Efficiency of four secondary enrichment protocols in differentiation and isolation of *Listeria* spp. and *Listeria monocytogenes* from smoked fish processing chains

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Abstract

Four secondary enrichment protocols (conventional methods: UVM II, Fraser 24 h and Fraser 48 h: Impedimetric method: *Listeria* electrical detection medium) were studied for their ability to isolate *Listeria* spp. and *Listeria monocytogenes* from fish and environmental samples collected along the processing chain of cold-smoked fish. From all methods, *Listeria* spp. and *L. monocytogenes* were respectively present in 56 and 34 of 315 samples analysed. Fraser broth incubated for 48 h gave the fewest false negative *Listeria* spp. results [4/56; (7.1%)], but concurrently only 15/34 (44.1%) samples were correctly identified as containing *L. monocytogenes*. *Listeria* electrical detection (LED) medium detected only 36/56 (64.3%) *Listeria* spp. positive samples. Despite this lower isolation rate, LED identified 20/34 (58.8%) *L. monocytogenes* positive samples correctly and gave fewer false positive results. The overall conclusion was that more than one isolation method is needed to accurately estimate *L. monocytogenes* contamination rates.

Keywords: Cold-smoked fish; *Listeria*; *Listeria monocytogenes*; Secondary selective enrichments; Conventional cultural and bactometer capacitance-based detection methods

1. Introduction

The genus *Listeria* includes six species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri* and *L. grayi* (Lovett and Twedt, 1988; Rocourt et al., 1992). The ingestion of *L. monocytogenes* in foods can pose a significant health risk, with a high reported mortality rate for foetuses and immunocompromised patients.

Is important to prevent the contamination of ready-to-eat products, like cold-smoked fish, with *L. monocytogenes*. The mild temperatures ($<30°C$) and low brine concentrations (NaCl, % in aqueous phase ca. 3.5%) used in the cold-smoking process are not sufficient to inactivate *L. monocytogenes* or

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to limit growth of the organism in the product during storage.

Traditional isolation methods for *Listeria* spp. from foods take more than one week to complete. For a food company operating a positive release system this leads to lengthy delays before the product is known to be ''safe''. As a result, there has been interest in developing sensitive and rapid methods for detecting *L. monocytogenes* in foods. Development of fully automated immunological and genetic based methods which are suitable for routine monitoring of food products has been made, though the balance between accuracy, time and the corresponding cost does not always make implementation of these methods commercially worthwhile.

This study was conducted to evaluate the efficacy of individual selective secondary enrichment protocols, three traditional growth media and a bactometer-based capacitance broth, for presumptive differentiation and isolation of *Listeria* spp. from environmental and fish samples taken from salmon, salmon-trout, tuna fish and swordfish cold-smoked processing chains.

2. Materials and methods

During a one-year period, a total of 183 fish samples and 132 environmental samples were collected from the production chain of three Portuguese cold-smoking factories and examined for *Listeria* spp. and *L. monocytogenes*.

The methodology of Rodrigues et al. (1995) and Capell et al. (1995) for *Listeria* isolation from food products, using UVM I as primary enrichment medium, Fraser and *Listeria* electrical detection (LED) broths as secondary enrichment media and selective plating on Oxford agar, was combined with the Jemmi and Keusch (1994) procedure for *Listeria* detection in the cold-smoked fish processing chain using UVM I and UVM II enrichment steps with isolation on Oxford and PALCAM agars. Swabs from fresh salmon and salmon trout skin, from eviscerated fresh salmon belly cavity and from working surfaces (10 cm^2, five swabs per sample) were placed in 25 ml of 0.1% (w/v) peptone water [tryptone, 1 g/l (Lab M MC5, Bury, UK); NaCl 5 g/l (Merck 6404, Darmstadt, Germany)] and transported to the laboratory in chilled boxes and then transferred to 225 ml of UVM I (Merck 110824.0500). Water and ice samples (500 ml) were filtered (0.45 μl sterile membrane filters, Ø 47 mm, Gelman Sciences, MI, USA) and the filters were placed in 20 ml of UVM I. A 25 g amount of processed fish and fish feed was placed in 225 ml of UVM I and homogenised in a stomacher for 2 min. All the UVM I samples were incubated at 30°C for 24 h and an aliquot (0.1 ml) then transferred to 10 ml of two secondary enrichment broths, UVM II [UVM I + 0.013 g/l acriflavine HCl (Sigma A8251, Aldrich, Madrid, Spain)] and Fraser broth (Merck 110398.0500), and incubated at 30°C for 24 h and 24–48 h, respectively. At the same time, 0.05 ml of the UVM I samples was added to 2 ml of LED medium (Rodrigues et al., 1995) that had been prewarmed to 30°C in bactometer module wells (BioMérieux 99052, BioMérieux Vitek, MO, USA). The modules were placed into the Vitek bactometer (Model 120 SC, BioMérieux Vitek), set to monitor capacitance for a test time of 48 h at 30°C. The samples showing presumptive positive characteristics (turbidity in UVM II, darkening in Fraser broth after 24 h or 48 h, and more than 30% change in capacitance within 30 h in LED medium measured by the bactometer) were considered as presumptive positives. All the samples. whether showing growth or not, were subcultured onto Oxford (Merck 107004.0500) and PALCAM (Merck 11755.0500) agars by streaking and incubated at 30°C for 48 h. Colonies presenting typical *Listeria* spp. characteristics were streaked on the tryptone soy yeast extract agar (TSAYE) [tryptone soy broth (Lab M LAB4) + 6 g/l yeast extract (Lab M MCl) + 12 g/l agar (Lab M MC2)] and incubated at 37°C for 24 h, before further confirmation tests.

