Evaluation of enhanced haemolysis agar for detection of *Listeria* spp. and *L. monocytogenes* from production lines of fresh to cold-smoked fish

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Abstract

Enhanced haemolysis agar (EHA) was compared to the two conventional *Listeria* isolation agars Oxford and PALCAM for its ability to detect *Listeria* spp. from production lines of fresh to cold-smoked fish. The ability of EHA for distinguishing *L. monocytogenes* colonies from other *Listeria* spp. was also evaluated.

A total of 243 fish and environmental samples were analysed. Overall, 42 samples were found to contain *Listeria* spp. Only 34 samples were positive simultaneously by the three plating media. Two samples considered to be negative by the two conventional agars were found to be positive after isolation on EHA. All three selective agars were shown to be less effective in recovering *Listeria* spp. after primary enrichment in half-Fraser broth, compared to secondary enrichment in Fraser broth after 24 and 48 h.

From 79 *Listeria* but presumptive negative *L. monocytogenes* colonies, EHA identified correctly 76 *Listeria* spp. and presented three false-negative results—three colonies further identified as *L. monocytogenes* but showing no noticeable haemolysis on EHA. Twenty-three of the thirty-three *L. monocytogenes* presumptive positive colonies, were confirmed positive and ten were identified as *L. seeligeri*.

Despite its ability of distinguishing *L. monocytogenes* from the other *Listeria* spp., unless it is produced as a commercial medium, EHA cannot be an alternative to time-consuming classical identification because the preparation of this medium is both time and labour intensive. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Haemolysis; *Listeria monocytogenes*; *Listeria* selective plating media; *Listeria* spp.

1. Introduction

Listeria monocytogenes is the only species of the genus *Listeria* that has been involved in known foodborne outbreaks of listeriosis. Only the haemolytic species of *Listeria*—*L. monocytogenes*, *L. ivanovii*, *L. seeligeri*—are associated with human pathogenicity. *L. ivanovii* has been reported to be involved in human pathology only rarely, and *L. seeligeri* has been reported only once to be the cause of meningitis in a non-immuno-compromised adult (Lovett and Twedt, 1988). The pathogen has been consistently isolated from production lines of fresh

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to cold-smoked fish (Farber, 1991; Hartemink and Georgsson, 1991; Dillon et al., 1992; Gibson, 1992; Hudson et al., 1992; Ben Embarek, 1994; Fuchs and Nicolaides, 1994; Jemmi and Keusch, 1994; Duarte et al., 1995; Eklund et al., 1995; Rørvik et al., 1995; Loncarevic et al., 1996; Jørgensen and Huss, 1998; Vaz-Velho et al., 1998; Autio et al., 1999). A suspected outbreak of listeriosis caused by cold-smoked salmon-trout and involving nine people was recently reported (Ericsson et al., 1997).

The commercially available plating media for *Listeria* can mask the presence of *L. monocytogenes*, and, even the selection of five suspect colonies at random from such agar media could lead to the detection only of non-pathogenic *Listeria*, even though a few *L. monocytogenes* were present on the plate (Beumer et al., 1997). Furthermore, *L. innocua* has been reported to grow faster than *L. monocytogenes* in selective enrichment broths, which increases the difficulty of isolating this last species (MacDonald and Sutherland, 1994). Therefore, the introduction of an isolation medium on which the pathogen can be differentiated from the non-pathogenic *Listeria* spp., can be achieved using enhanced haemolysis agar (EHA; Beumer et al., 1997). On EHA, *L. monocytogenes* and *L. seeligeri* can lyse ovine erythrocytes in the presence of the enzyme sphingomyelinase. *L. ivanovii* does not show a noticeable haemolysis on EHA.

