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Virulence and antimicrobial susceptibility profile of *Listeria monocytogenes* isolated from frozen vegetables available in the Egyptian market

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Listeria monocytogenes **is among the most important foodborne pathogens. It may enter food-processing environments through raw materials, handlers or equipment and may persist due to ineffective cleaning or sanitation. The bacterium can be isolated from both frozen vegetables and fresh food substances. This study aimed to estimate the prevalence of** ***L. monocytogenes*** **in spices and frozen vegetables and screen for some virulence factors and drug-resistance determinants of the isolated bacteria. First, conventional microbiological methods were used for the isolation and identification of bacteria. Next, the identity of isolated bacteria was confirmed by molecular techniques, and the virulence genes** ***iap*** **and** ***hlyA*** **were identified by real-time polymerase chain reaction (PCR). The hemolytic activity of the isolates was assessed by cultivation on sheep blood agar. Furthermore, the antimicrobial susceptibility of confirmed** ***L. monocytogenes*** **isolates was tested by the disk diffusion method against 10 antibiotics. Out of 331 vegetable samples, 47 isolates were confirmed to contain** ***L. monocytogenes*****, whereas none of 40 spice samples tested positive. All isolates were positive for** ***iap*** **and** ***hlyA*** **genes. Susceptibility testing indicated that all isolates were sensitive to trimethoprim/ sulfamethoxazole, but only 36% were sensitive to penicillin G, while 100% and 70% showed intermediate resistance to chloramphenicol and erythromycin, respectively. All tested isolates were resistant to amoxicillin, gentamicin and norfloxacin; on the other hand, 90, 86 and 84% of the tested strains were resistant to ciprofloxacin, ceftazidime/clavulanic acid and amikacin, respectively. In summary,** ***L. monocytogenes*** **isolates disseminated in frozen vegetable samples from the Egyptian market were highly virulent, entirely multiple-drug resistant and were enriched in iron-containing vegetables. Since** ***L. monocytogenes*** **is primarily pathogenic to humans and causes a life-threatening disease, there is a potential infection risk for people who usually deal with frozen vegetables before cooking. Hence, surveillance to** ***L. monocytogenes*** **in frozen products, together with implementation of tight measures would be valuable in preventing listeriosis, and are highly recommended.**

Key words: *Listeria monocytogenes,* virulence gene, antibiotic resistance.

INTRODUCTION

Bacteria of the genus *Listeria* are Gram-positive, facultative anaerobic and non-spore forming bacilli (Wong and Freitag, 2004). The genus is represented by eight major species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, *Listeria grayi*, *Listeria seeligeri, Listeria ivanovii, Listeria marthii* and *Listeria rocourtiae*; recently (Weller et al., 2015) added new species are *Listeria booriae* and *Listeria newyorkensis.* The most medically relevant species, *L. monocytogenes,* is classified into 13 serotypes. Serotypes 1/2a, 1/2b, 1/2c and 4b strains are associated with human infections (Graves et al., 2010; Leclercq et al., 2010). Almost all major outbreaks of invasive listeriosis are due to serotype 4b strains (Salcedo et al., 2003). The ability of these bacteria to survive and grow over a wide range of environmental conditions, including high salt concentration, refrigeration temperature, and low pH, which makes them a potential hazard in foods (Ryser and Marth, 2007) and the ability of *L. monocytogenes* to persist in the environment is due to their capacity to form biofilms (Colagiorgi et al., 2016). This organism is a recognized foodborne pathogenic bacterium that causes many diseases, from mild gastroenteritis to severe blood and/or central nervous system infections, as well as abortion in pregnant women. Many studies have detected *L. monocytogenes* in fresh product samples and even in some minimally processed vegetables (Lopez, 2008; Zhu et al., 2017). However, *L. ivanovii* and *L. seeligeri* have been also rarely associated with disease in humans (Lopez, 2008). Listeriosis was responsible for 30% of foodborne deaths from 1996 to 2005 and had a high case fatality rate of 16.9% according to Food Net US (Barton et al., 2011). *L. monocytogenes* expresses a highly conserved pore-forming toxin known as listeriolysin O (LLO). LLO is a member of a large family of cholesterol-dependent cytolysins (CDCs) found in several bacterial pathogens (e.g., streptolysin O of *Streptococcus pyogenes* and alveolysin of *Bacillus alvei*). It is the primary virulence factor in *L. monocytogenes* and is essential for its pathogenesis (Tweten, 2005; Cossart et al., 1989). The entire infection cycle of *L. monocytogenes* is governed by multiple proteins, such as internalin A and internalin B (encoded by *inlA* and *inlB*), hemolysin (encoded by *hly*), phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by *plcA*), phosphatidylcholine-specific phospholipase C (PC-PLC, encoded by *plcB)* and actin polymerization protein (encoded by *actA*) (Jaradat et al., 2002). *L. monocytogenes* is susceptible to many antibiotics; but multi-drug resistant isolates have been reported (Jaradat et al., 2002). Listeria species are generally susceptible to a wide range of antimicrobials, but the first multi resistant *L. monocytogenes* strain has been isolated in 1988. Since then, antibiotic-resistant *L. monocytogenes* isolates have been recovered from food, environment, and human listeriosis cases (Soni et al., 2014). Currently, a β-lactam antibiotic (e.g., ampicillin or penicillin) combined with an aminoglycoside (for example, gentamicin) is the reference therapy for human listeriosis, while the second choice of treatment is a combination of vancomycin, erythromycin and trimethoprim-sulfamethoxazole for pregnant women or patients allergic to β-lactams (Hof, 2004).