All the isolates were confirmed to the genus level by Gram staining, catalase and oxidase tests and tumbling motility (tryptone soy broth, 22°C, 24 h), and to the species level by API Listeria (BioMérieux 10300) and the CAMP (Christie et al., 1944, cit. Farber and Peterkin, 1991) test with *Staphylococcus aureus* ATCC 25923 (Food Quality Centre, Escola Superior de Biotecnologia, Porto, Portugal) and *Rhodococcus equi* NCTC 1691 [Leatherhead Food Research Association (LFRA), Surrey, UK] on sheep blood agar plates (BioMérieux 43041).

3. Results and discussion

Four secondary enrichment methods for *Listeria* spp. detection (three conventional and one electrical capacitance method) were simultaneously performed on 183 fish samples and 132 environmental samples.

The sample was considered positive whenever it was confirmed positive in one or more media and negative only if it was confirmed to be negative in all the media simultaneously.

Listeria spp. and *L. monocytogenes* were isolated from 56 and 34 of 315 fish and environmental samples, respectively.

The results from the environmental samples are shown in Table 1.

From a total of 132 samples, nine (7%) contained *Listeria* spp. Six of the nine positive samples (66.7%) contained only *L. monocytogenes* and three samples (33.3%) contained both *L. monocytogenes* and *L. innocua*.

The sensitivity [correct positives rate (%) = correct positives/(correct positives + false negatives); Flanders et al., 1995] of the four protocols was the same (6/9). The number of false presumptive negative results (3/9) obtained was also the same for the four protocols. Considering the individual analysis of each protocol, LED broth showed the highest specificity giving the lowest number of false positive results (32/123). UVM II, the least specific medium, gave the highest number of false positive results (84/123). Fraser 24 h and 48 h broths gave 48/123 and 53/123 false positive results, respectively.

The results from the fish samples are included in Table 1.

From the 183 fish samples, 47 (25.7%) were

Table 1
Detection of *Listeria* spp. in environmental (E.S.) and fish samples (F.S.) from production lines of cold-smoked fish[a]

Protocol	Single protocol results[b]				Combined results[c]	
	Confirmed as correct		Confirmed as incorrect		Confirmed positives/ total positives	
	E.S.	F.S.	E.S.	F.S.	E.S	F.S.
UTVM II						
P.P.	6/90[d]	41/176[d]	84/90[e]	135/176[e]	6/9[f]	47/47[f]
P.N.	42/42[g]	7/7[g]	0/42[h]	0/7[h]	3/9[i]	0/47[i]
Fraser 24 h						
P.P.	6/54[d]	44/133[d]	48/54[e]	89/133[e]	6/9[f]	45/47[f]
P.N.	78/78[g]	50/50[g]	0/78[h]	0/50[h]	3/9[i]	2/47[i]
Fraser 48 h						
P.P.	6/59[d]	45/136[d]	53/59[e]	91/136[e]	6/9[f]	46/47[f]
P.N.	73/73[g]	47/47[g]	0/73[h]	0/47[h]	3/9[i]	1/47[i]
LED						
P.P.	6/38[d]	30/66[d]	32/38[e]	36/66[e]	6/9[f]	31/47[f]
P.N.	93/94[g]	107/117[g]	l/94[h]	10/117[h]	3/9[i]	16/47[i]