2. Materials and methods

A total of 243 samples were analysed. Forty-two environmental and 191 fish samples were collected, respectively, at sites before the production head and along the process line of three Portuguese cold-smoking fish plants. All the smoking plants use fresh salmon, imported from Norway, salmon trout from two Portuguese trout farms and swordfish from different suppliers. The fresh salmon from the importer and the salmon trout, water, containers, ice, and polystyrene boxes of the trout farms were also analysed. Ten samples of smoked salmon and swordfish included in this study were collected from a retail outlet.

The fresh salmon and salmon-trout samples and the environmental samples from the trout farms were transported to the laboratory inside cold portable insulated boxes, refrigerated overnight and analysed the day after.

Due to the distance between the fish smoking plants and the laboratory (600 km), all the environmental and fish samples along the smoking processing chain of each factory were maintained in refrigerated conditions and were analysed up to a week after collection.

2.1. Sampling procedure

Ten centimetres square of the fresh fish skin and surfaces were swabbed (five swabs per sample) and the swabs were placed in 25 ml of 0.1% (w/v) peptone water (1 g/l Tryptone (LabM, MC5, Bury, UK) + 5 g/l NaCl (Merck, 6404, Darmstadt, Germany)). Water samples were collected in sterile 500-ml bottles for further filtration. Twenty-five grams of processed fish samples was collected in sterile plastic bags. The samples were transported to the laboratory inside cold portable insulated boxes.

2.2. Isolation procedure

The ISO 11290-1 (1996) analytical protocol was followed. For the swabbed samples, a pre-enrichment step was introduced in the protocol—0.1% peptone water was chosen according to Eklund et al. (1995) sampling procedure for cold-smoked fish processing equipment surfaces. Furthermore, it has been concluded that this pre-enrichment step improves the recovery of *L. monocytogenes* in the same type of samples as well as in fresh fish skin (Vaz-Velho et al., 2001). The water and ice samples were filtered (0.45 μm, ø 47-mm membrane filters, Gelman Sciences, MI, USA) and the filters were placed in 20 ml of primary enrichment broth, half-Fraser broth (Merck, 110398.0500) that contains half of the concentration of the selective supplement (Merck, 110399.0001) compared to Fraser broth. Swabs of the fresh fish and environmental samples in 25 ml of peptone water were transferred to 225 ml of primary enrichment broth and mixed. The 25 g of processed fish was placed in 225 ml of primary enrichment broth and homogenised in a stomacher (Seward 400) for 2 min. All the samples were incubated at 30°C for 24 h. Aliquots of primary enrichment cultures

(0.1 ml) were transferred to 10 ml of secondary enrichment Fraser broth and incubated at 35°C, 24–48 h. Primary and secondary enrichment cultures, whether showing growth or not, were sub-cultured by streaking onto Oxford (Merck, 107004.0500), PALCAM (Merck, 11755.0500) and EHA (Beumer et al., 1997) selective media. The Oxford and PALCAM plates were incubated at 30°C for 48 h and the EHA plates at 37°C for 48 h. EHA has the following composition: Trypticase soy blood agar base EH (Difco 0028-17-9) 40 g/l, 4-methylumbelliferyl-β-D-glucoside (MUβG, Sigma M3633) 50 mg/l, LiCl 5g/l, PALCAM supplement (Merck, 12122.0001) two vials, sphingomyelinase (Sigma S8633) 10 units, and sheep blood (sterile, defibrinated) 50 ml. The medium was prepared following the protocol of Beumer et al. (1997). The EHA plates were observed under daylight and under UV light at 366 nm. Typical *Listeria* colonies on EHA under UV light (366 nm) are surrounded by a very diffuse zone of light, whitish-blue fluorescence, with a yellow matt fluorescent centre after 48 h of incubation. Under daylight, they are translucent, shiny, entire, whitish to light buttery yellow, slightly raised, with a more prominent whitish centre after 48 h of incubation. *L. monocytogenes* colonies are surrounded by a distinct zone of haemolysis. *L. innocua* shows no haemolytic zone; *L. seeligeri* produces zones of haemolysis that are less prominent than those of *L. monocytogenes*. The other *Listeria* species react in the same way as *L. innocua* (Beumer et al., 1997).

