

Prevalence and serotyping of *Listeria monocytogenes* in Portuguese live bivalve molluscs sampled in various steps along the sanitary control process

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Abstract

The prevalence and contamination levels of *Listeria monocytogenes* were investigated in live bivalve molluscs for human consumption, collected in various steps of the commercial and sanitary circuits in the North of Portugal. Samples of different species were collected per lot before and after depuration treatment in two depuration units and further, when placed in retail markets. *Listeria monocytogenes* was isolated from 4% of the samples although with very low contamination levels (less than 100 MPN g⁻¹). None of the environmental (non-depurated) samples were contaminated with the food-borne pathogen. The positive samples involved depurated and commercialized samples from two lots, one in each circuit. Two serotypes (1/2a and 3b) were detected among the isolated strains. This study demonstrated the possibility of occurrence of *L. monocytogenes* contamination of live bivalve molluscs along the sanitary control circuit, including at the retail market level. As *L. monocytogenes* grows well at refrigerated temperatures and in high salt matrixes such as seafood its presence in these products should not be overlooked.

Keywords: *Listeria monocytogenes*, prevalence, serotyping, live bivalve molluscs, sanitary control

Introduction

Listeria monocytogenes is a non-sporing ubiquitous bacterium, Gram-positive facultatively anaerobic rod

(Farber & Peterkin 1991). The presence of this pathogen in foods, particularly ready-to-eat (RTE) foods, gained public health significance from several outbreaks of listeriosis in North America and Europe. It is known to be particularly hazardous for high-risk groups such as immuno-compromised people, the elderly, pregnant women and neonates, and has a high mortality rate in these individuals of 20–30% (Farber 1991a). A recent study showed that healthy individuals can also develop milder forms of gastrointestinal illness upon exposure (Dalton, Austin, Sobel, Hayes, Bibb, Graves, Swaminathan, Proctor & Griffin 1997).

Bivalve molluscs are among the most consumed seafoods in Portugal as in some other countries, preferably in the raw state or lightly cooked. The microbiological quality control of these RTE foods therefore assumes particular importance (Ben Embarek 1994). The main objective of sanitary control, characterized by a set of steps and procedures, is the guarantee of public health protection. The process depends on the production area classification and monitoring, and on purification and certification of the products in depuration centres. While shellfish has not caused any large-scale outbreaks of listeriosis, small outbreaks and sporadic cases have been caused by smoked mussels and raw oysters (Brett, Short & McLaughlin 1998; Monfort, Minet, Rocourt, Piclet & Cormier 1998; Plusquellec, Monfort, Piclet & Rio 1998). The scarcity of relevant data in our country and the need to provide new information about the potential of raw bivalves as a source of *L. monocytogenes* contamination led us

to carry out this work, performing a limited study on the prevalence of *L. monocytogenes* in Portuguese live bivalve molluscs, sampled in various steps of the sanitary control circuit. The serovar identity of the isolates was determined as an epidemiological indicator.

Material and methods

Samples and sites

A total of 75 live bivalve mollusc samples were collected from January to July 2004 from two production sites, and analysed for *L. monocytogenes* along the sanitary control process as shown in Fig. 1. Each lot of shellfish species was sampled in three steps of the circuit: production sites, after depuration treatment in two depuration units (DA and DB) and in two retail markets (MA and MB).

The harvesting sites are situated along the North coast of Portugal (from Aveiro to Vila Praia de Âncora). Bivalve samples, including oysters, mussels, clams, cockles and razor shells, of 1 kg were collected at each sampling site, stored and transported at 6–8 °C to the laboratory for analysis. Fifteen to 35 individuals according to the species analysed were scrubbed, rinsed with tap water and opened aseptically.

Listeria monocytogenes detection and quantification

Listeria monocytogenes detection was performed using the MINI-VIDAS-LMO2 test kit (bioMérieux[®], Marcy L'Etoile, France) following the manufacturer's instructions. This automated qualitative test has obtained AOAC International Official Methods of Analysis approval. The confirmation of positive samples was performed as described in the MINI-VIDAS-LMO2 protocol, streaking 10 µL of the Fraser broth on PALCAM (Merck, Darmstadt, Germany) and Oxford agar plates (Merck) and incubating at 37 °C for 24 h. Five to 10 typical colonies from each selective agar

were picked, purified on tryptic soy agar (Lab M, Bury, UK) with 0.6% wv⁻¹ yeast extract (Lab M; TSAYE), incubated at 37 °C for 24 h and maintained on TSAYE slants at 4 °C. The *L. monocytogenes* isolates were phenotyped and biochemically characterized by Gram stain, cell and colony morphology, catalase test, tumbling motility by microscopy, the ability to ferment rhamnose (1.0% wv⁻¹) but not xylose (0.5% wv⁻¹) or mannitol (0.5% wv⁻¹), haemolysis on Sheep Blood Agar (Merck) and CAMP test with *Staphylococcus aureus* (NCTC 25923) and *Rhodococcus equi* (NCTC 1621). For quantification of *L. monocytogenes* in the positive samples, the most probable number (MPN) method was used according to Vaz-Velho (2000).

