Turkish Journal of Veterinary & Animal Sciences

Volume 38 | Number 1 Article 9

1-1-2014

Listeria monocytogenes in mussels (Mytilus galloprovincialis) harvested from North Aegean coastal area

NIKOLAOS SOULTOS

ELENI IOSSIFIDOU

AMIN AMBRAHIM

EVDOXIOS PSOMAS

IOANNIS TZAVARAS

See next page for additional authors

Listeria monocytogenes in mussels (Mytilus galloprovincialis) harvested from North Aegean coastal area

Authors

NIKOLAOS SOULTOS, ELENI IOSSIFIDOU, AMIN AMBRAHIM, EVDOXIOS PSOMAS, IOANNIS TZAVARAS, DEMETRIOS KOUTSOPOULOS, and THOMAI LAZOU

TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2014) 38: 50-53
© TÜBİTAK
doi:10.3906/vet-1212-36

Listeria monocytogenes in mussels (*Mytilus galloprovincialis*) harvested from North Aegean coastal area

Nikolaos SOULTOS*, Eleni IOSSIFIDOU, Amin AMBRAHIM, Evdoxios PSOMAS, Ioannis TZAVARAS, Demetrios KOUTSOPOULOS, Thomai LAZOU

Laboratory of Hygiene of Foods of Animal Origin, Department of Hygiene and Technology of Food of Animal Origin, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

Received: 21.12.2012 • **Accepted:** 18.02.2013 • **Published Online:** 18.12.2013 • **Printed:** 20.01.2014

Abstract: One hundred and two samples of mussels (*Mytilus galloprovincialis*), harvested from approved shellfish coastal water in northern Greece, were screened for the presence and antimicrobial resistance of *Listeria monocytogenes*. *Listeria* spp. were isolated according to International Organization for Standardization method 11290-1: 1996/FDAM 1: 2004(E) and identified using a multiplex polymerase chain reaction (PCR) system. The serovar identity of *L. monocytogenes* isolates was also determined with a multiplex PCR assay. The antimicrobial profile of the isolates was determined by the disk diffusion method. *Listeria* spp. were present in 8 of 102 samples tested (8%) and only 1 (1%) yielded *L. monocytogenes*. The isolate identified as *L. monocytogenes* was defined as serogroup I and found to be resistant to nalidixic acid and streptomycin. In conclusion, this study demonstrated that *L. monocytogenes* is not commonly found in mussels harvested in the North Aegean Sea, whereas there is a higher possibility of mussels' contamination with other *Listeria* species.

Key words: *Listeria monocytogenes*, mussels, *Mytilus galloprovincialis*, antibiotic resistance, serotyping

1. Introduction

Mussels (*Mytilus galloprovincialis*) are a very popular seafood dish in Greece due to their exceptional nutritional value and delicious taste. The aquaculture production of mussels in Greece has rapidly increased over the last 2 decades and reached over 30,000 t in 2008 (1). Mussels are usually sold as shucked products packaged in plastic containers (pouches) and kept refrigerated or frozen in vacuum-sealed plastic containers, and they are preferably consumed, as in some other countries, in an almost raw state or lightly cooked.

Listeria monocytogenes is widely distributed in the environment and has been isolated from numerous sources, including the intestines of humans, domesticated animals, and birds and many foods and environmental samples (2,3). The bacterium has been isolated from molluscan shellfish and especially mussels, which are filter feeders and notoriously liable to become contaminated with pathogenic bacteria such as *Listeria* derived from polluted aquatic environments (4,5). Compared with other food products, very few studies have been carried out on the prevalence of *L. monocytogenes* in mussels (6,7). Its occurrence in raw shellfish is generally considered rare (4).

Serotyping of *L. monocytogenes* may have value as a virulence screening test. There are 13 *L. monocytogenes* serovars that can cause disease, but only 4 of these serovars, specifically 1/2a, 1/2b, 1/2c, and 4b, account for the majority of foodborne listeriosis outbreaks (2,8,9). 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 (10,11).

