

Identification and serotyping of *Listeria monocytogenes*, isolated from various salmon products, sold in retail market in Lithuania

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Abstract

Cold smoked salmon products (belly flaps, pieces, fillet, and loin) obtained from the retail market in Lithuania were tested for the presence of *L. monocytogenes*. It was found that contamination of the cold smoked fish products with *Listeria* spp. depends on the type of the product. Contamination with listeria in salmon belly flaps was 7.5 times higher than in the loin ($P < 0.05$), 1.8 times higher than in the pieces ($P < 0.05$) and 30 times higher than in the fillet ($P < 0.05$). Microbiological analysis showed that 32.5% ($P < 0.05$) of the fish product samples were infected with *L. monocytogenes*, while multiplex PCR confirmed 31.25% positive samples ($P < 0.01$). According to the study results, *L. monocytogenes* strains were divided into two serotypes: 4b (94.6%) and 1/2a (5.4%). High contamination of the products with *Listeria* spp. showed that cold smoked salmon products, sold in local market, can be a reason of human listeriosis in Lithuania.

Introduction

Listeriosis is a food-borne zoonosis, affecting animals, birds, fish, crustaceans and humans (Dhama *et al.*, 2015). The number of human listeriosis cases has increased during 2009-2018 year period in European Union and European Economic Area countries and 2,549 cases were reported in 2018 (EFSA, 2019). The death rate of listeriosis could reach up to 20-30% of infected patients (Jeyaletchumi *et al.*, 2010). It usually affects susceptible groups of young, old, pregnant or immunodeficient people (EFSA, 2016). Listeriosis during pregnancy and infant listeriosis account for 10-20% of cases (McLauchlin *et al.*, 2004).

Additionally, patients with AIDS or leukemia is 600 and 1000 times more susceptible to listeriosis infection in comparison to healthy people, respectively (Miettinen *et al.*, 2006). It was confirmed that wild and domestic animals are a major source of infection (Atil *et al.*, 2011). However, *Listeria* spp. may be found in human feces, but it is not considered an important source for food contamination (Sauders *et al.*, 2005).

Lithuania is among nine EU member countries (Germany, Estonia, Finland, Italy, Latvia, Lithuania, Poland, Romania and Spain) with a significantly increasing trend of human listeriosis cases during 2014-2018 period (EFSA, 2019). The country has reported 20 domestic (not travel related) human listeriosis cases in 2018, two-fold higher number in comparison to 9 and 10 cases in 2017 and 2016 years, respectively (EFSA, 2019). Also, listeriosis can be deadly: 3 people died in Lithuania in 2016 out of 10 infected (www.ulac.lt).

The majority of listeriosis cases during the last 30 years in European Union were related to milk and its products, soft cheeses, paté, sausages, smoked fish, salad, deli meat and ready to eat products (Garrido *et al.* 2010). Cold smoked fish products cause a main concern because of a lack of heat during thermal processing (Gombas *et al.*, 2003).

It is known that *L. monocytogenes* and *L. ivanovii* are pathogenic bacteria, however *L. monocytogenes* is the main cause of human and animal listeriosis (Liu, 2006). However, there are reports related to disease, caused by *L. seeligeri*, *L. ivanovii*, *L. innocua* (Jeyaletchumi *et al.*, 2010).

According to Garrido *et al.* (2010), the majority of human listeriosis cases is caused by serotype 4b, isolated from contaminated food products, while serogroup 1/2 has the highest prevalence. Additionally, there is an increasing trend of food contamination with serotype 1/2a (Garrido *et al.*, 2010).

Materials and methods

Experimental samples were collected from major and minor fish producers in Lithuania during one year period with no notice in advance. Four different types of cold smoked salmon were tested: belly flaps, loins, pieces and fillets. Each type of product consisted of 40 samples, making a total of 160 tested units. Samples were tested each week immediately upon collection according to LST EN ISO 11290-1:2017. Primarily, the first enrichment was done: 25 g of sample were cultivated in 225

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ml of Half-Fraser broth (Oxoid Ltd., England) for 25±1 h at 30±1°C, then 0.1 ml of culture from the first enrichment was transferred to 10 ml of Fraser broth (Oxoid Ltd., England) and cultivated for 24±2 h at 37±1°C temperature. Cultures from the first and secondary enrichments were transferred to ALOA (Agar *Listeria* according to Ottaviani and Agosti) and PALCAM selective agar, incubated for 24±2 h at 37±1°C and additionally 24 h if needed. Presumptive *Listeria* colonies were plated on nutrient Tryptic Soy Agar (Oxoid Ltd., England) and cultivated for 24±2 h at 37±1°C in order to obtain well-separated colonies. Not less than 5 colonies or all colonies present (if less than 5 on the plate) were taken for confirmation tests: microscopic aspect, catalase. Then pure culture of confirmed *Listeria* spp. was stored in Brain Heart Infusion broth, enriched with 30% of glycerol at -80°C temperature. Further study was proceeded only with samples, confirmed positive for *Listeria* spp.

