

Prevalence of *Listeria monocytogenes* in ready-to-eat seafood marketed in Thessaloniki (Northern Greece)

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Received: 15-09-2014, **Revised:** 25-10-2014, **Accepted:** 27-10-2014, **Published online:** 27-11-2014

doi: 10.14202/vetworld.2014.1004-1009. **How to cite this article:** Soultos N, Iossifidou E, Tzikas Z, Sergelidis D, Lazou Th, Drakopoulos G, Konstantelis I (2014) Prevalence of *Listeria monocytogenes* in ready-to-eat seafood marketed in Thessaloniki (Northern Greece), *Veterinary World*, 7(11): 1004-1009.

Abstract

Aim: In the current study, a contribution to the knowledge on the prevalence and level of contamination of *Listeria monocytogenes* in ready-to-eat (RTE) seafood marketed in Thessaloniki (Northern Greece) was provided; the serovar identity of the *L. monocytogenes* isolates was also determined.

Materials and Methods: A total of 132 RTE seafood samples consisting of 74 smoked fish products, 18 salted fish products, 16 dried fish products, 9 raw marinated fish products, 10 cooked marinated cephalopods and 5 surimi crab stick products were analyzed. *L. monocytogenes* were isolated and enumerated based on ISO 11290-1/A1 and ISO 11290-2/A1 protocols, respectively, and identified using a multiplex polymerase chain reaction (PCR) system utilizing genus and species specific primers. For the identification of serotypes a second multiplex PCR assay was used which clusters *L. monocytogenes* strains into four major serogroups.

Results: Of the samples examined, 11 (8.3%) proved positive for *Listeria* spp. with 8 (6.1%) yielding *L. monocytogenes*. Only in one sample of smoked mackerel the level of *L. monocytogenes* exceeded the legal safety limit of 100 cfu/g set out in Commission Regulation (EC) No. 1441/2007. Serotyping showed higher percentages of isolates belonging to PCR serogroup 3:1/2b, 3b, 7 (46.7%) and serogroup 1:1/2a, 3a (40%) followed by serogroup 4:4b, 4d, 4e (13.3%).

Conclusion: This study demonstrated that *L. monocytogenes* can be isolated from processed RTE seafood products at retail in Thessaloniki (Northern Greece) in low concentrations. However, the presence of this human pathogen in RTE seafood should not be overlooked, but it should be considered as having significance public health implications, particularly among the persons who are at greater risk. Therefore, RTE seafood should be produced under appropriate hygienic and technological conditions since the product does not undergo any treatment before consumption.

Keywords: level, *Listeria monocytogenes*, Northern Greece, prevalence, ready-to-eat seafood, serotyping.

Introduction

Listeria monocytogenes, the causative agent of listeriosis, is a well-known foodborne pathogen which continues to be of major concern for food industry, public health authorities and consumers. Listeriosis represents a severe public health problem because *L. monocytogenes* can produce life-threatening infections with high mortality rate, especially among susceptible populations. In some risky groups i.e. the young, the old, the pregnant women and the immunocompromised individuals, the so called "YOPI" [1], the lethality is as high as 20-30% [2].

Many outbreaks as well as isolated cases of listeriosis occur worldwide. Although various foods can serve as sources of food-borne listeriosis, typically, the disease is associated with the consumption of prepared RTE foods that have extended shelf life and are stored in chilled environment [3]. This category of food does not undergo any treatment to ensure its safety before consumption and therefore risk of food-borne listeriosis must be considered if the pathogen is present in

the food. RTE seafood products, in particular, have been sources of infection with *L. monocytogenes* and lightly preserved fish products are classified as high risk merchandise [4]. Epidemiological evidence has suggested that listeriosis has been caused by smoked mussels [5], cold-smoked rainbow trout [6] and vacuum-packed gravad trout [7].

In the Community Summary Report [8] the highest frequencies of positive samples in RTE foods were found in fish products in 2012; 10.3% for fish products and clearly lower 2.1% for meat products and 0.47 for cheese products. In European Countries, the number of infections from *L. monocytogenes* showed an increasing trend over the 5 year period 2008-2012, with 1642 confirmed human cases, 198 deaths (case fatality rate 17.8%) and annual incidence 0.41 cases per 100,000 inhabitants in 2012 [8]. The observed increasing incidence of listeriosis cases in humans within European Union could be attributed mainly to the extended consumption of ready-to-eat (RTE) products [8,9] and the increase of vulnerable population, especially the elderly individuals [8,10,11].

