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RESEARCH ARTICLE

OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN SOME MARINE FRESH AND FROZEN FISH MARKETED IN DAMIETTA, EGYPT

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ABSTRACT

The objectives of this study were to determine the incidence of *Listeria* spp. and *Listeria monocytogenes* isolated from two types of imported frozen and fresh locally marine fish samples obtained from fish markets in Damietta, Egypt. A total of 400 fish samples, comprising 100 samples each of fresh *Saurus,* fresh Sardines*,* frozen *Saurus* and frozen Sardines, were collected from different public fish markets at Damietta Governorate. *Listeria* isolation was performed according to FDA, (2011) protocols. Standard microbiological techniques were confirmed to *L. monocytogenes* by PCRAmplification of a fragment of *hyla* gene. The present data revealed that the overall incidence of *Listeria* spp. in was (13.75%), whereas the overall incidence of *L. monocytogenes* was (4.5%). *Listeria* spp. were isolated from fresh *Saurus* fish, fresh Sardine*s*, frozen *Saurus* and frozen Sardine*s* as 25(25%), 16(16%), 8(8%), 6(6%), respectively. The highest prevalence (25%) of *Listeria* spp. were observed in fresh *Saurus*, while the lowest prevalence was detected in frozen Sardine*s* (6%). Ten isolates (10%) from fresh *Saurus* fish samples were confirmed to *L. monocytogenes*, while *L. Monocytogenes* was not isolated from frozen Sardines. The highest incidence of *L. monocytogenes* was in Kafr Saad 7/128 (5.4%) and Damietta city 9/44 (5.11%). Serovar determination of *L. monocytogenes*18 isolates revealed that the serovar 1/2a was the predominant *L. monocytogenes* serovar in the samples tested in the present study. It was isolated from the examined fresh fish and frozen *Saurus* while Serovar 1/2c was isolated from fresh Sardine only and Serovar 4b was isolated from fresh *Saurus* only. In conclusion, this study showed that *L.monocytogenes* were common contaminant of fish obtained from Damietta fish markets, and this may pose serious public health implications.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that is distributed in a variety of environments. Listeria monocytogenes can survive in a wide range of pH, temperature and osmolarity conditions (Liu *et al.,* 2005). *L. monocytogenes*is commonly distributed throughout the environment such as soil, cultivated and uncultivated fields, feeding grounds, wild life, faeces of animals and birds. Moreover, the bacterium has also been isolated from different kinds of fish, squid and crustaceans (Miettinen and Wirtanen, 2005). *L.monocytogenes* has emerged as a significant food borne zoonosis in recent decades. It can cause a serious food borne illness called listeriosis which is an atypical foodborne disease with a high fatality rate, ranging from 25 to 30% in susceptible populations. Pathogenic infections by *L.monocytogenes* usually affect individuals predisposed through an underlying disease affecting the immune system, such as cancer or AIDS, and also other susceptible persons such as the elderly, pregnant women and newborn babies.. Symptoms of the disease are flu-like in normal people, yet may result in severe complications in immune compromised people, such as meningitis, septicaemia, abortion, and new born listerios is (Wing and Gregory, 2000, Laer *et al.,* 2009). *L. monocytogenes* is divided into at least 12 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7). The virulence of *L. monocytogenes* appears to be serotype dependent with serotypes 1/2a, 1/2c, 1/2b and 4b being found in 98% of recorded human listerios is cases. The 4a and 4c serotypes are rarely associated with outbreaks of disease although isolated frequently from a variety of food and environmental samples

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(Wiedmann *et al.,* 1997). *Listeria* spp. are components of the indigenous micro flora in surface water and other water bodies connected to rivers. Therefore, *L. monocytogenes* are most likely found on the external surface of fish that swim in contaminated water. *L. monocytogenes* has been observed on the fish surface and in the stomach, gills, and intestines, but the flesh is commonly free of *Listeria* unless it has been contaminated from different sources. *L. monocytogenes* is prevalent in raw fresh fish in several countries, but the rate of contamination ends to be low and varies between 0% and approximately 30% of the fish products (Miettinen and Wirtanen 2005). Food safety issues are of critical concern to society, governments, and industry.