Serotyping

Listeria monocytogenes serotyping was performed using two methods, i.e. the *L. monocytogenes* Seiken kit (Denka Seiken, Tokyo, Japan) following the manufacturer's instructions and, multiplex polymerase chain reaction (PCR) according to Doumith, Buchrieser, Glaser, Jacquet and Martin (2004) using the conditions described by the authors.

Results

Table 1 shows the prevalence of *L. monocytogenes* detected in the live bivalve molluscs, i.e. 4% (three of 75 samples).

None of the bivalve molluscs collected at the harvesting sites contained the pathogen. The three positive samples were found only in depurated clams, although of two different species (*Venerupis pullastra* and *Tapes philippinarum*), demonstrating very low contamination levels (less than 100 MPN g⁻¹; data not shown). Two lots were involved (one in each circuit), two of the positive samples being from the same lot (circuit A).

The serotypes of *L. monocytogenes* were also identified. The multiplex PCR assay clusters *L. monocyto-*

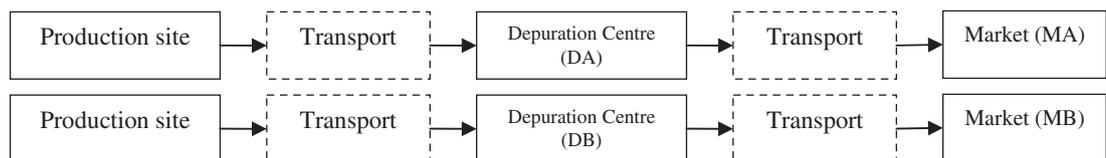


Figure 1 Simplified sanitary control process of live bivalve molluscs (sampling sites are indicated in full boxes).

Table 1 *Listeria monocytogenes* prevalence in shellfish

Circuit	Bivalve species	No. of lots	No. of samples	No. of positive samples for <i>L. monocytogenes</i>		
				Production sites	After depuration	Market
A	Oysters (<i>Crassostrea angulata</i> , Lamarck, 1835)	1	3			
	White clam (<i>Spisula solida</i> , L.)	3	9		1	1
	Japanese Clam (<i>Tapes philippinarum</i> , Reeve, 1864)	1	3			
	Clam (<i>Venerupis pullastra</i> , Montagu, 1803)	4	12			
	Cockle (<i>Cerastoderme edule</i> , L.)	2	6			
	Mussel (<i>Mytilus galloprovincialis</i> , Lamarck, 1819)	1	3			
	Razor Shell (<i>Solen marginatus</i> , Pulteney, 1799)	2	6			
B	Oysters (<i>Crassostrea angulata</i> , Lamarck, 1835)	1	3			
	White clam (<i>Spisula solida</i> , L.)	2	6			
	Japanese Clam (<i>Tapes philippinarum</i> , Reeve, 1864)	4	12			1
	Clam (<i>Venerupis pullastra</i> , Montagu, 1803)	2	6			
	Cockle (<i>Cerastoderme edule</i> , L.)	1	3			
	Mussel (<i>Mytilus galloprovincialis</i> , Lamarck, 1819)	1	3			
	Razor Shell (<i>Solen marginatus</i> , Pulteney, 1799)	0	0			
Total	25	75	0	1	2	

Table 2 Serogrouping of the isolated *Listeria monocytogenes* strains

Shellfish species	Circuit	<i>L. monocytogenes</i> serotype group		
		Production site	After depuration	Market
Clams (<i>Venerupis pullastra</i>)	A	–	1/2a (3a)	1/2a (3a)
Clams (<i>Tapes philippinarum</i>)	B	–	–	3b

genes strains into four major groups (group 1: serotypes 1/2a, 3a; group 2: 1/2b, 3b, 7; group 3: 1/2c, 3c; and group 4: 4b, 4d, 4e; Doumith *et al.* 2004). Owing to the inability to serotype the two positive isolates from the circuit A by conventional serotyping, the authors used the multiplex PCR (Doumith *et al.* 2004). The two strains isolated in sanitary circuit A belonged to the serotype group 1, i.e. 1/2a (3a) identified by multiplex PCR and, the strain from circuit B belonged to the serotype group 2, i.e. 1/2b (3b). In this case, as it was possible to use the Seiken kit, the serotype 3b was confirmed (Table 2).