In Greece, listeriosis is not common and only 11 confirmed cases were reported in 2012 [8]; however,

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this number is likely an underestimate of the actual number of cases since many cases of food-borne illness are not reported to public health officials [12]. Although *L. monocytogenes* has been detected from different types of foods in Greece, there is a lack of information on the occurrence of *L. monocytogenes* in RTE seafood products.

Thus, the purpose of the present study was to generate information on the prevalence and the level of contamination of this pathogen in RTE seafood marketed in Thessaloniki (Northern Greece); the serovar identity of the *L. monocytogenes* isolates was also determined.

Materials and Methods

Sample collection

A total of 132 RTE seafood samples consisting of 74 smoked fish products, 18 salted fish products, 16 dried fish products, 9 raw marinated fish products, 10 cooked marinated cephalopods and 5 surimi crab stick products (Table-1) were analyzed. All samples were packaged (sliced or unsliced), with packaging sizes ranging from 100 to 250 g and extended shelf-life >20 days and purchased at retail from five different supermarket chains in Thessaloniki (Northern Greece). Immediately following purchase, samples were transported to the laboratory inside a portable ice-chest and examined within 1 h of arrival.

Detection and enumeration of *L. monocytogenes*

Procedures based on ISO, 11290-1/A1 [13] and ISO 11290-2/A1 [14] were used to detect and enumerate *L. monocytogenes*, respectively, in seafood samples. Briefly, 25 g of each sample were added to 225 ml of half Fraser broth base (without supplements) and homogenized in a stomacher 400 Lab-blender (Seward Medical, London, UK) for 2 min. The suspensions were incubated at 20°C for 1 h, in order to recover stressed microorganisms. For enumeration of *L. monocytogenes*, 0.1 ml of each suspension was spread over Agar *Listeria* Ottaviani Agosti

(ALOA) (Biolife, Milan-Italy) and incubated at 37°C for 24-48 h. For the detection of *L. monocytogenes*, the prepared suspension was supplemented by Fraser half selective supplement (primary enrichment) and incubated (24 h, 30°C). Following this, a secondary enrichment was prepared by inoculating an aliquot (0.1 ml) of the primary culture into 10 ml Fraser broth (48 h, 30°C). Afterward, a loopful (10 µl) of the primary and secondary enriched cultures were streaked onto ALOA (Biolife, Milan-Italy) and Oxford agar and examined after 24 and 48 h (37°C). Five suspect *Listeria* spp. colonies from each plate were streaked to purity on tryptone soya agar with yeast extract (24 h, 37°C).

Identification

For the identification of *Listeria* spp., pure cultures in tryptone soya agar were submitted to a multiplex polymerase chain reaction (PCR) procedure, according to Lawrence and Gilmour [15], using the conditions described by authors. This assay uses a combination of genus-and-species specific primers (U1, U2, LI1, LM1 and LM) and gives three results: A band indicative of bacterial DNA (U1, U2), *Listeria* spp. (LI1, U1) and *L. monocytogenes* (LM1, LM2).

Serotyping

Strains identified as *L. monocytogenes* were further serotyped using a second multiplex PCR assay, as described by Doumith *et al.* [16]. This system uses four set of primers specific for *L. monocytogenes*, (*lmo737*, *lmo1118*, ORF2819, ORF2110) and an addition set of primers (*prs*) specific for *Listeria* spp. Applying this method, *L. monocytogenes* strains are classified in four PCR groups of serotypes (Group 1: Corresponding to conventional serotypes 1/2a, 3a; Group 2: Corresponding to conventional serotypes 1/2c, 3c; Group 3: Corresponding to conventional serotypes 1/2b, 3b, 7; and Group 4: Corresponding to conventional serotype serotypes 4b, 4d, 4e).

Appropriate positive and negative controls were included in all assays.

Results

In the present study, a total of 132 RTE seafood samples were analyzed. Table-1 lists the various retail RTE seafood products that were analyzed, the number of samples of each product tested and the values of *Listeria* spp. and *L. monocytogenes* contamination. Of the samples examined, 11 (8.3%) proved positive for *Listeria* spp. With 8 (6.1%) yielding *L. monocytogenes*. The pathogen was not detected (<1 organism in 25 g) from the 8 cooked marinated cephalopods and the 5 surimi crab sticks tested samples.