[a] P.P. = Presumptive positives; P.N. = presumptive negatives.
[b] Individual analysis of each protocol.
[c] Simultaneous analysis of the four protocols.
[d] Correctly identified and confirmed presumptive positive results after isolation on Oxford and/or PALCAM.
[e] Total presumptive positive results confirmed to be negatives after isolation on Oxford and PALCAM.
[f] Total presumptive positive results confirmed as positive by any of the methods.
[g] Correctly identified presumptive negative results after isolation on Oxford and PALCAM.
[h] Presumptive negative results subsequently confirmed to be positive after isolation on Oxford rind/or PALCAM.
[i] Total presumptive negative results subsequently confirmed to be positive by any of the methods.

confirmed positive for *Listeria* spp. Thirteen of these 47 positive samples (27.7%) contained only *L. innocua*; five (10.6%) contained only *L. monocytogenes*; 20 (42.6%) contained both *L. monocytogenes* and *L. innocua*. In addition, five samples (10.6%), three samples (6.4%) and one sample (2.1%) contained only *L. seeligeri*, both *L. innocua* and *L. welshimeri*, and both *L. innocua* and *L. seeligeri*, respectively.

In terms of sensitivity, LED broth was poor, giving the highest number of false negative results (16/47). However, LED broth exhibited the highest specificity [correct negatives rate (%) = correct negatives/(correct negatives + false positives); Flanders et al., 1995] with the smallest number of false positive results (36/136). Fraser 24 h and 48 h broths gave 88/136 and 91/136 false positive results and 2/47 and 1/47 false negative results, respectively. UVM II presented the lowest specificity giving the highest number of false positives (135/136) but no (0/47) false negative results. Despite *Listeria* spp. being isolated from 40 of the LED method tests, the bactometer only gave 31/47 curves with 30% change within 30 h but no *Listeria* spp. were isolated from one of these presumptive positive LED broths that was confirmed as being positive by other methods; further 10 LED tests, from which *Listeria* spp. were isolated, were considered as presumptive negatives as they gave either less than 30% change in capacitance after 3 h or atypical curves.

The LED method seems to be the best method for environmental samples showing the highest specificty. The reduction in false presumptive positives was achieved without increasing the number of false negatives and the presumptive positive results could be obtained up to 18 h before the traditional methods.

The LED method appears unsuitable for screening of fish samples, due to the high number of false negative results (curves $< 30\%$ change within 30 h). UVM II, although giving no false negative results, seems not to be appropriate as a differential medium, since it gave 219/259 false positive results (using turbidity as a presumptive test). Despite producing 91/136 false positive results for fish samples, Fraser 48 h broth gave a low number of false negative results (1/47) which makes it the elective method of choice for these types of products. This 66% false presumptive positive rate is similar to other reported results for Fraser broth with other foods (70.7%, Warburton et al., 1991; 65.5%, Rodrigues et al., 1995). Commercial application of expensive rapid confirmation techniques to Fraser 48 h broth would suffer from the high false presumptive positive rate.

The isolation rates of *L. monocytogenes* were not equal between the four secondary enrichment protocols (Tables 2 and 3). More than one species was isolated from 26 (46%) of the 56 samples found to contain *Listeria* spp. Despite having the lowest *Listeria* spp. isolation rate, LED medium gave the highest isolation rate of *L. monocytogenes*.

Ravomanana and Rosec (1993) examined 140 food samples and found Fraser enrichment to be more effective and rapid, giving more positive results for both *L. monocytogenes* and *Listeria* spp. after both 24 h and 48 h incubation than UVM II. Kornacki et al. (1993) compared the recovery of *L. monocytogenes* from Fraser broth incubated for 26 h versus 48 h and found 3.3% false negative results at 26 h. In the study of Walker et al., (1990), 23% of

Table 2
Identification of *L. monocytogenes* from test samples found to contain *Listeria* species

Protocol	Oxford	PALCAM	Either agar	Both agars
UVM II	14/47	19/46	20/47	13/46
Fraser 24 h	12/50	11/49	17/50	6/48
Fraser 48 h	11/51	12/51	15/51	8/51
LED (all isolates)	24/46	19/46	15/47	18/45
LED (only presumptive positives)	19/35	16/35	20/36	5/34
Total confirmed	31/56 (55%)	30/56 (54%)	34/56 (61%)[a]	24/56 (43%)[b]
L. monocytogenes				5/56 (9%)[c]

[a] Confirmed *L. monocytogenes* result from any plate of any test.
[b] Confirmed *L. monocytogenes* result from both plates of any test.
[c] Confirmed *L. monocytogenes* result from all plates of all protocols.