Listeriosis is a rare human disease, but it may have life-threatening clinical manifestations, requiring antibiotic therapy for the resolution of infections (5,12). The annual incidence in the Europe Union for 2010 was 0.35 cases per 100,000 inhabitants (13). In some susceptible population groups, i.e. the young, the old, pregnant women, and immunocompromised individuals, the so-called YOPI group (14), 20% to 30% of the cases are fatal (2). It is worth noting that there is growing evidence of the emergence of multidrug-resistant strains as a result of the constant use of antimicrobial agents. Although most isolates of *L. monocytogenes* and other *Listeria* spp. are susceptible to antibiotics active against gram-positive bacteria, there are several reports on the occurrence of antibiotic resistance in *L. monocytogenes* isolated from a variety of food items

* Correspondence: soultos@vet.auth.gr

such as dairy, meat, and seafood products (5,15–17). Since the food origin of human listeriosis is now recognized, the presence of antimicrobial-resistant *L. monocytogenes* strains in foods may be of public health concern.

Because of the scarcity of relevant data in Greece, we carried out this work determining the presence of *L. monocytogenes* in mussels harvested in shellfish growing areas of northern Greece and the resistance of the isolates to various antimicrobial agents; the serovar identity of the isolates was also determined.

2. Materials and methods

2.1. Sample collection and preparation

One hundred and two samples of aquacultured mussels (*Mytilus galloprovincialis*) were provided by the National Reference Laboratory on Marine Biotoxins Center of the Veterinary Institution of Thessaloniki and analyzed for *L. monocytogenes*. Fifty-nine samples were collected from 7 approved coastal sites in the Thermaikos Gulf (Northwest Aegean Sea) and 43 samples came from 2 approved production sites in the Gulf of Kavala (Northeast Aegean Sea). Mussels were placed into foamed polystyrene boxes containing ice and transported to the laboratory. All specimens were stored under refrigerated conditions (4 °C) and examined within 1–2 days after harvesting**.** Broken or dead mollusks were discarded.

From each sample, 25–30 live mussels (approximately 100–180 g) were taken. After scrubbing under tap water, mussels were allowed to dry, disinfected with 70% ethanol, and shucked aseptically using a sterilized knife. The flesh with the intervalvular fluids (mussel inner content) were homogenized for 2 min using a stomacher 400 Lab Blender (Seward Medical, London, UK).

2.2. Isolation, identification, and serotyping of *L. monocytogenes*

Isolation of *L. monocytogenes* was carried out according to procedures detailed in ISO 11290-1: 1996/FDAM 1: 2004 (E) (18). Briefly, a portion (25 g) of blended sample was added to 225 mL of the primary enriched medium half Fraser broth and incubated (24 h, 30 °C). Afterwards, a secondary enrichment was prepared by transferring an aliquot (0.1 mL) of the primary enriched culture to 10 mL of the secondary enriched medium Fraser broth (48 h, 30 °C). A loopful (10 µL) of the primary and secondary enriched cultures were streaked onto Agar Listeria Ottaviani Agosti (Biolife, Milan, Italy) and Oxford agar and colonies examined after 24 h and 48 h (37 °C). Five presumptive *Listeria* spp. colonies from each plate were selected at random and checked for purity on Tryptone Soya agar with yeast extract (24 h, 37 °C) before identification and serotyping. All media used were obtained from Merck KGaA (Darmstadt, Germany) unless otherwise stated.

For the identification of *Listeria* spp., pure cultures in Tryptone Soya agar were submitted to a multiplex polymerase chain reaction (PCR) assay, as described by Lawrence and Gilmour (19). This method uses genus- and species-specific primers and gives 3 results: a band indicative of bacterial DNA, *Listeria* spp., and *Listeria monocytogenes*.

Strains identified as *L. monocytogenes* were further serotyped using a second multiplex-PCR assay, as described by Doumith et al. (10). This system uses 4 primer pairs specific for *L. monocytogenes* in addition to 1 primer pair specific for *Listeria* spp. This method clusters the 4 major *L. monocytogenes* serotypes (1/2a, 1/2c, 1/2b, and 4b) into 4 molecular serogroups (group 1: serotypes 1/2a, 3a; group 2: 1/2c, 3c; group 3: 1/2b, 3b, 7; and group 4: 4b, 4d, 4e). Appropriate positive and negative controls were included in all assays.