When possible, five typical colonies from the three plating media were streaked on Tryptone soy yeast extract agar (Tryptone soy broth (Lab M LAB4) + 6 g/l yeast extract (Lab M, MC1) + 12 g/l agar (Lab M, MC2) and incubated at 37°C for 24 h.

The sample was considered positive for *Listeria* spp. if it was confirmed positive from at least one medium, and negative if it was confirmed negative from all the plating media.

2.3. Confirmation and identification procedures

All the isolates were confirmed to the genus level by Gram, catalase and oxidase tests, and tumbling motility (Tryptone soy broth, 25°C, 24 h), and to the species level by API *Listeria* (BioMérieux, 10300) and by the Christie–Atkins–Munch–Peterson (CAMP) (Christie et al., 1944, cited by 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, Surrey, UK) on sheep blood agar plates (BioMérieux, 43041).

Fifty-seven *L. monocytogenes* isolates were serotyped according to Seeliger and Hohne (1979) in the Food Safety Microbiology Laboratory in London in collaboration with Dr. James McLauchlin, using in-house produced anti-sera.

3. Results and discussion

A total of 201 fish and 42 environmental samples were analysed. *L. monocytogenes* was recovered from the fresh swordfish, fresh salmon-trout, vacuum packed cold-smoked salmon-trout, fresh salmon, vacuum packed cold-smoked salmon and from the water of the lake where the salmon trout are farmed. The overall frequency of *Listeria* spp. found in this experiment was 17% (42/243). *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. grayi* and *L. welshimeri* were present respectively in 12, 22, 11, 2, and 1 samples. Twenty-one strains (37%), isolated from in-processing fish were serovar 4b, while thirty-six strains (63%), isolated from fresh salmon-trout, from the lake where the trout is farmed and from fresh salmon were serovar 1/2a.

It is important to remark that the elective ability of EHA to differentiate *L. monocytogenes* and *L. seeligeri* from the other *Listeria* spp., must be accomplished by the guarantee that this medium is also a good alternative to traditional Oxford and PALCAM selective plating media, showing at least, equal capability for recovering *Listeria* spp. from food samples. For this purpose, colonies should be randomly picked up from the three plating media based respectively on esculin hydrolysis differentiation (Oxford and PALCAM) and appearance under UV and daylight based on the description of Beumer et al. (1997) (EHA). Of course it was impossible to ignore the differentiation among *Listeria* spp. in EHA based on the occurrence of haemolysis when picking up the presumptive positive *Listeria* colonies. This fact did not matter when just one species was

noticeable in the plate. When more than one *Listeria* species were present on the EHA plate, the five *Listeria* spp. colonies, were picked up independently of showing or not haemolysis, to include all the different species present on the plate. For this last reason, in this study, it was possible to compare the ability of the three plating media to detect the genus *Listeria* but not to evaluate their ability for detecting each particular species. The ability of EHA for distinguishing *L. monocytogenes* from the other *Listeria* spp. colonies was also evaluated.

The recovery of *Listeria* spp. by the three plating media is shown in Table 1. The abilities of the three media to detect *Listeria* spp. varied depending on whether the samples were enriched in half-Fraser broth or in Fraser from 24 or 48 h. All the media were shown to be less effective when isolating from Fraser primary enrichment where the competitive microbiota can inhibit or mask the *Listeria* colonies. Oxford medium was the best recovery medium from half-Fraser. EHA was the best recovery medium from Fraser broth after 24 h of incubation. Although, EHA detected one less positive sample than the other agars from Fraser after 48 h, overall, the abilities of the three agars for recovering *Listeria* spp. were considered not markedly different. However, it should be remarked that only 34 of the 42 *Listeria* spp. confirmed samples (at least positive by one method) were simultaneously positive by all the plating media. This finding emphasises the importance of using more than one protocol for the detection of *Listeria* spp. The same conclusion was reported by Duarte et al. (1999), after comparing four different protocols for detection of *Listeria* spp. in the same type of products. Although *L. monocytogenes* was found in 34 samples after isolation from either Oxford or PALCAM agars, only five samples 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 tested. These differences between methods underline the probability of underestimating the occurrence of *L. monocytogenes* when just one protocol is used for its detection.