The level of *L. monocytogenes* in the 7 out of 8 positive samples did not exceed the legal safety limit of 100 cfu/g set out in Commission Regulation (EC) No. 1441/2007 on microbiological criteria for food-stuffs [17]. Only in one sample of smoked mackerel higher levels of contamination (110 cfu/g) were found.

Table-1: Prevalence of *Listeria* spp. and *L. monocytogenes* in RTE seafood.

Product type	Number of samples	Number of positive samples (%)	
		<i>Listeria</i> spp.	<i>L. monocytogenes</i>
Smoked fish	76	3 (3.9)	3 (3.9)
Dried fish	16	2 (12.5)	2 (12.5)
Salted fish	18	3 (16.7)	2 (11.1)
Raw marinated fish	9	3 (33.3)	1 (11.1)
Cooked marinated cephalopods	8	0 (-)	0 (-)
Surimi crab stick	5	0 (-)	0 (-)
Total	132	11 (8.34)	8 (6.1)

L. monocytogenes=*Listeria monocytogenes*,
RTE=Ready-to-eat

Altogether, 15 isolates of *L. monocytogenes* were obtained from the 8 positive RTE seafood samples; of which 8 were isolated from the 3 smoked fish, 3 from 2 salted fish, 3 from 2 dried fish and 1 from 1 raw marinated fish. The 15 *L. monocytogenes* isolates were classified in three serovar groups (Table-2). Most isolates tested 7 (46.7%) belonged to serogroup 3 (1/2b, 3b, 7), 6 (40%) were identified as serogroup 1 (1/2a, 3a) and only 2 (13.3%) were identified as serogroup 4 (4b, 4d, 4e). Multiple serogroups were not observed in any of the samples tested.

Discussion

The general occurrence of *L. monocytogenes* in various RTE food groups, including RTE seafood products and in various food processing environments is well documented in many countries, and seem to enhance the general view that *L. monocytogenes* is an ubiquitous bacterial pathogen. The microorganism because of its ubiquity in nature may be introduced into food-processing environments and ultimately may result in the contamination of food products [18]. The association of *L. monocytogenes* with RTE seafood is most likely due to environmental contamination occurring during processing. The raw fish material as well as food handlers may be sources of *L. monocytogenes* introducing it into the factory environment [19]; however product contamination is often contamination during processing rather than by survivors from the raw material [20]. *L. monocytogenes* is capable of colonizing food processing environments and readily forms biofilms allowing the pathogen to adhere and survive on different surfaces of food-processing settings [21,22]. Pieces of biofilms incorporating *L. monocytogenes* may be dislodged either continuously or occasionally and contaminate food products during processing.

Unlike some other food-borne pathogens, the organism has the ability to survive and grow in stress conditions such as refrigeration temperatures, high salt concentrations, low concentration of oxygen, low water activity as well as low pH values [2,23,24]. These properties of the pathogen appear to be the main cause of its occurrence in RTE seafood products with extended shelf lives.

The results of the current study demonstrate that 8.3% of the retail RTE seafood samples tested were contaminated with *Listeria* spp. and 6.1% were

contaminated with *L. monocytogenes*. The incidence of *L. monocytogenes* observed in our study is in accordance with published data in Malaysia, which reported the presence of the pathogen in 6.7% of RTE seafood-based products [25]. Previous studies, carried out in other countries, revealed prevalence of *L. monocytogenes* in RTE seafood products ranging from 1.4% to 66.7% [9]. High *L. monocytogenes* isolation rates 34.1%, 27.8% and 21.6% were reported in smoked fish products obtained from retail market in Italy [26], Belgium [27] and Ireland [28], respectively. Two recent studies carried out in Israel by Vasilev *et al.* [29] and in Iran by Fallah *et al.* [30] revealed approximately similar rate of *L. monocytogenes* contamination 15% and 14.5% in RTE seafood products, respectively. In Italy, in a survey of the prevalence of *L. monocytogenes* in RTE foods, smoked fish were found to harbor the pathogen at a rate 12% [31]. Similar occurrence (12%) was also observed by Lambertz *et al.* [32] in a study conducted in RTE fish products at retail markets in Sweden. However, much lower incidences of *L. monocytogenes* have been found in smoked seafood (4.31%) and seafood salads (1.4%) samples from retail markets in the United States [33].