2.3. Antimicrobial resistance testing

The antimicrobial resistance testing of the isolates was performed using the disk diffusion method on Mueller-Hinton agar plates (BBL) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). The isolates were tested against the following antimicrobial agents: ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamycin (10 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), streptomycin (10 µg), sulfamethoxazole+trimethoprim (23.75/1.25 µg), tetracycline (30 µg), and vancomycin (30 µg). Because there are currently no interpretative criteria provided for *Listeria* by the CLSI, with the exception of susceptibility breakpoints for ampicillin and penicillin, the CLSI criteria for staphylococci were applied. *E. coli* ATCC 25922 and *St. aureus* ATCC 29213 were used as control strains in the present study.

3. Results

A total of 102 samples of aquacultured mussels (*Mytilus galloprovincialis*), collected from 9 approved production sites in the North Aegean sea, were examined for the presence of *L. monocytogenes*. *Listeria* spp. were found in 8 out of the 102 samples tested (approximately 8%) and only 1 (approximately 1%) yielded *L. monocytogenes*. The unique strain of *L. monocytogenes* isolated was identified as serotype 1/2a (or 3a) belonging to serogroup I. The only isolate of *L. monocytogenes* recovered in this study was found resistant to nalidixic acid and streptomycin and susceptible to cephalothin, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, ampicillin, gentamicin, and chloramphenicol. It showed intermediate resistance to penicillin, vancomycin, and neomycin.

4. Discussion

In our study, the incidence rates of *Listeria* spp. (approx. 8%) and *L. monocytogenes* (approx. 1%) in mussels harvested in the North Aegean Sea are very low. Our results can be compared with findings reported in a study carried out on mussels along the Moroccan southern Atlantic coast by El Marrakchi et al. (21), who found that 4.5% and 0.6% of the mussels they examined were contaminated with *Listeria* spp. and *L. monocytogenes*, respectively. In Spain, higher contamination rates were observed by de Simon et al. (6); proportions of 22.5% and 7.5% of 40 fresh mussel samples collected at market contained organisms of *Listeria* spp. and *L. monocytogenes*, respectively. Ferrer and de Simon (22) also found that 8.6% of the Spanish mussels contained *L. monocytogenes*. A much higher prevalence (90%) was reported for mussels harvested from the Goyen River (Brittany), an area not approved for shellfish rearing (7). Failure to detect *L. monocytogenes* has also been documented. The pathogen was not detected in samples obtained from mussels suspended in the Adriatic Sea adjacent to Cattolica (Rivini, Italy) (23). Nevertheless, the diversity in results reported by several authors may be attributed to certain factors such as seawater conditions in the shellfish, which differ markedly from country to country; moreover, the sampling plan and the methodology used must be taken into account.

Generally, the incidence of *L. monocytogenes* in shellfish harvested from monitored grouping sites is very low. On the rare occasions when *L. monocytogenes* contamination has occurred, improper harvesting, handling, processing, or sanitation has often been the cause (24). It is interesting to note that all *Listeria* isolates were detected in samples obtained from mussels suspended in the Thermaikos Gulf and this might be attributed to the bacterial loads of the main rivers in Greece and the drainage ditches that flow in Thermaikos Gulf. In addition, the prevailing currents within the area of the gulf could disperse the discharge of the bacteria from the river mouth to the mussel breeding area, leading to the contamination of the mussels.

Molecular serotyping in the present study, using a rapid multiplex PCR assay described by Doumith et al. (10), showed that the only *L. monocytogenes* isolate belonged to the molecular serogroup I that contains serotypes 1/2a and 3a. Although the reported multiplex PCR method does not differentiate serovar 1/2a from 3a (10), this disadvantage would not decrease the efficiency of the multiplex PCR assay in long-term epidemiological studies of *L. monocytogenes* (11). Previous studies have reported the presence of serotypes 1/2a and 3b in bivalve shellfish (25–27), whereas de Simon et al. (6) found serovars 1/2c and 4b in bivalve market samples. According to the literature, serotype 1/2a is the most frequently isolated strain from contaminated foods (2,8,9).