This study aimed to estimate the prevalence of *L. monocytogens* in spices and frozen vegetables, and screen for some virulence factors and drug-resistance determinants of the isolated bacteria.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Forty spices and 331 frozen food samples (45 okra, 16 carrot, 20 green beans, 57 artichoke, 36 Molokia, 8 spinach, 11 green peas, 61 strawberry, 11 grape leaves, 2 broad bean, 4 broccoli, 14 grape, 2 peach, 29 salad, 6 mixed vegetables, 7 pomegranates and 2 cauliflowers) were collected from the Egyptian market. *L. monocytogenes* was isolated according to the ISO 11290 method (ISO 11290, Technical committee ISO iTC 34, Food products).

Twenty-five grams of each food sample was weighed and mixed with 225 ml of half Fraser primary enrichment medium. The mix was incubated at 30 ±1°C for 24 ± 2 h. 0.1 ml of primary enrichment was transferred to a tube containing 10 ml Fraser broth. Then, this inoculated medium was incubated at 37°C for 4 ± 2 h. From the primary enrichment culture, a loopful (10 µl) was inoculated on the surface of Listeria Agar according to Ottaviani and Agosti medium (MERCK) (Ottaviani et al., 1997) and chromogenic listeria agar medium (OXOID) and were observed for typical *L. monocyogenes* colonies. The identity of the isolated colonies was further confirmed biochemically following the Microbact 12L scheme (Table 1).

Molecular identification of *L. monocytogenes* and detection of virulence genes

Real-time PCR was used to identify *Listeria* genus. DNA was extracted by Prep Man® Ultra according to manufacturer's protocol. Ten microliters of the supernatant was transferred to a new tube containing 90 µl of ultra-pure water, and then vortexd. The mixture was used as a DNA template for PCR. Real-time PCR mixture solution was prepared Using Promag™ custom kit (PROMAGA GMBH, Berlin, Germany) according to manufacturer procedure and then added into PIKO 96-well PCR (Thermo Fisher Scientific, Vantaa, Finland). Primers and probes used for the detection of *hlyA* and *iap* genes are listed in Table 2.

Hemolytic activity assay

Haemolysin was detected by culturing *L. monocytogenes* isolates on blood agar base supplemented with 5% defibrinated sheep blood. Blood agar plates were then incubated at 37°C for 24 h. Colonies producing clear zones of haemolysis were classified according to zone diameter of haemolysis as strong, intermediate and weak (ISO 11290-1-(2014).

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Table 1. Substrates and reactions in Microbact 12L system used to identify *Listeria monocytogenes*.