Listeria spp. identification by multiplex PCR

Listeria species were identified by a method described in Bubert *et al.* (1999)

study. *iap* DNA sequence was chosen to identify one group of *Listeria*, consisting of *L. monocytogenes* and *L. innocua* and another group of *L. seeligeri*, *L. welshimeri*, *L. ivanovii* detected together with *L. grayi*, *L. grayi* subsp. *Murrayi*. Therefore, combinations of primers (Table 1) were chosen according to the comparison of *iap* DNA sequences. Primer Lis1B was chosen as a fixed primer in multiplex PCR mix with four other primers. The combination of primers MonoA and Lis1B was used to identify *L. monocytogenes* when 600 bp product was formed. Primers Ino2 and Lis1B was used for identification of *L. innocua* with a product length of 870 bp. *L. ivanovii*, *L. seeligeri* and *L. welshimeri* were identified by the combination of Siwi2 and Lis1B primers, while Mugra1 and Lis1B was used to identify *L. grayi*, *L. grayi* subsp. *Murrayi*, when product length was 480 bp. All reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA)

DNA extraction

Bacterial culture was cultivated for 24±3h – 48h±3h at 37°C temperature on ALOA agar. Pure culture (0.5 µl) was transferred to 500 µl of sterile distilled water, mixed well with Vortex. Then suspension is heated in thermocycler at 99±1°C temperature for 10 min. Afterwards, sample was centrifuged for 5 min at 14000 rpm/min. Supernatant was transferred to a new tube and stored at 20°C until further testing.

Multiplex PCR and serotyping

PCR amplification was done in 25 µl reaction mix, consisting of 2.5 µl 10 x PCR buffer, 2.5 µl (2 mM) dNTP mix, 0.4 (x5) µl of each primer, 0.3 µl Maxima Hot Start Taq polymerase, 1.5 µl (25Mm) MgCl₂, 2 µl genomic DNA and additional 14.2 µl of ddH₂O. PTC-100 (Programmable thermal controller) (MJ Research Inc., Waltham, MA, USA) was chosen for DNA amplification with subsequent reaction conditions: 30 cycles, denaturation at 95°C for 15 s, oligonucleotide adhesion at 58°C for 30 s, DNA extension at 72°C for 50 s. *L. monocytogenes* reference strain ATCC 7644 was used as a control. PCR products (each 11 µl) were analysed in 1.2% TopVision™ LM GQ electrophoresis gel with ethidium bromide. PCR results were analysed under UV light.

L. monocytogenes serotypes were identified according to the method, described by Doumith *et al.* (2004). Primer sequences, chosen after *prs* gene specific to all *Listeria* species, are listed in Table 2. This multiplex PCR method could identify the main four *L. monocytogenes* serotypes – 1/2a, 1/2b, 1/2c, 4b. Reaction mix used in this study was 100 µl and consisted of 2 U Maxima Hot Start Taq polymerase, 0.2 mM deoxynucleoside triphosphate, 50 mM 10X Tris-HCl buffer, 10 mM KCl, 50 µM (NH₄)₂SO₄, 2 µM Mg Cl₂. Primer used in this study: 1 µM *Imo*0737, ORF2819 and ORF2110; 1.5 µM *Imo*1118; 0.2 µM *prs*. Reaction conditions: initial denaturation at 94°C for 3 min; 35

cycles: denaturation at 94°C for 15 s; oligonucleotide adhesion at 53°C for 75 s; DNA extension at 72°C for 75 s; final extension at 72°C for 7 min in thermocycler. Products were analysed in 2% agarose gel with ethidium bromide.

Statistical analysis

Results were evaluated by Microsoft Office Excel 2010 program. The sum of the amounts (sum), mean values (x), standard deviations (sd), correlation dependency (r) were calculated. To determine the accuracy and reliability of the statistical estimation, the reliability of the average differences was calculated (P). Student's criterion (td) was used to determine reliability (statistically reliable when P<0.05).

