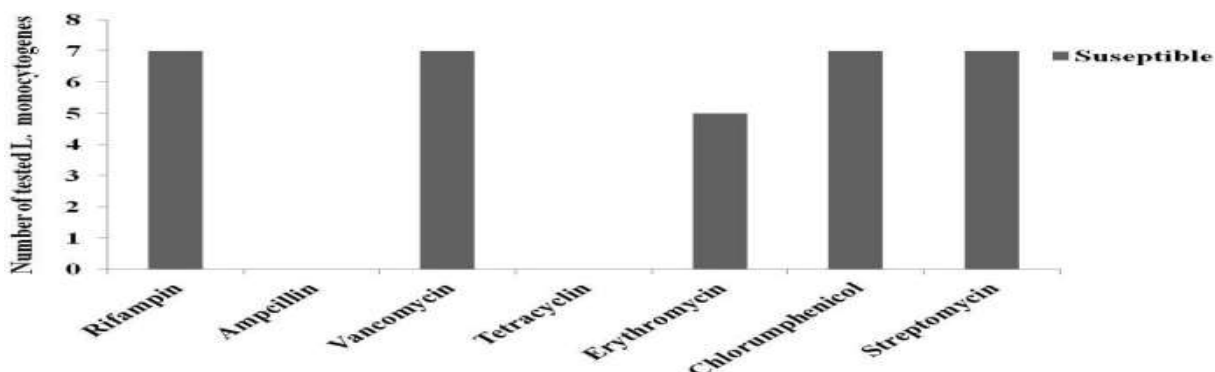


Figure 3. Antimicrobial susceptibility test of *L. monocytogenes*. All *L. monocytogenes* isolates were resistant to ampicillin and tetracycline. Two isolates were resistant to erythromycin. However, they were susceptible to rifampicin, vancomycin, chloramphenicol and streptomycin.

3.4. Virulence associated genes

The distribution of virulence genes in *L. monocytogenes* and other *Listeria* spp. is shown in **fig. 4**. All the isolates, irrespective of the species were positive

for *prfA* gene confirming the genus in all the isolates studied. All *L. monocytogenes* isolates were positive for *prfA*, *hlyA*, *actA* and *inlA* genes. On the other hand, all other *Listeria* spp. isolates were negative for these virulence genes.

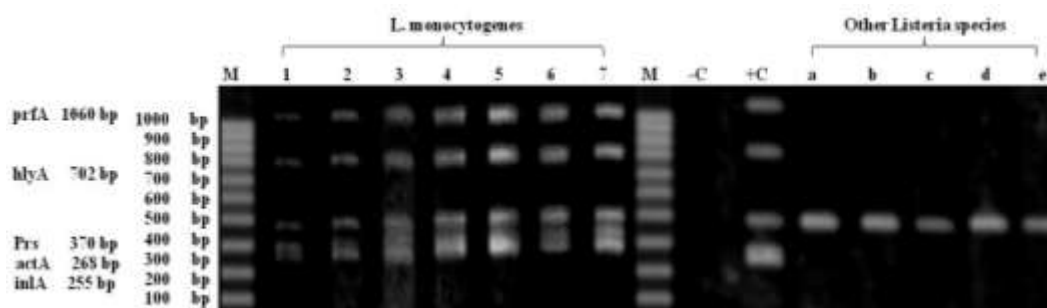


Figure 4. Agarose gel electrophoresis of DNA fragments generated by multiplex PCR with *L. monocytogenes* and other *Listeria* spp. M, DNA molecular weight marker (100 bp); **Lanes 1 to 7**, *L. monocytogenes* strains; **Lanes -C**, Negative control; **Lanes +C**, Positive control; **Lanes a to e**, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. grayi sub grayi* and *L. grayi sub murayi* respectively. Genes corresponding to the amplified fragments are indicated on the left. Molecular sizes are given (in base pairs) at the left.

4. DISCUSSION

Although *L. monocytogenes* is found ubiquitously in the environment, it is an emerging bacterial foodborne pathogen and represents a major public health concern for consumers, the food industry, and regulatory agencies. Listeriosis results in an estimated 1600 cases per year and accounts for approximately 19% of all foodborne disease-related deaths in the U.S. (Scallan et al., 2011). In 2011, a multistate listeriosis outbreak associated with cantaloupe caused 146 invasive illnesses, one miscarriage and 29 deaths. It was one of the deadliest foodborne-disease outbreaks in the U.S. (Laksanalamai et al., 2012). Based on the reported fatality rates and the total numbers of reported confirmed cases, it is estimated that in the European Union in 2009, there were approximately 270 human deaths due to listeriosis (EFSA, 2011 and 2012). In Egypt, recent studies reported a high prevalence of *L. monocytogenes* in patients with liver cirrhosis associated with ascites (El sayed et al., 2011) and in food (El-malek et al., 2010; El-shabacy, 2008; Zaki et al., 2011). In the present study, 29(56.9%) and 1(0.96%) of catfish samples and mackerel samples respectively were positive for *Listeria spp.* (Fig. 2). In catfish, *L. welshimeri* (13.7 %) was the most commonly isolated species followed by *L. monocytogenes* (11.8 %), *L. innocua* (9.8 %), *L. grayi subsp. murrayi* (9.8 %), *L. grayi subsp. grayi* (7.8 %), and *L. ivanovii* (3.9 %). However, in mackerel, only *L. monocytogenes* was detected in one sample. No *Listeria* was detected in herring and horse mackerel. Such a high occurrence of *L. monocytogenes* and other *Listeria spp.* in frozen catfish indicates the inadequacy of current intervention strategies for *L. monocytogenes* control (Kozempel et al., 2001; Silva et al., 2003; Soni et al., 2009). Our findings are consistent with the observations of other researchers who reported high prevalence of *L. monocytogenes* in catfish. A study by Chou et al. (2006) showed that 25% to