Recent preventative and control measures have led to a substantial reduction in the frequency of listerios is, but listerios is remains a majorhealth issue. Several studies have observed significant strain/serotype heterogeneity in the virulence and pathogen city of *L. monocytogenes* strains. Therefore, improving the discriminatory capacity of current sub typing methodologies will enable tracking and elimination of contamination sources in the food industry. The virulence potential of a representative set of *L. monocytogenes* strains should be examined (FDA, 2011 b). The present study was undertaken to investigate the incidence of *Listeria* spp. and *L. monocytogenes* that isolated from fresh and imported frozen fish samples collected from retail fish markets in three localities in Damietta governorate, Egypt.

MATERIAL AND METHODS

Sampling area

Damietta Governorate is located in the northeastern part Egypt. Its capital is the city of Damietta city. It is located at the point where the Nile meets the Mediterranean Sea. The total area of Damietta Governorate is 910,30km^2, equivalent to about 0.10% of Egypt's total area and its population density is 1.40 per km^2.The governorate is divided into five cities: Damietta, El-Zarka, Faraskor, Kafrsaad, Kafr Al-Buteekh.

Sample collections

A total of 400 fish samples were collected as following:100 samples from each fresh and frozen *Saurus* (*Synodus saurus*) and fresh and frozen Sardines (*Sardina pilchardus*) in the period between March 2014 and November 2015.All fresh samples were caught locally, where as all frozen samples were imported. Fish samples in the first third of their shelf life were collected randomly from different fish markets in the early morning from three main cities of Damietta Governorate that were Damietta city (176), Kafr Saad (128) and Kafr Al-Buteekh (96).

Each sample consisted of a number of fishes weighting100 grams were separately collected, identified and labelled in sterile bags to avoid further contamination and transferred in an ice box as rapid as possible to Damietta sea port laboratory for food inspection. Fresh samples were immediately examined and frozen samples stored at -20^o C until examination.

Sampling, Isolation and Identification of *L. monocytogenes*

Listeria isolation was performed according to the protocols of FDA (2011a). The tissues were homogenized and analysed together as one sample. Each composite tissue sample was prepared by aseptically sampling (removing of apiece of skin surface by using surface quarterisation by flame) 15 grams from 5 fish portion proportionate of the same spp. and similar size. Twenty five grams of composite sample were taken from muscles of nape portion under aseptic conditions, and then transferred into a sterile blender jar (Stomacher lab. Blender 400, seward lab. Serial no 30469 type BA 7021 London) 225 ml buffered *Listeria* Enrichment Broth (BLEB) (Oxoid, CM 897), supplemented by (*Listeria* selective enrichment supplement, Oxoid, SR141) were aseptically added, then the blender was operated to give 3000 r.p.m. for not more than 2.5 minutes, then incubated for 24-48 h at 30°C. Enriched samples were streaked on to modified Oxford agar plate (Oxoid, CM 856) with listeria selective supplement (Oxoid, SR 140), and incubated at 35ºC for 24-48 h. *Listeria* spp. appeared as small black, grey or brown colonies that were surrounded by a black zone on modified Oxford agar. After 48 h, they become darker with a hollow black centre andsurrounded by black zones. Colonies suspected to be *L. monocytogenes* were identified according to (Margolles *et al.,* 2000) by Gram's stain, tumbling motility, V.P, Catalase, oxidase, haemolysis of sheep blood agar and CAMP test.

Biochemical tests

The isolated *listeria* spp. was identified following the protocols of by Gram's staining, motility test and biochemical tests. Biochemical tests including Indole, methyl red, Vogus Proskauer, citrate test, catalase, oxidase, TSI, nitrate reduction, carbohydrate fermentation test of mannitol, rhamnose and xylose and bile escul in hydrolysis test were performed using the isolated spp.of bacteria. Three typical single colonies were streaked onto Tryptone Soya Yeast Extract Agar (TSYEA) (*Merck, Germany*), incubated at 35ºC for 24 h, and submitted to biochemical identification to *Listeria* species.