Discussion

Compared with other food products, very few studies have been carried out on the prevalence and growth

of *L. monocytogenes* in shellfish. The limited sampling of bivalves along the circuit accomplished in this study demonstrated that these RTE seafoods could be contaminated with *L. monocytogenes*, including at the retail market level. In previous studies conducted in Portugal, Pedro (1996) isolated *Listeria* spp. and *L. monocytogenes* in 23% and 20%, respectively, from 35 shellfish samples; Pereira, Guerra and Bernardo (2001) found *L. monocytogenes* in 13.1% of 61 shellfish samples; and Mena, Almeida, Carneiro, Teixeira, Hogg and Gibbs (2004) did not find any shellfish contaminated with the pathogen. Other studies cited in the literature indicate some similar prevalences: Weagant, Sado, Colburn, Torkelson, Stanley, Krane, Shields and Thayer (1988), Buchanan, Stahl, Bencivengo and Corral (1989), Motes (1991) and Jeyasekaran, Karunasagar and Karunasagar (1996) did not find *Listeria* spp. including *L. monocytogenes* in shellfish; Colburn, Kaysner, Abeyta and Wekell (1990) found *Listeria* spp. with frequencies of 1–5%; De Simón, Tarragó and Ferrer (1992) detected *L. monocytogenes* in 7.5% (three of 40) of the bivalve samples collected at markets and Monfort *et al.* (1998) obtained a higher prevalence of *L. monocytogenes* in live shellfish of 11.2%. In general, the levels per gram of the organism in fish products (with the exception of cold-smoked salmon) appear to be lower than 10^2 – 10^3 CFU g⁻¹ (Farber 1991b). Nevertheless, it is important to take into account that shellfish have been implicated in listeriosis outbreaks (e.g. Monfort *et al.* 1998).

The inability to serotype the positive isolates from the circuit A by conventional serotyping led the authors to use the multiplex PCR (Doumith *et al.* 2004). In fact, Graves, DeWitt, Mauro, Bordoni and Swaminathan (2005) observed agreement between multiplex PCR and conventional serotyping methods of 94% and suggested multiplex PCR as an alternative method for untypable strains by conventional methods. Cordano and Rocourt (2001) as well as Graves *et al.* (2005) also found strains untypable by conventional serotyping.

Brett *et al.* (1998) and Inoue, Nakama, Arai, Koku-bo, Maruyama, Saito, Yoshida, Terao, Yamamoto and Kumagai (2000) found the same serovars in bivalve shellfish, whereas De Simón *et al.* (1992) found serovars 1/2c and 4b in bivalve market samples. Most human listeriosis cases (> 95%) are caused by strains belonging to serotypes 1/2a, 1/2b and 4b (Farber & Peterkin 1991); however, other factors influence the occurrence of listeriosis.

More information dealing with *L. monocytogenes* contamination in bivalve molluscs should be collected. Many questions are still unanswered. It appears important to understand the behaviour of *Listeria* spp. including *L. monocytogenes* in aquatic environments, the role of depuration in pathogen reduction/elimination, its relationship with faecal indicators, the ecology of the organism in the shellfish-processing environment, seasonal variations, etc. Moreover, as a psychrotrophic bacterium, *L. monocytogenes* can grow at low temperatures and, under conditions of high salt content (Handa, Kimura, Takahashi, Koda, Hisa & Fujii 2005). Thus, refrigerated storage, placement in ice or water used to cool shellfish in markets, among other factors such as transport and handling, could contribute to the introduction and persistence of the organism in the sanitary control and commercial circuits.

In conclusion, this study demonstrated the presence of *L. monocytogenes* in depurated and commercialized live bivalve molluscs in Portuguese markets. Given the low levels of the pathogen in the analysed samples and their relatively short shelf-life (rarely consumed after 1 week of refrigerated storage), unless these shellfish are temperature abused, *L. monocytogenes*-contaminated bivalve molluscs should not represent a serious health hazard. Nevertheless, the need for consumer education in Portugal, regarding safe food-handling practices with respect to *Listeria* spp. contamination, is evident (Azevedo, Regalo, Mena, Almeida, Carneiro, Teixeira, Hogg & Gibbs 2005).