Table 3
Species composition of the 56 *Listeria* spp. positive samples

Agar	Protocol	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	Totals
Oxford	UVM II	14	29	4	0	47/56
	Fraser 24 h	12	29	7	2	50/56
	Fraser 48 h	11	32	6	2	51/56
	LED	24 (19)[a]	16 (14)[a]	5 (1)[a]	1 (1)[a]	46 (35)[a]/56
PALCAM	UVM II	19	20	5	2	46/56
	Fraser 24 h	11	32	5	2	49/56
	Fraser 48 h	12	31	6	2	51/56
	LED	19 (16)[a]	21 (17)[a]	4 (0)[a]	2 (2)[a]	46 (35)[a]/56

[a] Only bactometer ''typical curves'' (>30% a change in capacitance in <30 h).

the samples that were positive in Fraser after 48 h incubation were negative after only 24 h. In our study, one extra *Listeria* spp. positive sample was found after 48 h in Fraser broth. However, after 48 h, fewer identifications of the presence of *L. monocytogenes* were made from Oxford or PALCAM agars (Table 2, 24 h: 17/50, 48 h: 15/51). This result could be due to the variability resulting from confirmation of only one colony from each Oxford and PALCAM agar from each secondary enrichment medium. The overgrowth of *L. monocytogenes* by other *Listeria* spp. is a more probable explanation, as the two samples identified as containing *L. monocytogenes* after 24 h but not after 48 h were identified as containing *L. innocua* after 48 h.

Patel and Beuchat (1995) reported Fraser broth to show lower recovery of heat-injured *Listeria* spp., probably due to the presence of lithium chloride. In the LED medium, the concentration of lithium chloride (1.75%) is nearly six-times higher than in Fraser broth. Even so, greater isolation rates of *L. monocytogenes* were obtained from LED than from the other broths when plated onto Oxford and PALCAM agars. Moreover, Cox et al. (1997) found that *L. monocytogenes* could grow at concentrations up to 2% LiCl, but above this level inhibition occurred.

MacDonald and Sutherland (1994), using an impedance technique for *Listeria* detection, never found *L. innocua* and *L. monocytogenes* in the same milk sample, and when they inoculated both species together into the same sample, *L. innocua* outgrew *L. monocytogenes*. In the present study, both species were found together in 23 samples. Curiale and Lewus (1994), reported that *L. innocua* has a shorter generation time than *L. monocytogenes* and that recovery of *L. monocytogenes* from foods using selective broths was lower when *L. innocua* was present. Beumer et al. (1997) stated that enrichment broths having lower acriflavine concentrations and an adequate buffer favoured the isolation of *L. monocytogenes*. The LED medium contained half the acriflavine concentration of UVM II and Eraser broths. As shown in Table 3, the LED medium recovered more *L. monocytogenes* than *L. innocua*. The other broths recovered more *L. innocua* than *L. monocytogenes* which suggests the incidence of *L. monocytogenes* to be underestimated when only high acriflavine media are used and if samples contain both these *Listeria* spp.

The present results. as well as those from several other studies, make it clear that no enrichment broth by itself can assure a 100% recovery rate of *Listeria* spp. or more significantly *L. monocytogenes*. In this study, only 39 of the 56 *Listeria* spp. confirmed positive samples (at least positive by one method) were simultaneously positive by all methods examined.

Although *L. monocytogenes* was found in 34 samples after isolation from either Oxford or PALCAM agars, only five samples (Table 3) were simultaneously positive in all the protocols. If the isolation on either of the selective agars was considered, only nine samples would be simultaneously positive for *L. monocytogenes* in all four broths. These differences between methods underline the probability of underestimation of the occurrence of *L. monocytogenes* when just one protocol is used for its detection. Rather than promoting a single method for detection of *Listeria* spp. and *L. monocytogenes*,

the benefit of using a combination of methods should be stressed.

Acknowledgements

The FAIR project CT95-1207 ‘‘Spoilage and Safety of Cold-Smoked Fish’’ and JNICT for the financial support of Gabriela Duarte, are gratefully acknowledged.

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