Motility test

Colonies were examined on semi-solid medium for tumbling motility at 22ºc (typical umbrella motility).

Haemolytic activity

L.monocytogens strain to produce haemolys in was tested on blood agar supplemented with 5% sheep blood with anticoagulant. All the selected cultures were seeded in blood agar plates, and the plates were incubated at 37C for 24 hours. Haemolytic activity was determined.

The Christie-Atkins-Munch-Peterson (CAMP) test

Synergistic lyses of erythrocytes (CAMP reaction) were performed following the procedures of (Kerr and Lacey, 1991). Strain of *S.aureus* was inoculated on sheep blood agar. The suspected colony was inoculated perpendicular to *S.aureus* incubated at 37ºc for 24 hours. Positive results for

L.monocytogenes indicated by a typical haemolytic zone were occurred at the junction of the two inoculums as a half circle.

Serotyping of *L. monocytogenes*isolates

The detected *L. monocytogenes* isolates were serotyped using the commercially prepared *Listeria* antisera against somatic (O) and flagellar (H) antigens according to the manufacturer (Denka-Seiken Co. Ltd., Tokyo, Japan).

DNA extraction and PCR examination

In order to confirm the isolates to the *L. monocytogenes* sp., 161bp fragment of *hyla*gene was amplified using PCR reactions. DNA was extracted from the cultured pure colonies using boiling method described by (Bansal *et al.,* 1996). Briefly, a single colony of bacteria was cultured in 5-6 ml *Listeria* enrichment broth for 24-48 h. Three millilitres of grown bacteria were centrifuged for 5 min at 10,000g. Bacterial pellets were washed once with 1 ml phosphate buffered saline pH 7.4, re-suspended in a 200 ul of distilled water and boiled in a water bath for 10 min. The clear supernatants obtained after 5 min centrifugation at 12000 g were used for PCR reaction. For PCR amplification two primers (Forward: TTA CGA ATT AAA AAG GAG CG and Reverse: TTA AAT CAG CAG GGG TCT TT) found in downstream of *hyla* gene and targeting a fragment of 161bp was employed as described by (Moon *et al.*, 2004). Amplification reactions were performed in a final volume of 25 µl of the 2X Master mix (Jena, Germany), containing 2.5 units of Taq DNA Polymerase in reaction buffer, 4 mM MgCl2, 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and about 100 ng of extracted DNA as template. Amplification was performed in Mastercycler (Eppendorf, Germany) with an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 40 s, 53°C for 75 s, and 72°C for 75 s, and one final cycle of 72°C for 7 min. Ten ul of the amplified mixture were separated on 1.5% agarose gel in a TBE buffer. The PCR product was visualized by ethidium bromide staining in Gel documentation system.

RESULTS

Phenotypic characteristics of *Listeria* spp. and *L. monocytogenes* spp. isolates

Results revealed that *Listeria*Isolates including *L. monocytogenes* isolates showed typical characteristics by microbiological tests. *L monocytogenes* isolates showed positivity for CAMP test and motile and haemolytic activities. In addition, *L. monocytogenes* isolates were further confirmed by PCR amplification of 161 pb of the downstream of *hyla* gene.

Incidence of isolated *Listeria*spp. and *L. monocytogenes*

The present data tabulated in Table (1) revealed that the overall incidence of *Listeria*spp. in was 55/400 (13.75%), whereas the overall incidence of *L. monocytogenes* was 18/400 (4.5%). The incidence of *Listeria* contamination of *Saurus* fish was much higher than Sardine fish collected from markets. *L. monocytogenes* was detected in 10% in fresh *Saurus*, 2% in frozen *Saurus* matched with 6% in fresh Sardine while *L. monocytogenes* cannot be detected in frozen Sardines.