47% of fresh channel catfish were contaminated with *L. monocytogenes*. Furthermore, another recent study by Pao et al. (2008) reported that *L. monocytogenes* was present in 23.5% of catfish that was obtained from various retail stores. The difference in the isolation rate of *L. monocytogenes* could be partly due to the sizes of samples, the sampling season, and the isolation methods (Hansen et al., 2006). The 7 *L. monocytogenes* isolates were serologically typed as 3 type 1; 3 type 4 and 1 isolate was non typeable. The 6 serotypes were isolated from catfish while the non-typeable one was isolated from mackerel. Our results indicate that frozen catfish is a predominant type that can be contaminated with *L. monocytogenes* and other *Listeria spp.*, presumably owing to the aquaculture setting or processing and handling under unhygienic conditions. The presence of *L. monocytogenes* in these samples cannot be neglected, and we have not to underestimate the risk of listeriosis, due to the consumption of this type of food. Although there are no documented cases of listeriosis associated with the consumption of cooked catfish (Soni et al., 2009), potential risks remain for that type of food due to a high prevalence of *L. monocytogenes*. Further, there is a possibility for other foods to become cross contaminated by *L. monocytogenes* from catfish.

Although, the *Listeria spp.* are thought to be naturally susceptible to antibiotics active against Gram-positive bacteria (Rota et al., 1996; Teuber, 1999; Yücel et al., 2005), *L. monocytogenes* has been reported to be resistant to a number of these antibiotics, including ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nail-dixic acid, suphamethoxazole/trimethoprim, and tetracycline (Charpentier and Courvalin, 1999; Ennaji et al., 2008; Rahimi et al., 2010). The frequency of using antibiotics as supplements in fish feed, as growth

promoters and for therapeutic treatments has contributed to the increasing emergence of multidrug-resistant bacteria (Adzitey, 2011; Charpentier and Courvalin, 1999). In the present study, AMR profiling (Fig. 3) showed all *L. monocytogenes* isolates resistant to ampicillin and tetracycline. Two isolates were resistant to erythromycin. These results might be in part due to the excessive use of these antibiotics in catfish aquaculture. Other sources may also contribute to the evolution of antibiotic-resistance include runoffs from farms, feces from animals and workers in catfish processing lines, which carry antibiotics and antibiotic-resistant micro-organisms (An Expert Report, Funded by the IFT Foundation 2006). Presence of ampicillin and tetracycline resistant strains of *L. monocytogenes* in imported raw catfish could cause major public health concerns as each of ampicillin and tetracycline is often the first choice of drug used in Egypt for the treatment of human and animal infections respectively. All *L. monocytogenes* isolates were susceptible to rifampicin, vancomycin, chloramphenicol and streptomycin. Ennaji et al. (2008) reported that *L. monocytogenes* was resistant to cefotaxime (100%) nalidixic acid (100%), cephalothin (10%), and susceptible to ampicillin, chloramphenicol and gentamicin. More recent studies have provided consistent evidence that resistance of *L. monocytogenes* to antimicrobial drugs is substantially increased, but geographically varies in its prevalence. The differences in resistance rates can be influenced by the country involved and regulations for the use of antibiotics, farming and processing practices, and the type of samples. Ruiz-Bolivar et al., (2011) found the susceptibility of *L. monocytogenes* was 100% for ampicillin, amoxicillin/clavulanic acid, vancomycin and chloramphenicol, whereas for trimethoprim/sulfamethoxazole, azithromycin, erythromycin, tetracycline, penicillin,

ciprofloxacin, rifampin, meropenem and clindamycin, it was 98%, 96%, 91%, 82%, 97.8%, 84.4%, 64.4%, 71.1% and 22.2% respectively. In contrast, Soni et al. (2013) reported that all isolates were resistant to ampicillin, except two isolates, and showed variable resistance to gentamicin, cotrimoxazole, ofloxacin, rifampicin and tetracycline. Therefore, we recommend that the use of antibiotics in fish production should be reduced or maintained in order to prevent the incidence of increasing multidrug-resistant *L. monocytogenes* associated with fish. Attention also needs to be focused on monitoring the antimicrobial resistance of *L. monocytogenes* in humans and animals to understand changes in the patterns of resistance to commonly used antimicrobials, to implement pro-active measures to control the use of antimicrobial agents and to prevent the spread of multi-drug resistant strains, which can have many undesired consequences (Conter et al., 2009; Elseisy et al., 2010; Mahmoud et al., 2013; Mousa et al., 2010).