From the food-safety point of view, bivalve mollusks are very interesting organisms because they have some distinctive characteristics that magnify bacterial hazards. Bacterial contamination of mussels has been described as a rapid phenomenon when mussels are active; in the process of filter feeding, bivalve shellfish are able to filter large amounts of water and subsequently accumulate in their digestive tract (28) human pathogens naturally occurring in aquatic environments and/or derived from sewage-polluted waters (29). In addition to bioaccumulation, immediately after the harvest, the loose texture of the flesh, the high water activity ($a_w > 0.95$), the high glycogen and free amino acids content, and high pH (6.7–7.1) of mussels make them an ideal substrate for the growth of microorganisms (3). The above hazards are compounded by the traditional consumption of the entire animal, including the viscera, in the raw state or after minimal heat treatment (29).

Antimicrobial resistance in *L. monocytogenes*, as a result of the uptake of antibiotic resistance genes from other gram-positive bacteria, is of major public health importance due to the high mortality rates associated with listeriosis. Interestingly, the one isolate of *L. monocytogenes* recovered in our study was found susceptible to antibiotics most commonly used for the resolution of infections both in human (such as a β-lactam antibiotic alone or in combination with an aminoglycoside, or the association of trimethoprim with a sulfonamide) and veterinary (ampicillin or tetracycline) medicine (30). Our isolate was found surprisingly sensitive to cephalothin while *Listeria* spp. have been reported to be naturally and intrinsically resistant to cephalosporins (16). Resistance detected to streptomycin has been also reported in other studies involving *Listeria* isolated from various sources (15).

In conclusion, this study demonstrated that *L. monocytogenes* is not commonly found in mussels harvested in the North Aegean Sea, whereas there is a higher possibility of mussels' contamination with other *Listeria* species. In addition, the presence of *L. monocytogenes* serogroup I in our study is of public health concern, because of the possibility of causing human listeriosis. Public health protection involves both the preventing actions to ensure the safety of shellfish growing areas and the recommendation to people that have a high risk of infection to avoid consumption of raw or inadequately cooked molluscan shellfish.

Acknowledgments

The authors thank the National Reference Laboratory on Marine Biotoxins, Centre of Veterinary Institutions of Thessaloniki, for their contribution to sampling.