Table 1. Incidence of total isolated *Listeria* spp. and *L. monocytogenes* among fresh and frozen marine fish in Damietta Governorate

Fish samples (No =100 each)	*Listeria* species	*L. monocytogenes*
	No. (%)	No. (%)
Fresh *Saurus*	25 (25%)	10 (10%)
Sardines	16 (16%)	6 (6%)
Frozen *Saurus*	8(8%)	2(2%)
Sardines	6(6%)	0(0%)
Total No=400	55/400 (13.75%)	18/400 (4.5%)

Table 2. Incidence of isolated *Listeria* spp. from different fish markets in Damietta governorate

Fish samples	Damietta city No= 176	KafrSaad No=128	Kafr Al-Buteekh No=96
	No. (%) (each 44)	No. (%) (each 32)	No. (%) (each 24)
Fresh *Saurus*	13/44 (29.5%)	8/32 (25%)	4/24 (16%)
Fresh Sardines	7/44 (15.9%)	5/32 (15.6%)	4/24 (16%)
Frozen *Saurus*	4/44 (9%)	4/32 (12.5%)	0 (0%)
Frozen Sardines	3/44 (6.8%)	3/32 (9.3%)	0 (0%)
Total	27/176 (15.3%)	24/128 (18.7%)	7/96 (7.2%)

Table 3. Incidence of isolated *L. monocytogenes* from different fish markets in Damietta governorate

Fish samples	Damietta city	KafrSaad	Kafr Al-Buteekh
	No. (%) (each 44)	No. (%) (each 32)	No. (%) (each 24)
Fresh *Saurus*	4/44 (9%)	4/32(12.5%)	2/24 (8%)
Fresh Sardines	3/44 (6.8%)	3/32 (9.4%)	0 (0%)
Frozen *Saurus*	2/44 (4.5%)	0 (0%)	0 (0%)
Frozen Sardines	0(0%)	0 (0%)	0 (0%)
Total	9/176 (5.11%)	7/128 (5.4%)	2/96 (2%)

Table 4. Incidence of *L. monocytogenes* serovars in different fish samples

Fish samples	Number of examined samples	*L. monocytogenes* Serovars		
		1/2a	1/2c	4b
Fresh *Saurus*	10	7 (70%)	3 (30%)	0(0%)
Fresh Sardines	6	4 (66.7%)	0(0%)	2 (33.3%)
Frozen *Saurus*	2	2 (100%)	0(0%)	0(0%)
Frozen Sardines	0	0	0	0
Total	18	13(72.22%)	3(16.67%)	2 (11.11%)

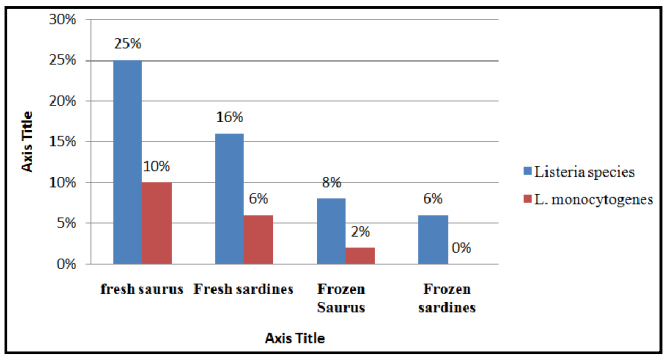

Figure 1. Incidence of isolated *Listeria* spp. and *L. monocytogenes* from different fish markets in Damietta governorate

Incidence of *Listeria* spp. and *L. monocytogenes* among three markets in Damietta governorate

As tabulated in Table 2, the total incidence of *Listeria* spp. was highest in KafrSaad city 12/128 (18.7%) followed by Damietta city 27/176 (15.3%) and Kafr Al-Buteekh7/96 (7.2%). Whereas, the highest incidence of *L. monocytogenes* was nearly similar in Dameitta city 9/44 (5.11%) and KafrSaad 7/128 (5.4%), which was much higher than Kafr Al-Buteekh (2/96) 2% (Table 3).