Well no.	Designation	Reaction principle	Negative	Positive
1	Esculin	Hydrolysis of Esculin	Yellow	Black
2	Mannitol			
3	Xylose			
4	Arabitol			
5	Ribose			
6	Rhamnose			
7	Trehalose	Utilization of specific sugars resulting in the production of acidic end products	Purple	Yellow
8	Tagatose			
9	Glucode-1-phosphate			
10	Methyl-D-glucose			
11	Methyl-D-mannose			
12	Haemolysis	Haemolysis of red blood cells	Red cell deposit	Clear zone

Table 2. Primers used in RTi-PCR assays for *L. monocytogenes* (Rodriguez et al., 2004).

Name	Target gene	Type	Sequence
hlyQF		Forward primer	5'-CAT GGC ACC ACC AGC ATC T-3'
hlyQR	*hlyA*	Reverse primer	5'-ATC CGC GTG TTT CTT TTC GA-3'
hlyQP		TaqMan_ Probe	5'-FAM-CGC CTG CAA GTC CTA AGA CGC CA-TAMRA-3'
iapQFa		Forward primer	5'-AAT CTG TTA GCG CAA CTT GGT TAA-3'
iapQRa	*iap*	Reverse primer	5'-CAC CTT TGA TGG ACG TAA TAA TAC TGT T-3'
iapQP		TaqMan probe	5'-FAM-CAA CAC CAG CGC CAC TAC GGA CG-TAMRA-3'

Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by the Kirby-Bauer disc diffusion method (Bauer et al., 1966) as previously recommended by the National Committee for Clinical and Laboratory Standards (NCCLS, 2012). Four to five colonies were picked up from overnight cultures; then, a loopful was inoculated into sterile TSB (about 3-4 ml/tube), incubated for 2 to 4 h. The culture turbidity was adjusted to 0.5 McFarland (equal to 0.08 – 1 absorbance at wavelength 624 nm). Using a sterile cotton swab, the bacterial broth culture was streaked on Muller Hinton agar surface. The inoculum was left to dry for 3 to 5 min. Discs were placed individually on the agar surface with sterile forceps and then gently pressed down onto agar surface to provide uniform contact. Plates were allowed to diffuse for 2 h in a refrigerator then incubated at 37 ± 2°C for 18 to 24 h. The susceptibility of the *Listeria* isolates was detected by a clear zone around the discs. Results were interpreted according to the standardized interpretive chart by NCCLS (NCCLS, 2012). The antibiotics used were as follows: Penicillin G (PG 10), trimethoprim 1.25 µg + sulfamethaxazole 23.75 µg (TS25), erythromycin (E15), ciprofloxacin (CIP5), Amoxicillin (AML10), amikacin (AK30), norofloxacin (NOR 10 µg), gentamycin (GM 200 µg), ceftazidime + clavulanic acid (CAL40) and chloramphenicol (C30) (MAST Diagnostics-UK).

RESULTS

Distribution of *L. monocytogenes* in tested food samples

When forty spice samples and 331 frozen samples were examined for *L. monocytogenes,* 47 out of the 331 vegetable samples (14.2%) were positive for the presence of *L. monocytogenes* (Figure 1), while none of the spice samples were positive.

Haemolytic activity and frequency of virulence genes among *L. monocytogenes* isolates

All 47 *L. monocytogenes* isolates were PCR-positive for *iap* and *hlyA* genes. *L. monocytogenes* isolates showing haemolytic activity were classified according to their potency as shown in Figure 2.

Antimicrobial susceptibility of the *L. monocytogenes* isolates

The *in vitro* susceptibility of 47 *L. monocytogenes* strains isolated from different kinds of foods was tested against 10 antibiotics. All tested strains were sensitive to Trimethoprim/Sulfamethoxazole, while 36% of tested strains were sensitive to Penicillin G. Moreover, 100 and 70% of the samples showed intermediate resistance to Chloramphenicol and Erythromycin, respectively. All tested strains were resistant to Amoxicillin, Gentamicin and Norfloxacin, while 90, 86, 84% of tested strains were