Failure to detect *L. monocytogenes* in certain types of RTE seafood products has also been documented. In the current study, none of the 8 cooked marinated cephalopods and the 5 surimi crab sticks tested samples were contaminated with the pathogen. This pathogen was not isolated from raw marinated products [31] and smoked salmon [34] marketed in Italy or in any of the 125 surimi tested samples in a survey undertaken in Spain [35]; the microorganism was not detected in domestic or imported dried seafood (shrimps, squids) and smoked mussels in a survey conducted in Korea, as well [36]. The discrepancies in the prevalence of *L. monocytogenes* among different studies and countries may be due to type of seafood sample, differences in food-processing environment, source of the samples (retail level and factories), sampling season, isolation method, human activity etc.

The significance of listeriosis with regard to level infectious dose and serotype of *L. monocytogenes* is well known. Currently, there is insufficient data for the dose-response relationship to be determined, however, it has been established that only exposure to high levels of *L. monocytogenes* (>1000 cfu/g) causes listeriosis [37]. According to International Commission on Microbiological Specification of Foods [37,38], foods in which *L. monocytogenes* levels do not exceed 100 cfu/g at the time of consumption are acceptable for a healthy human population. Therefore, the European Commission Regulations (EC) No. 2073/2005 [39] and No. 1441/2007 [17] on microbiological criteria for foodstuffs established a limit of 100 cfu/g for RTE foods unable to support the growth of *L. monocytogenes* throughout their shelf-life. For RTE foods that are able to support the growth of bacterium, *L. monocytogenes* is required to be absent in 25 g at the time

Table-2: Distribution of the serotypes in the 15 strains of *L. monocytogenes* isolated from RTE seafood.

Serogroup (serotypes)	Number of samples (isolates)			
	Smoked fish	Salted fish	Dried fish	Raw marinated fish
1 (1/2a, 3a)	2 (6)			
3 (1/2b, 3b, 7)	1 (2)	2 (3)	1 (2)	
4 (4b, 4d, 4e)			1 (1)	1 (1)

L. monocytogenes=*Listeria monocytogenes*,
RTE=Ready-to-eat

leaving the production plant, if the producer is not able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout its shelf-life.

Data regarding levels of *L. monocytogenes* in RTE seafood are very limited, since, in previously published surveys, most often only prevalence rates have been reported. In the present study, the microbial load of *L. monocytogenes* in the 7 out of the 8 positive samples was <100 cfu/g, in conformity with the food safety criteria provided by the Commission Regulation (EC) No. 1441/2007 for RTE foods [17] able to support the growth of the pathogen. Only in one sample of smoked mackerel were found higher levels of contamination (110 cfu/g).

These results are in agreement with those reported in previous studies carried out in Spain [35], in Sweden [32] and in Belgium [27]; the observed percentages of unsafe samples were very low ($\approx 0.4\%$). However, higher concentrations of the pathogen have been reported for certain types of RTE seafood products. Contamination levels >100 cfu/g were reported for smoked and gravid fish, smoked seafood and seafood salads [30,33,40]. Dominguez *et al.* [41], in a survey carried out in Spain, reported that of the 38 positive retail smoked fish samples, 18 contained 100-1000 cfu/g *L. monocytogenes* and two samples (vacuum-packaged cold-smoked salmon and rainbow trout) contained the organism at levels exceeding 1000 cfu/g. In the European Union, the highest proportion of non-compliant units were observed in RTE fishery products, at levels of 8.0% and 0.5% in single samples, at processing and at retail, respectively, in 2012 [8].

Serotyping of *L. monocytogenes* has been widely used for epidemiological monitoring and may have value as a virulence screening test. Furthermore, for the food industry, where the presence of the pathogen is a big concern, tracing contamination strains within the food chain, and the plant environment is of primary importance. *L. monocytogenes* is made up of 13 serotypes showing varied virulence potential. It has been confirmed through studies that only four of these serovars (1/2a, 1/2b, 1/2c and specifically 4b) account for the majority of foodborne listeriosis outbreaks, providing additional evidence that certain serotypes have greater potential to cause illness [2,8,42,43]. Other serotypes (i.e. 3a, 3b, 3c, 4a, 4c, 4e, 4d and 7) are very infrequent in food and rarely responsible for human *L. monocytogenes* infections [16,44].