Results

Prevalence of *Listeria* spp. in cold smoked salmon products

Based on the culture medium results, *Listeria* spp. was present in 52 (32.5%) out of 160 tested cold smoked salmon products (P<0.05). Standard method showed that 30 positive for *Listeria* spp. samples were salmon belly flaps, 17 positive samples of salmon pieces, 4 positive samples of the loin and 1 – fillet. Therefore, cold smoked salmon belly flaps are the most contaminated tested product, because they there were

Table 1. Primers used for *Listeria* species identification (Bubert *et al.*, 1999).

DNA sequence	Primer	Fixed primer	Primer sequence (5 – 3)
<i>iap</i>	MonoA	Lis1B	-C AAACGCTAACACAGCTACT -
	Mugra1		-TTATACGCGACCGAAGCCAAC-
	Ino2		-ACTAGCACTCCAGTTGTTAAAC-
	Siwi2		-TAACTGAGGTAGCGAGCGAA-

Table 2. Primer sequences and target genes used in this study (Doumith *et al.*, 2004).

Target genes	Primer sequences (5' – 3') Forward / Reverse	Product length (bp)	Specificity of serotypes
<i>Imo</i> 0737	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	691	<i>L. monocytogenes</i> serotypes 1/2a, 1/2c, 3a and 3c
<i>Imo</i> 1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCCGCATACTTA	906	<i>L. monocytogenes</i> serotypes 1/2c and 3c
ORF2819	AGCAAAATGCCAAAACCTCGT CATCACTAAAGCCTCCCATTTG	471	<i>L. monocytogenes</i> serotypes 1/2b, 3b, 4b, 4d, 4e
ORF2110	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	597	<i>L. monocytogenes</i> serotypes 4b, 4d, 4e
<i>prs</i>	GCTGAAGAGATTGCCAAAAGAAG CAAAGAAACCTTGATTTGCCG	370	All <i>Listeria</i> species

7.5, 1.8 and 30 times more positive for *Listeria* spp. samples in comparison to salmon loin samples, pieces and fillet, respectively ($P < 0.05$).

Multiplex PCR confirmed 50 positive samples, a reduction by 1.04 times ($r = 0.99$). Therefore, the prevalence rate was reduced to 31.25% with a strong positive dependency between two methods – culture medium and multiplex PCR. According to the study, 37 samples (74%) of 50 PCR confirmed cold smoked fish products were contaminated with *L. monocytogenes*. The remaining samples were distributed as follows: 3 samples (6%) were infected with *L. innocua* and 10 samples (20%) – with *L. ivanovii*, *L. seeligeri* and *L. welshimeri*. The differences between groups were statistically significant ($P < 0.01$, Figure 1).

According to product type, the highest contamination with *L. monocytogenes* in cold-smoked fish products was determined in belly flaps (21 sample of 30, 70%), one sample of 30 (3.3%) was contaminated with *L. innocua* and 8 samples (26.7%) out of 30 were contaminated with *L. ivanovii*, *L. seeligeri* and *L. welshimeri*. Four samples of cold smoked salmon loin were contaminated with *L. monocytogenes*.

Out of 15 samples analyzed by PCR method, 12 (80%) were confirmed as *L. monocytogenes*, 1 sample (6.66%) as *L. ivanovii*, *L. seeligeri* and *L. welshimeri* and 2 samples (13.3%) as *L. innocua*. One sample

of cold smoked salmon fillet was tested, which proved to be *L. ivanovii*, *L. seeligeri* and *L. welshimeri* ($P > 0.05$).

Listeria monocytogenes serotyping

Afterwards, 37 positive for *L. monocytogenes* samples were serotyped. Results showed predominant 4b (90.5%) serotype in cold-smoked salmon belly flap samples, while serotype 1/2a (9.5%) was found in only 2 samples. Based on the data of the study (Table 3), we see that serotype 4b in cold-smoked salmon belly flaps was 9.5 times more common than serotype 1/2a. Only serotype 4b was determined in cold-smoked salmon loin and salmon pieces ($P > 0.05$; $r = 0.80$ positive dependency between features). Overall, serotype 4b was 17.5 times more frequent than serotype 1/2a (Figure 2).