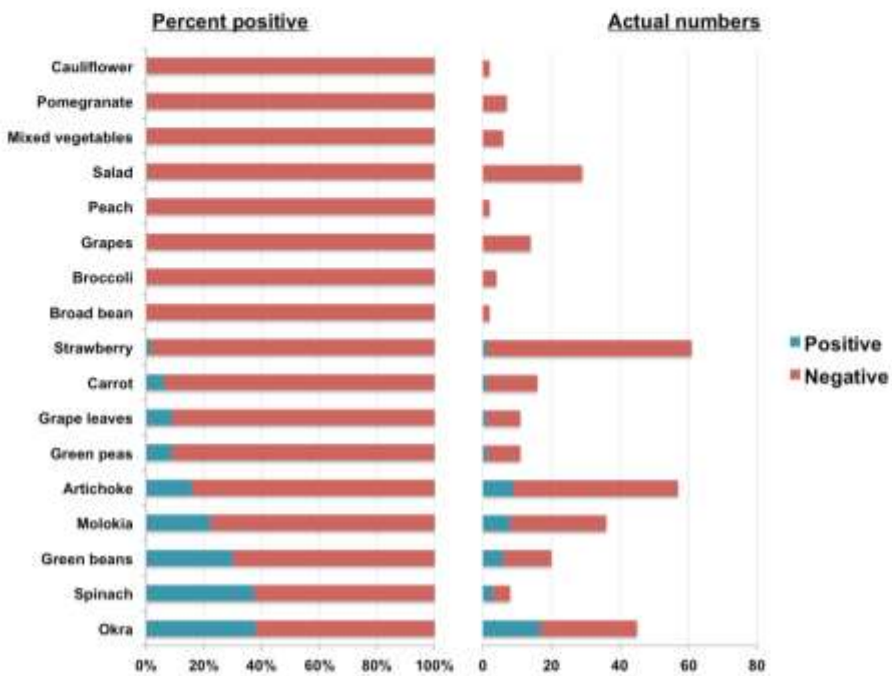

Figure 1. Distribution of isolated *L. monocytogenes* among vegetable samples.

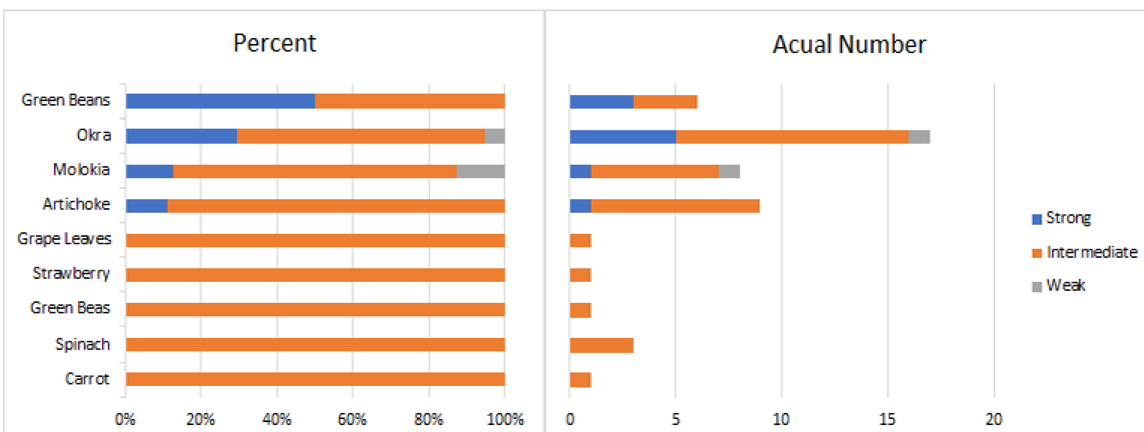

Figure 2. Hemolytic activity among *L. monocytogens* isolates.

resistant to Ciprofloxacin, Ceftazidime/clavulanic acid and Amikacin, respectively.

Statistical analysis

Chi-square tests were used to determine significant trends in the data. First, it was obvious from the culture results that *L. monocytogenes* cannot be isolated from spices (0% in 40 spices samples as opposed 14.2% of 331 frozen food samples).