The species composition of the 42 *Listeria* spp.-positive samples is shown in Table 2. On EHA, most of *L. monocytogenes* colonies were easily distinguished from the other *Listeria* spp. colonies. The *L. monocytogenes* colonies were surrounded by a distinct zone of haemolysis, whereas *L. innocua* and *L. grayi* showed no haemolysis and *L. seeligeri* showed a very faint zone of haemolysis. *L. welshimeri* was only recovered from PALCAM agar. As mentioned above, purposefully not only *L. monocytogenes* typical colonies but also the other *Listeria* spp. typical colonies were picked up from EHA to assure the diversity of the species recovered, and thus to omit the differentiation capability at species level, just highlighting its selectivity behaviour at the genus level. If only presumptive positive *L. monocytogenes* colonies were picked up from EHA, it would be impossible to observe the similarity of species composition in the positive samples detected by the three selective agars (Table 2).

For evaluation of the most important feature of EHA—the elective ability of distinguishing *L. monocytogenes* from the other *Listeria* spp. colonies

Table 1
Comparison of Oxford, PALCAM and EHA selective plating media for isolation of *Listeria* spp.

Enrichment broth	Oxford	PALCAM	EHA	Only Oxford[a]	Only PALCAM[b]	Only EHA[c]	Three media[d]	Either medium[e]
Half-Fraser	34/38	31/38	32/38	3/38	0/38	3/38	27/38	38/42
Fraser, 24 h	36/42	38/42	40/42	1/42	0/42	3/42	34/42	42/42
Fraser, 48 h	38/42	38/42	37/42	1/42	1/42	2/42	33/42	42/42
Total positives	40/42	40/42	41/42	0/42	0/42	2/42	23/42	42

[a] *Listeria* spp.-positive samples detected only by Oxford agar.
[b] *Listeria* spp.-positive samples detected only by PALCAM agar.
[c] *Listeria* spp.-positive samples detected only by EH agar.
[d] *Listeria* spp.-positive samples detected by the three media simultaneously.
[e] *Listeria* spp.-positive samples detected by any of the three media.

Table 2
Species composition of the 42 *Listeria* spp.-positive samples

Medium	Enrichment broth	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. grayi*	Total
Oxford	Half-Fraser	9	17	8	0	0	34/42
	Fraser, 24 h	10	20	8	0	0	38/42
	Fraser, 48 h	9	20	9	0	0	38/42
PALCAM	Half-Fraser	8	16	7	0	0	31/42
	Fraser, 24 h	9	18	10	1	1	39/42
	Fraser, 48 h	10	20	8	0	0	38/42
EHA	Half-Fraser	8	17	7	0	0	32/42
	Fraser, 24 h	8	22	10	0	0	40/42
	Fraser, 48 h	9	19	9	0	1	38/42

based on the haemolytic activity—33 suspect *L. monocytogenes* colonies (presumptive positive *L. monocytogenes* (P.P.)) and 79 suspect *Listeria* spp. colonies (presumptive negative *L. monocytogenes* (P.N.)) from EHA plates were identified by the procedures described above. These results are shown in Table 3. EHA identified correctly 76 *Listeria* spp. and presented only three false-negative results (*L. monocytogenes* colonies showing no noticeable haemolysis on EHA but CAMP-positive). Twenty-three of the thirty-three *L. monocytogenes* presumptive positive colonies, were confirmed to be positive and ten were confirmed to be *L. seeligeri*.