Discussion

According to our study, more than one third of cold smoked salmon products supplied to the local Lithuanian market is contaminated with *Listeria* spp. bacteria. These results are related to other authors studies: in Italy *L. monocytogenes* was found in 34.1% of tested samples (Pinto *et al.*, 2009), Spain – 25% of tested samples (Garrido *et al.*, 2010), Canada – 20%

(Mesak *et al.*, 2012), Ireland – 21.6% (Nissreen Abu-Ghannam *et al.*, 2011). However, Klæboe *et al.* (2010) reported less contaminated cold smoked and other fish products in Norway, where the prevalence of *Listeria* spp. in various fish products varied from 5 to 15%.

Similar relation between *L. monocytogenes* serotypes was found in other studies. For example, Doumith *et al.* (2004) determined that serotypes 1/2a, 1/2b, 1/2c and 4b formed 95 % of *L. monocytogenes* strains, isolated from humans and food products. Jeyaletchumi *et al.* (2010) found out that serotypes 1/2a, 1/2b and 4b accounted for 98% of *L. monocytogenes* isolated from humans and animals. According to Narkevicius *et al.* (2004), serotype 4b causes the majority of *L. monocytogenes* cases in Europe, Canada and USA. Liu (2006) states that serotype 4b is mainly isolated during human listeriosis outbreaks, while serotypes 1/2a and 1/2b cause sporadic listeriosis cases.

Additionally, Braga *et al.* (2017) determined that *L. monocytogenes* serotype 4b was one of the most frequent serotypes in various food samples like frozen, deli meats, ready-to-eat products and cheese. Nonetheless, Lopez-Valladares *et al.* (2018) emphasizes the shift from serotype 4b to 1/2a as a cause of human listeriosis from the middle of 1990s and early 2000s. On the contrary, Zhang *et al.* (2019) found out that

Table 3. Serotypes of *L. monocytogenes* strains detected in cold smoked fish products.

Product type	N. identified <i>L. monocytogenes</i> strains	Serotype 1/2a	Serotype 4b	Serotype 1/2a, %	Serotype 4b, %
Belly flaps	21	2	19	9.5	90.5
Loin	4	0	4	0	100
Fillet	0	0	0	0	0
Pieces	12	0	12	0	100
Total	37	2	35	5.4	94.6

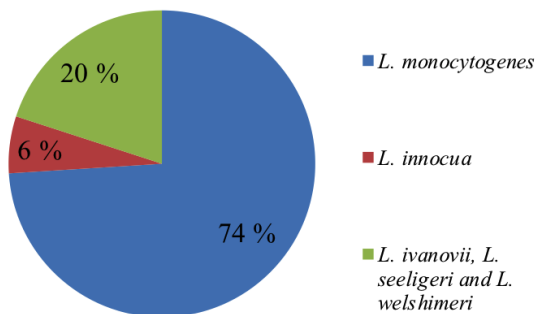


Figure 1. The prevalence of *L. monocytogenes* in cold-smoked salmon products (based on PCR method).

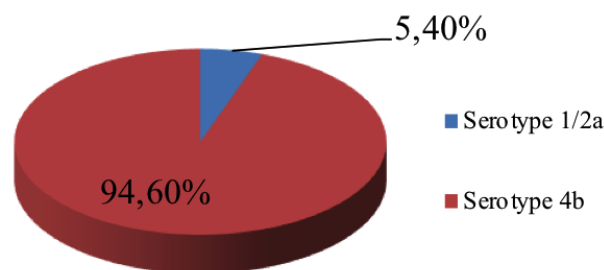


Figure 2. The prevalence of *L. monocytogenes* serotypes 4b and 1/2a in cold-smoked salmon products.

the majority (47.9%) of *L. monocytogenes* contaminated various retail food samples (ready-to-eat foods, raw meat, poultry, raw seafood) belonged to 1/2a serotype, while salmon sashimi had a relatively high prevalence of *L. monocytogenes* (6.9%).

Conclusions

This study showed that more than thirty percent (31.25%) of tested cold smoked salmon products in retail market of Lithuania were contaminated with *Listeria* spp. The prevalence of *Listeria* spp. depended on the type of the salmon product with belly flaps being the most contaminated ($P < 0.05$). PCR showed a higher testing accuracy with 23.13% of tested samples containing *L. monocytogenes*, while serotype 4b was the main serotype found in tested samples. High prevalence of *Listeria* spp. in fish products shows that fish product suppliers should improve hygiene in salmon processing and marketing places. These measures would help to avoid the risk to acquire human listeriosis.

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