Among the food samples, however, a clear over representation of *L. monocytogenes* was observed in okra, spinach, and artichoke with $p < 0.05$ which indicates statistically significant relationship between the categorical variables.

DISCUSSION

This study aimed to isolate *L. monocytogenes* from different kinds of spices and frozen vegetables. Overall, 40 spices and 331 vegetable samples were examined for the presence of *L. monocytogenes*. It was found that 47

(14.2%) samples out of 331 (17 okra,1 carrot, 6 green beans, 9 artichoke, 8 molokia, 3 spinach, 1 green peas, 1 strawberry and 1 grape leaves) were positive for *L. monocytogenes*. Meanwhile, surprisingly none of the spice samples showed any positive results for the pathogen. The absence of *Listeria* in spices may suggest a potential antimicrobial activity of these spices, and this will need confirmation in further studies. Even though reports on the sensitivity of *L. monocytogenes* to spices such as ginger, finger-root and turmeric were studied (Thongson et al., 2005), the current search for *L. monocytogenes* in spices was based on recent reports of detection of a number of food pathogens including *L. monocytogenes* in spices and herbs (Thongson et al., 2005; Kara et al., 2015).

Previous studies among the analyzed categories showed variation in occurrence of *L. monocytogenes*. For instance, Byrne et al. (2016) studied the occurrence and antimicrobial resistance patterns of listeria isolated from vegetables in Brazil and found that 3% of the samples were contaminated with *L. monocytogenes*, including 2% raw vegetables and 5.5% ready-to-eat vegetables. They confirmed the virulence potential of the isolates and antimicrobial susceptibility, revealing 50% of the isolates were susceptible to antibiotics (Byrne et al., 2016).

In Uruguay, on the other hand, 11.2% of different food samples were positive for *L. monocytogenes.* The highest percentage was among frozen food samples (38%) followed by cheese (10%). The same study discussed the serotype distribution among the samples and concluded on the prevalence of serotype 1/2b and 4b. These results highlight the role that frozen foods may play in the spread of this pathogen (Braga et al., 2017).

Moreover, the prevalence of *L. monocytogenes* in frozen burger patties was studied by Wong et al. (2012) in Malaysia. *L. monocytogenes* was detected in 33% of the chicken burger patties, 22.9% of the beef patties and 10% of fish patties; their results suggest that burger acts as a potential source of listeriosis if adequate cooking is not involved.

Finally, the prevalence of *Listeria* species in fresh and frozen fish and shrimp was studies in Iran by Rahimi et al. (2012). *Listeria* species were isolated in 7.5, 4.2, 11.7 and 6.6% of fresh fish, frozen fish, fresh shrimp and frozen shrimp, respectively. Almost 2% of identified species were *L. monocytogenes* which led to the conclusion that consumption of sea food either raw or frozen may lead to food borne illnesses in Iran (Rahimi et al., 2012).

L. monocytogenes detected in this study were positive for both *iap* and *hlyA* genes. Isolates showing haemolytic activity were classified according to their degree of heamolysis, into strong, intermediate and weak. Previous studies reported isolates positive for the virulence genes *inlA, inlB, prfA, iap, actA, plcB* and *hlyA*; their results suggest that all *L. monocytogenes* isolates have the potential to cause listeriosis in humans (Xiaolong et al., 2017). Various genes such as *hlyA* and *iap* genes have been targeted for detection of *L. monocytogenes* using PCR (Aznar et al., 2003). Pulsed field gel electrophoresis (PFGE) methodology is recommended in the identification protocol to identify the food implicated in an outbreak which is considered a key point for public health.

From previous reports, it is evident that differences in prevalence of *L. monocytogenes* in different types of food reflect the effect of geographical location, demography, and food type and hygiene standards among other factors. Food containing only spices or high levels of them, like Indian food, almost lack *L. monocyotogenes* (Suriyapriya et al., 2016). As indicated above, none of our 40 spice samples collected from the Egyptian market contained *Listeria*, agreeing with what was found in Indian spicy food (Suriyapriya et al., 2016).

Susceptibility testing results (Figure 3) indicates that all tested strains were multi drug resistant as they were resistant to amoxicillin, gentamicin and norfloxacin. Moreover, 90, 86 and 84% of the tested strains are resistant to ciprofloxacin, ceftazidime + clavulanic acid and amikacin, respectively.