Serotyping in the present study, using a rapid and practical multiplex PCR assay described by Doumith *et al.* [16], showed higher percentages of isolates belonging to PCR serogroup 3 (7 isolates, 46.7%) and serogroup 1 (6 isolates, 40%) followed by serogroup 4 (2 isolates, 13.3%). *L. monocytogenes* belonging to molecular serogroup 3, containing serotypes 1/2b, 3b and 7, could be isolated from most RTE seafood categories (smoked fish, salted fish and dried fish). Two isolates of serogroup 4, corresponding to serotypes

4b, 4d and 4e, were detected from two samples of dried fish and raw marinated fish respectively, while all isolates of serogroup 1 that includes serotypes 1/2a and 3a were found in two samples of smoked fish (Table-2). Simultaneous existence of isolates belonging to different serogroups in one single seafood product was not observed.

A disadvantage of Doumith's multiplex PCR assay used for serotyping in this study is that it is less discriminating than agglutination method [45]. This assay separates *L. monocytogenes* isolates into four distinct molecular serogroups, in which each molecular serogroup contains one of the four principal serotypes (1/2a, 1/2b, 1/2c and 4b) associated with human listeriosis [16]. As it was reported by Doumith *et al.* [16], multiplex PCR profiles do not distinguish, within the species of *L. monocytogenes*, serotype 1/2a from 3a, serotype 1/2b from 3b and 7 and serotype 4b from 4d and 4e. However, this drawback would not decrease the efficiency of the multiplex PCR assay in long-term epidemiological studies [44] as this method may be used as a first level in discriminating of *L. monocytogenes* strains of different origin. Indeed, serotypes 3a, 3b, 7, 4d and 4e are relatively rare in foods and particularly rarely reported as implicated in human listeriosis [16,44]. In contrast, previous studies from different countries revealed that serotypes 1/2a, 1/2b and 4b tend to be predominant in seafood products and production plants [29,30,31,46-49]. Thus, in this study, the isolated strains from RTE seafood samples assigned into serogroups 1(1/2a, 3a), 3(1/2b,3b,7) and 4 (4b, 4d, 4e) may be presumptively serotypes 1/2a, 1/2b and 4b, respectively. Specifically, it has been reported that serotype 1/2a was prevalent in RTE seafood in Italy and Estonia [31,47]; serotype 4b was predominant in seafood products in Israel and China [29,49] and serotype 1/2b was the dominant serotype in Turkish seafood products [48]. Furthermore, a survey conducted in Iran indicated that serotypes 1/2a and 4b were the dominant serotypes in seafood products and environmental samples during warm and cold seasons, respectively [30]. In addition, a high percentage rate of serogroup 1 (1/2a, 3a) has been found in RTE seafood products in Malaysia [50].

Conclusion

In conclusion, this study demonstrated that *L. monocytogenes* were isolated from processed RTE seafood products at retail in Thessaloniki (Northern Greece) in low concentrations. Nevertheless, considering that the bacterium has the potential to grow well at refrigerated temperatures and in high salt matrixes such as seafood, its presence in these products should not be overlooked. Therefore, more attention should be focused by the consumers on the product's shelf life and the cold chain.

Due to the ubiquitous nature of *L. monocytogenes* it is extremely difficult to produce RTE seafood

products without sporadic occurrence of the bacterium in low levels. Therefore, it is of great importance for the producer to introduce effective control procedures throughout the production of RTE seafood in order to avoid colonization of the processing environment and subsequent spread to the finished product. This should be done by implementation of a comprehensive control system involving a combination of strategies that are compatible with hazard analysis critical control point, good hygiene practices and good manufacturing practices; the application of these measures will help to reduce the presence of the pathogen in the factory and consequently assure the microbiological safety and quality of the finished product.

Authors' Contributions

NS designed the plan of work and wrote the manuscript. EI and ZT collected the relevant samples and assisted in writing the manuscript. DS, TL, GD and IK carried out the laboratory work. All the authors have read and approved the manuscript. NS and ZT revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This study was financially supported by the Greek Military Land Forces - Medical Service.

Competing Interests

The authors declare that they have no competing interests.

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