Table 3
Ability of EHA to distinguish *L. monocytogenes* colonies from the colonies of other *Listeria* spp. based on haemolytic properties

Enrichment broth	Confirmed as correct (C.C.)	Confirmed as incorrect (C.I.)
Half-Fraser		
P.P. (14)	9 (a)	5 (b)
P.N. (20)	20 (c)	0 (d)
Fraser, 24 h		
P.P. (10)	6 (a)	4 (b)
P.N. (30)	28 (c)	2 (d)
Fraser, 48 h		
P.P. (9)	8 (a)	1 (b)
P.N. (29)	28 (c)	1 (d)
Total		
P.P. (33)	23 (a)	10 (b)
P.N. (79)	76 (c)	3 (d)

P.P.: presumptive positive (showing haemolysis); P.N.: presumptive negative.

a: Correctly identified presumptive positive and confirmed as *L. monocytogenes*.

b: Presumptive positive *L. monocytogenes* colonies confirmed to be other *Listeria* spp.

c: Correctly identified presumptive negative and confirmed to be other *Listeria* spp.

d: Presumptive negative results confirmed to be positive for *L. monocytogenes*.

The three false-negative colonies were never recovered from half-Fraser broth, whereas 5 of the 10 false-positive colonies were recovered from this primary enrichment. The major problem of distinguishing *L. monocytogenes* colonies from *L. seeligeri* colonies in plates of Fraser primary enrichment, was due to the greater growth of competitive microbiota, and consequently increasing difficulty of differentiating the corresponding typical haemolysis zone. From Fraser after 48 h, only one *L. seeligeri* was misidentified as *L. monocytogenes*.

The presence of acriflavine might lead to decreased recovery of *L. monocytogenes* from EHA (Beumer et al., 1997). As this selective agent is known to repress haemolysis, *L. monocytogenes* colonies might be misidentified as other *Listeria* spp. However, studies with *L. monocytogenes* blood agar (LMBA), another *Listeria* spp. selective plating medium based on haemolytic differentiation where the selectivity of the medium was reduced compared to EHA (no acriflavine), stated that a slightly higher selectivity would have been required for cold-smoked fish samples with high levels of competitive bacteria (Johansson, 1998).

The results of the present study confirm the elective ability of EHA to distinguish *L. monocytogenes* from the other *Listeria* spp., a finding that can improve the detection of the pathogen earlier than by using the standard media Oxford and PALCAM. As

reported by Beumer et al. (1997) and referred previously, the selective plating media used in traditional cultural procedures are not designed to differentiate *Listeria* spp. and the selection of five suspect colonies at random from such media could lead to the detection only of *Listeria* spp. other than *L. monocytogenes*, even though the latter was present on the plate. However, as the ability of EHA to distinguish *Listeria* spp. is based on the occurrence of haemolysis, false-negatives (due to non-haemolytic *L. monocytogenes* strains) and false-positives (due to the presence of *L. seeligeri*) might occur (Beumer et al., 1996). In this study, no non-haemolytic *L. monocytogenes* were found but a few non-haemolytic *L. monocytogenes* strains were found in the same smokeries in a previous survey (Duarte et al., 1995). As in this case where EHA cannot differentiate non-haemolytic *Listeria* spp. from *L. monocytogenes* colonies, further additional tests are required. Thus, colonies isolated by culturing methods must always be confirmed even when EHA is used.

However, despite the advantage of using this selective differential isolation medium as a rapid screening alternative to time-consuming classical identification, unless it is produced as a commercial medium, the preparation is both time- and labour-consuming.

Furthermore, as referred previously, rather than promoting a single method for isolation of *Listeria* spp., the benefit of using a combination of methods should be stressed.

Acknowledgements

The authors gratefully acknowledge EU FAIR Project CT95-1207 “Spoilage and Safety of Cold-Smoked Fish” for financial support.

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