In previous studies, all *L. monocytogenes* isolates were sensitive to most of the commonly used antibiotics, such as ampicillin, penicillin G and vancomycin. However, some multidrug-resistant *L. monocytogenes* isolates had been reported, which were resistant to ampicillin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole or rifampin. For example, a *L. monocytogenes* strain isolated from a meningoencephalitis patient was resistant to chloramphenicol, erythromycin, streptomycin and tetracycline (Charpentier et al., 1999).

These antibiotics have been increasingly used as supplements in animal feed, as growth promoters and for the treatment of human disease (Adzitey et al., 2013). Some common antibiotics, such as ampicillin, that are commonly used to treat clinical listeriosis, represent a high drug resistance phenomenon in *L. monocytogenes* strains. In recent years, with extensive use and abuse of antibiotics, multi-drug resistant strains have been detected from a variety of food samples (Ling et al., 2006). These findings confirmed that the prevalence of antibiotic resistance in *L. monocytogenes* might be increasing (Chen et al., 2014).

Conclusion

The findings of this study revealed a relatively high prevalence of virulent *L. monocytogenes* in frozen food in Egypt, which could potentially cause human disease. Thus, it is necessary to take precautions in the food factories, and periodical inspection must be performed on frozen food, which would be valuable to prevent human infection during consumption of this kind of food. All isolates recovered in this study were multi-drug resistant to most available antimicrobial agents, which represents

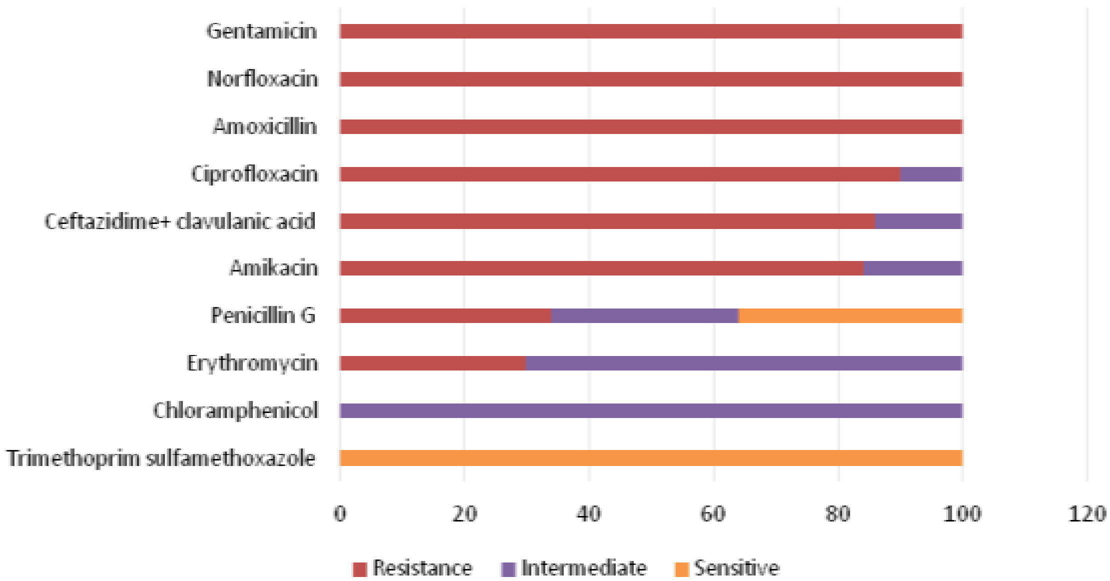

Figure 3. Percentage of sensitivity and resistance against 47 *L. monocytogenes* isolates.

a public health concern; thus, searching for alternatives is required.

This study is a full-scale, systematic investigation of the prevalence of *L. monocytogenes* in frozen foods in Egypt and the contamination of these foods, and it provides baseline information for Egyptian regulatory authorities to allow the formulation of a regulatory framework for controlling *L. monocytogenes* and to improve the microbiological safety of frozen foods.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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