Differences of *Listeria* incidences among between fresh and frozen fish samples

As illustrated in Fig.1, Results showed higher isolation rates of *Listeria* species. From fresh *Saurus* (25%) and fresh Sardines fish (16%) compared with isolation rated of (8%) and (6%) among frozen *Saurus* and Sardines, respectively. Consequently, *L. monocytogenes* was detected by higher in fresh samples (10%) and (6%) in fresh *Saurus* and Sardines respectively matched to (2%) in frozen *Saurus* whereas it was not detected in frozen Sardines.

Serotype analysis of *L. monocytogenes* isolates

As tabulated in Table 4, results revealed that of the 18 *L.monocytogenes* isolates from fish samples, 13 (72.2 %), 3 (16.7%), and 2 (11.1 %) were serovar 1/2a, 4b, and 1/2b, respectively. The Serotype 1/2a was the predominant *L. monocytogenes* serovar in the samples tested in the present study. It was isolated from the examined fresh fish and frozen *Saurus* while serovar1/2c was isolated from fresh Sardine only (30%), and serovar4b was isolated from fresh *Saurus* only (33.3%).

DISCUSSION

Ingestion of foods contaminated with *L. monocytogenes*can causelisterios is which consider a severe infectious disease characterized by meningoencephalitis, include septicaemia (Armstrong and Fung, 1993). The disease also causes intrauterine infections in pregnant women, which may result in spontaneous abortion or stillbirth abortion and a high fatality rate 30% (Franciosa *et al*., 2005). Listerios is predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immune compromised patients (Kathariou, 2002 and Mclauchlin *et al*., 2004). However, non-invasive form of listerios is can affect healthy persons by causing febrile gastroenteritis (Franciosa *et al*., 2005). Egypt has coastlines on both the Mediterranean Sea and the Red Sea. It is estimated that landings in the Mediterranean Sea represented about 62% of the total marine catch in 2009. Marine fisheries produce a wide variety of species. The most important are: Sardine (15.0 % of landings in 2009), shrimp (8.9 %), anchovy (5.8%), *Saurus* (4.7%), mullets (3.1%), bogue (2.7%), and round scade (6.2%) (FAO, 2009). Fish is considered as a major source of *Listeria* contamination. Fresh and marine water fish could be sources of human infection via eating raw or undercooked fish. *Saurus* and Sardine fish are a cheap fish sold as fresh in retail markets as well as imported as frozen fish. These fish could be contaminated by bacteria particularly *Listeria* from public health perspectives, *Listeria* contamination considered great public health significance. The Egyptian standards for food safety regulations tolerate zero limits for *L.monocytogenes* in frozen and fresh fish (EOS, 2005). The present data revealed that the overall incidence of *Listeria* spp. in was (13.75%), whereas the overall incidence of *L. monocytogenes* was (4.5%). Davies *et al.* (2001) isolated 10.4% *Listeria* spp. from marine fish samples in Turkey. On other hand a study of marine fresh fish samples collected from the east coastlines of Egypt had an incidence of *listeria* spp. 37%and *L.monocytogenes* 17. 3% (El-Shenawy and El-Shenawy, 2006). *L.monocytogenes* (44.5%) and *L. murrayi* (83.5%) were the most commonly isolated spp. from freshwater and marine fish samples, respectively (Yucel and Balci, 2010). *L. monocytogenes* contamination rate in fresh fish was 4.1% (Wang *et al.,* 2011). The present data of fresh *Saurus* and Sardine seems in good harmony with previous data recorded by (Jeyasekaran Karunasagar, 1996 and Moharem *et al.*, 2007). Lower findings were 7.5% of *Listeria* spp. and 1.9% *L.monocytogenes* from fresh fish samples (Ebrahim *et al.,* 2012). While, higher results were reported by who could isolate 44.5% *L. monocytogenes* from fresh samples (Soultos *et al.,* 2007). On other side, frozen *Saurus* and Sardine*s* results were coincided with these recorded by (Ghazi, 2010 and Harydi, 2010). However, lower findings were 4.2% of *Listeria* and 1.9% *L. monocytogenes*from frozen fish samples (Ebrahim *et al.,* 2012).