References

1. General Secretariat of National Statistical Service of Greece. Quantity of Seafoods. Table B4. Athens, Greece: General Secretariat of National Statistical Service of Greece; 2009.
2. Swaminathan B, Cabanes D, Zhang W, Cossart P. *L. monocytogenes*. In: Doyle MP, Beuchat LR, editors. Food Microbiology: Fundamentals and Frontiers. Washington, DC, USA: ASM Press; 2007. pp. 457–491.
3. Jay JM, Loessner MJ, Golden DA. Modern Food Microbiology. 7th ed. New York, NY, USA: Springer Science and Business Media; 2005.
4. Croonenbergs RE. Contamination of shellfish-growing waters. In: Martin RE, Carter EP, Flick GJ Jr, Davis LM, editors. Marine and Freshwater Products Handbook. Lancaster, PA, USA: Technomic Publishing Company Inc.; 2000. pp. 665–693.
5. Rodas-Suárez OR, Flores-Pedroche JF, Betacourt-Rule JM, Quinõnes-Ramírez EI, Vazquez-Salinas C. Occurrence and antibiotic sensitivity of *L. monocytogenes* strains isolated from oysters, fish, and estuarine water. Appl Environ Microb 2006; 72: 7410–7412.
6. de Simon M, Terrago C, Ferrer MD. Incidence of *L. monocytogenes* in fresh foods in Barcelona (Spain). Int J Food Microbiol 1992; 16: 153–156.
7. Monfort P, Minet J, Rocourt J, Piclet G, Cormier M. Incidence of *Listeria* spp. in Breton live shellfish. Lett Appl Microbiol 1998; 26: 205–208.
8. Jacquet C, Gouin E, Jeannel D, Cossart P, Rocourt J. Expression of ActA, Ami, InlB, and listeriolysin O in *L. monocytogenes* of human and food origin. Appl Environ Microb 2002; 68: 616–622.
9. Kathariou S. *L. monocytogenes* virulence and pathogenicity, a food safety perspective. J Food Prot 2002; 65: 1811–1829.
10. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major *L. monocytogenes* serovars by multiplex PCR. J Clin Microbiol 2004; 42: 3819–3822.
11. Chen Y, Knabel SJ. Multiplex PCR for simultaneous detection of the genus *Listeria*, *Listeria minocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. Appl Environ Microb 2007; 73: 6299–6304.
12. Aureli P, Ferrini AM, Mannoni V, Hodzic S, Wedell-Weergaard C, Oliva B. Susceptibility of *L. monocytogenes* isolated from food in Italy to antibiotics. Int J Food Microbiol 2003; 83: 325–330.
13. European Food Safety Authority (EFSA). The European Union summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in 2010. EFSA J 2012; 10: 2597.
14. de Cesare A, Manfreda G, Macri M, Cantoni C. Application of automated ribotyping to support the evaluation of the *L. monocytogenes* source in a Taleggio producing plant and to predict the risk human health linked to the accidental sale of contaminated cheese. Arch Vet Ital 2006; 57: 231–240 .
15. Antunes P, Reu C, Sousa JC, Pestana N, Peixe L. Incidence and susceptibility to antimicrobials agents of *Listeria* spp. and *L. monocytogenes* isolated from poultry carcasses in Porto, Portugal. J Food Prot 2002; 65: 1888–1893.
16. Yusel N, Citak S, Onder M. Prevalence and antibiotic resistance of *Listeria* species in meat products in Ankara, Turkey. Food Microbiol 2005; 22: 241–245.
17. Arslan S, Özdemir F. Prevalence and antimicrobial resistance of *Listeria* spp. in homemade white cheese. Food Control 2008; 19: 360–363.
18. International Organization for Standardization. Microbiology of food and animal feeding. Horizontal method for the detection and enumeration of *L. monocytogenes*. ISO 11290-1: 1996/ FDAM 1: 2004 (E). Geneva, Switzerland: ISO; 2004.
19. Lawrence LM, Gilmour A. Incidence of *Listeria* spp. and *L. monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. Appl Environ Microb 1994; 60: 4600–4604.
20. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; 14th Informational Supplement M100–S14. Vol. 24, No 1. Wayne, PA, USA: CLSI; 2004.
21. El Marrakchi A, Boumhandi R, Hamama A. Performance of a new chromogenic plating medium for the isolation of *L. monocytogenes* from marine environments. Lett Appl Microbiol 2005; 40: 87–91.
22. Ferrer MD, de Simon M. Isolation of *Salmonella* spp., *V. parahemolyticus*, *Y. enterocolytica* and *L. monocytogenes* in bivalve mollusks. Alimentaria 1993; 30: 33–35.
23. Vernocchi P, Maffei M, Lanciotti R, Suzzi G, Gardini F. Characterization of Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Adriatic Sea (Italy). Food Control 2007; 18: 1575–1583.
24. Bremer PJ, Fletcher GC, Osborne C. *L. monocytogenes* in seafood. Auckland, New Zealand: New Zealand Institute for Crop & Food Research Limited; 2003.
25. Brett MSY, Short P, McLauchlin J. A small outbreak of listeriosis associated with smoked mussels. Int J Food Microbiol 1998; 43: 223–229.
26. Inoue S, Nakama A, Arai Y, Kokubo Y, Maruyama T, Salto A, Yoshida T, Terao M, Yamamoto S, Kumagai S. Prevalence and contamination levels of *L. monocytogenes* in retail food in Japan. Int J Food Microbiol 2000; 59: 73–77.
27. Pinto AL, Teixeira P, Castilho F, Felicio MT, Pombal F, Eibbs PA. Prevalence and serotyping of *L. monocytogenes* in Portuguese live bivalve molluscs sampled in various steps along the sanitary control process. Aquac Res 2006; 37**:** 1112–1116.
28. Kueh CSW, Chan K. Bacteria in bivalve shellfish with special reference to the oyster. J Appl Bacteriol 1985; 59: 41–47.
29. International Commission on Microbiological Specifications for Foods. Microorganisms in Foods 6. Microbial Ecology of Food Commodities. 2nd ed. New York, NY, USA: Kluwer Academic; 2005.
30. Aarestrup FM. Other pathogens. In: Aarestrup FM, editor. Antimicrobial Resistance in Bacteria of Animal Origin. Washington, DC, USA: ASM Press; 2006. pp. 249–267.