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References

- Azevedo I., Regalo M., Mena C., Almeida G., Carneiro L., Teixeira P., Hogg T. & Gibbs P.A. (2005) Incidence of *Listeria* spp. in domestic refrigerators in Portugal. *Food Control* **16**, 121–124.
- Ben Embarek P.K. (1994) Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. *International Journal of Applied Bacteriology* **68**, 189–198.
- Brett M.S.Y., Short P. & McLauchlin J. (1998) A small outbreak of listeriosis associated with smoked mussels. *International Journal of Food Microbiology* **43**, 223–229.
- Buchanan R.L., Stahl H.G., Bencivengo M.M. & Corral F.D. (1989) Comparison of lithium chloride-phenylethanol-moxalactam and modified Vogel Johnson agars for detection of *Listeria* spp. in retail-level meats, poultry and seafood. *Applied and Environmental Microbiology* **52**, 59–63.
- Colburn K.G., Kaysner C.A., Abeyta C. & Wekell M.M. (1990) *Listeria* species in a California coast estuarine environment. *Applied and Environmental Microbiology* **56**, 2007–2011.
- Cordano A.M. & Rocourt J. (2001) Occurrence of *Listeria monocytogenes* in food in Chile. *International Journal of Food Microbiology* **70**, 175–178.
- Dalton C.B., Austin C.C., Sobel J., Hayes P.S., Bibb W.E., Graves L.M., Swaminathan B., Proctor M.E. & Griffin P.M. (1997) An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* milk. *The New England Journal of Medicine* **336**, 100–106.
- De Simón M., Tarragó C. & Ferrer M.D. (1992) Incidence of *Listeria monocytogenes* in fresh foods in Barcelona (Spain). *International Journal of Food Microbiology* **16**, 153–156.
- Doumith M., Buchrieser C., Glaser P., Jacquet C. & Martin P. (2004) Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *Journal of Clinical Microbiology* **42**, 3819–3822.
- Farber J.M. (1991a) *Listeria monocytogenes*. *Journal of Association of Official Analytical Chemists* **74**, 701–704.
- Farber J.M. (1991b) *Listeria monocytogenes* in fish products. *Journal of Food Protection* **54**, 922–934.
- Farber J.M. & Peterkin P.I. (1991) *Listeria monocytogenes* a foodborne pathogen. *Microbiology Reviews* **55**, 476–511.
- Graves L., DeWitt W., Mauro L., Bordoni P. & Swaminathan B. (2005) Comparison of a multiplex PCR assay and conventional serotyping for sero-classification of *Listeria mono-*

- cytogenes* isolates. In: *9th Annual PulseNet Update Meeting*, 9–11 May 2005, Seattle, WA, USA. http://www.cdc.gov/pulsenet/events/pages/pn_update_2005.htm
- Handa S., Kimura B., Takahashi H., Koda T., Hisa K. & Fujii T. (2005) Incidence of *Listeria monocytogenes* in raw seafood products in Japanese retail stores. *Journal of Food Protection* **65**, 411–415.
- Inoue S., Nakama A., Arai Y., Kokubo Y., Maruyama T., Saito A., Yoshida T., Terao M., Yamamoto S. & Kumagai S. (2000) Prevalence and contamination levels of *Listeria monocytogenes* in retail food in Japan. *International Journal of Food Microbiology* **59**, 73–77.
- Jeyasekaran G., Karunasagar I. & Karunasagar I. (1996) Incidence of *Listeria* spp. in tropical fish. *International Journal of Food Microbiology* **31**, 333–340.
- Mena C., Almeida G., Carneiro L., Teixeira P., Hogg T. & Gibbs P.A. (2004) Incidence of *Listeria monocytogenes* in different food products commercialized in Portugal. *Food Microbiology* **21**, 213–216.
- Monfort P., Minet J., Rocourt J., Piclet G. & Cormier M. (1998) Incidence of *Listeria* spp. in Breton live shellfish. *Letters of Applied Microbiology* **26**, 205–208.
- Motes M.L. (1991) Incidence of *Listeria* spp. in shrimp, oysters, and estuarine waters. *Journal of Food Protection* **54**, 170–173.
- Pedro S. (1996) *Occurrence of Listeria in seafood and its ecore-sistence in aquatic environment*. Thesis submitted to the Universidade Técnica de Lisboa to attain the degree of Master in Food Science and Technology, 123pp.
- Pereira F., Guerra M.M. & Bernardo F.A. (2001) Natural occurrence of *Vibrio* spp. and *Listeria monocytogenes* in bivalve molluscan in Portugal. *Journal of Shellfish Research* **20**, 1229–1233.
- Plusquellec A., Monfort P., Piclet G. & Rio L. (1998) *Listeria innocua* and *Salmonella panama* in mussels: a comparative study. *Journal of Food Protection* **61**, 1137–1142.
- Vaz-Velho M.M. (2000) *Cold-Smoked Fish-Processing and Safety*. PhD thesis in Biotechnology, Food Science and Engineering, Universidade Católica Portuguesa, Portugal, 382pp.
- Weagant S.D., Sado P.N., Colburn K.G., Torkelson J.D., Stanley M.H., Krane S.C., Shields S.C. & Thayer C.F. (1988) The incidence of *Listeria* species in frozen seafood products. *Journal of Food Protection* **51**, 655–657.