In general, there are two possible routes for contamination of fish with *Listeria*; The first is the spread of *Listeria* from the intestinal contents to other fish tissues, especially if the period between death and viscera removal is greater than a few hours, and the second is crosscontamination due to manipulation of fish using contaminated equipment and to inappropriate transport (Gudmundsdottir *et al.* 2006, Souza *et al.* 2008). Therefore, contaminated raw materials can also affect the final products (Miettinen and Wirtanen 2005). Also, the data revealed that *Saurus* was more contaminated than Sardine, and that may be due to its predatory behaviour during feeding, however the diet of the *Saurus* mainly includes other spp. of fish, also feed occasionally on other animals and live mostly on sandy bottoms in island waters (Sulak, 1986). As tabulated in Table (2), the total incidence of *Listeria*spp. was highest in KafrSaad city and lowest in Kafr Al-Buteekh. Whereas, the highest incidence of L. *monocytogenes*was nearly similar in Damietta city and KafrSaad. The location of *Listeria* in fish carcasses and the place of sampling are considered to affect *Listeria* screening results. Several studies have used samples from the fish surface to generate prevalence data; however, this approach may not always yield accurate results. There is evidence that gill-filtered water containing small quantities of *L. monocytogenes* may concentrate the cells on the gas-exchange surfaces of the gill. Miettinen and Wirtanen (2005) observed that among 510 fish, 43 gills tested positive for *L.monocytogenes*, whereas only 1 skin sample and 1 visceral sample tested positive for L. *monocytogenes*. Conversely, in Turkey, L. *monocytogenes*has been frequently isolated from both gill (25%) and skin (52%) samples of raw freshwater and marine fish (n = 30) (Yucel and Balci 2010). As illustrated in fig.1, results showed thatthe isolation rates of *Listeria* spp. and

*L. monocytogenes*from fresh samples were higher than from frozen one whereas *L. monocytogenes*was not detected in frozen Sardines. *L. monocytogenes*is a psychrotrophic pathogen and have the ability to grow at low temperatures, and resistant to diverse environmental conditions, so also found in frozen samples and this could be due to post contamination after processing. The pathogen may be acquired from foodcontact surfaces and/or by secondary contamination from site equipment, the prevalence of this type of contamination varies from very low levels to 14% (Mena *et al.* 2004, Handa *et al.*, 2005, Parihar *et al.*, 2008). This difference is most likely caused by differences in sampling procedures and analytical methods. Furthermore, the rate of contamination of raw fish might vary among different geographical areas and processing plants. In several U.S. market surveys, the prevalence of L. *monocytogenes* ranged from 0% to 12.5% (Pao *et al.*, 2008). As tabulated in Table 4, The Serotype 1/2a was the predominant L. *monocytogenes*serovar in the samples tested in the present study. It was isolated from the examined fresh fish and frozen *Saurus*, while serovar 1/2c was isolated from fresh Sardine only and serovar 4b was isolated from fresh *Saurus* only. The diversity of L. *monocytogenes*strains is often specific to the processing environment (Tocmo *et al.,* 2014). Furthermore, there is a significant association between serotypes and listeriosis in patients (McLauchlin, 2004). Studies of *L. monocytogenes* isolated from seafood outbreaks and sea food processing environments have shown that >90% of human listerios is cases are caused by serotypes 1/2a, 1/2b, 1/2c, and 4b.

Conclusion

In conclusion, this study showed that *L. monocytogenes* were common contaminant of fish obtained from Damietta fish markets, and this may pose serious public health implications. Therefore, it is always recommended to inform consumers of the possible health hazards related with the consumption of fish since careful handling of fish, prevention of cross contamination in preventing infections associated with pathogens, and consumer health is adequately protected. Inappropriate handling of food products by consumers can also play a major role in increasing the prevalence of *Listeria*, resulting in noncompliance with the Food Safety Objective in the final RTE seafood products. Therefore, it is important to improve consumer education regarding food safety practices during the purchase, transport, storage, and handling of food.

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