L. monocytogenes has been successfully confirmed, typed and/or characterized using PCR assay, random amplification of polymorphic deoxyribonucleic acid (RAPD), repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) by several authors (Blatter et al., 2010; Ferreira et al., 2011; Jersek et al., 1999; Keeratipibul and Techaruwichit, 2012; Osaili et al., 2011). The choice of the target gene is important for detection of virulent strains of *L. monocytogenes* by PCR. However, it is plausible that some *L. monocytogenes* strain may lack one or more virulence determinants because of some mutations (Cooray et al., 1994). Previous studies have also revealed that using PCR assay for detection of single virulence associated gene is neither sufficient to identify the *L. monocytogenes* isolates nor to reveal its true pathogenic potential as majority of *L. monocytogenes* isolates showed different gene profiles (Rawool et

al., 2007; Shakuntala et al., 2006). MPCR is a variation of the traditional PCR in which multiple sets of primers are used in a single reaction to amplify multiple genes of interest simultaneously. The MPCR assay has the potential to be used in routine diagnostic laboratories and also might be very useful as a rapid screening tool in food testing laboratories to quickly identify food samples. Bubert et al. (1999) have developed MPCR to differentiate *Listeria spp.* using 5 primer pairs designed based on *iap* gene that is common to all *Listeria spp.* However, they failed to discriminate *L. ivanovii*, *L. seeligeri* and *L. welshimeri* simultaneously. Also, Doumith et al. (2004a) have discriminated total 222 *L. monocytogenes* strains, including nine serovars isolated from foods and patients into four groups by MPCR. Ryu et al. (2013) developed a MPCR for detection of six *Listeria spp.* including *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri*. They utilized one *Listeria* genus specific and each *Listeria spp.* specific primer pairs. One more specific aim of the present study was to assess the virulence potential of *L. monocytogenes* isolated from various imported frozen fish. The isolates were analyzed for the presence of virulence associated genes employing the MPCR. Fig. 4 shows the MPCR amplifications obtained with all isolated *L. monocytogenes* (seven isolates) and other *Listeria spp.* (one isolate from each *L. welshimeri*, *L. ivanovii*, *L. innocua*, *L. grayi subsp. grayi* and *L. grayi subsp. murrayi* isolates). All isolates, irrespective of the species amplified the *prfA* gene fragment (370 bp) confirming the genus in all the isolates studied. The MPCR profile in the present study did not distinguish, within the *L. monocytogenes* isolates, serotype 1 and/ or serotype 4 from non-typeable isolate. However, the amplifications of the four chosen virulence genes allowed separation of *L. monocytogenes* isolates from all other *Listeria spp.* All *L. monocytogenes*

isolates were positive for *prfA*, *hlyA*, *actA* and *inlA* genes. This result indicates that these isolates possess the properties of virulent strain and their sequences may be further investigated to explore the differences between pathogenic and less pathogenic strains. On the other hand, non of other *Listeria spp.* isolates amplified these tested virulence genes, which can be explained as the genetic information of these genes in other *Listeria spp.* could be divergent from that of *L. monocytogenes* (Gerstel et al., 1996; Haas et al., 1992; Kreft et al., 1989; Mengaud et al., 1988).

In conclusion, to the best of the author's knowledge, this is the first description of antibiotic resistance and the virulence genes of *L. monocytogenes* in imported frozen fish to Egypt. The study indicates that catfish can represent a significant source of *L. monocytogenes*, and particular attention should be paid to this type of fish food. Unfortunately, our isolates were resistant to ampicillin, which is the most common antibiotic used in the treatment of human infections in Egypt, suggesting that handling of contaminated catfish, cross-contamination, or eating raw or undercooked represent a potential danger to public health, especially in immunosuppressed individuals, elderly people, and children. Egypt, like most countries, applied a zero-tolerance policy regarding the presence of *L. monocytogenes* in food. Therefore, sanitation standard operating procedure (SSOP), controls for aquaculture drugs, good manufacturing practice (GMP) and hazard analysis and critical control points (HACCPs) programs shall all be implemented in the fish industry to prevent fish products from being contaminated with foodborne pathogens, including *L. monocytogenes* during handling and processing. Moreover, proper storage of raw fish products and avoiding cross-contamination during handling at the retail levels also helps to minimize risk of human infection

associated with RTE products. Furthermore, there is a great need for a surveillance program in Egypt to monitor epidemiological information on *L. monocytogenes* diffusion in different sources.

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