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ORIGINAL ARTICLE

Performance testing of six chromogenic ALOA-type media for the detection of *Listeria monocytogenes*

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Abstract

Aims: The aim of the study was to test the performance of commercially available chromogenic plating media for detection and enumeration of the food-borne pathogen *Listeria monocytogenes*. A wide range of chromogenic media similar to Agar Listeria according to Ottaviani and Agosti (ALOA) were compared using PALCAM agar, according to van Netten *et al.*

Methods and Results: Six chromogenic media similar to ALOA were challenged for inclusivity and exclusivity. Additionally, the ability of chromogenic agars to facilitate growth of stressed *L. monocytogenes* strains and mixed cultures with competitive non-Listeria strains was estimated. Finally, we tested the detection and enumeration of *L. monocytogenes* in artificially inoculated and naturally contaminated food samples. The results of this study indicated that chromogenic media are a good supplementation to PALCAM agar. A single application is not advisable, as the specificity of chromogenic agars is frequently insufficient (50·0–88·9%), particularly in food samples with a complex microflora.

Conclusions: The competitive flora of food samples is able to overgrow low numbers of *L. monocytogenes*, especially in half-Fraser enrichment. This might lead to the underestimation of *L. monocytogenes* positive samples.

Significance and Impact of the Study: Although many evaluation studies of chromogenic agar have been published recently, harmonized validation strategies are lacking. This survey provides a new concept for stepwise testing of plating media.

Introduction

Listeria monocytogenes is a persistent foodborne pathogen. The high-risk group susceptible to this organism includes neonates, pregnant women, the elderly and immunosuppressed individuals (Slutsker and Schuchat 1999). This bacterial organism can be frequently recovered from a wide range of food types and is able to adapt to refrigeration temperatures, vacuum packaging and other stress conditions during and after food processing (Lou and Yousef 1999). The detection and enumeration of *L. monocytogenes* in food and feeding stuffs is generally considered to be a time-consuming procedure (Anon 1996, 1998). Hence, chromogenic media have been developed to facilitate confirmation of, and differentiation between, pathogenic and non-pathogenic Listeria species. This study is focused on chromogenic plating media similar to ALOA (Agar Listeria according to Ottaviani and Agosti). The detection system of ALOA is based on 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, which can be cleaved by β-D glucosidase produced by all *Listeria* spp. Furthermore, the two pathogenic species *L. monocytogenes* and *Listeria ivanovii* can be distinguished from non-pathogenic *Listeria* spp. by their phosphatidylinositol-specific phospholipase C (PI-PLC) activity (Notermans *et al.* 1991). The typical colony morphology of *Listeria* spp. is reported to be turquoise blue. Pathogenic *Listeriae* are additionally surrounded by a translucent halo (Ottaviani *et al.* 1997).

Previous studies compared ALOA with a single other chromogenic medium and standard plating medium PALCAM and/or Oxford (Vlaemynck *et al.* 2000; Leclercq

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2004; El Marrakchi *et al.* 2005; Becker *et al.* 2006; Hegde *et al.* 2006; Willis *et al.* 2006). The purpose of the present survey was to compare a large variety of commercially available ALOA-type plating media with the standard plating medium PALCAM. We developed a comprehensive experimental design based on six consecutive steps, including tests with (i) pure bacterial cultures, (ii) heat-stressed *L. monocytogenes* strains, (iii) challenge tests on mixed cultures, (iv) artificial inoculation of soft cheese and (v) the detection of *L. monocytogenes* in naturally contaminated food samples.

Materials and methods

Test media

The following six chromogenic ALOA-type plating media (T1–T6) from different manufacturers were evaluated. (i) CHROMagar™ Listeria agar (CHROMagar, Paris, France) T1; (ii) OCLA – Oxoid Chromogenic Listeria agar (Oxoid Ltd., Basingstoke, UK) T2; (iii) Compass L. mono ready-to-use agar plates (Biokar Diagnostics/Solabia, Pantin Cedex, France) T3; (iv) OAA – Ottaviani Agosti ready-to-use agar plates (bioMérieux, Marcy l'Etoile, France) T4; (v) Chromoplate® Listeria Selective ready-to-use agar plates according to Ottaviani and Agosti (Merck KGaA, Darmstadt, Germany) T5 and (vi) Listeria ready-to-use agar plates according to Ottaviani and Agosti (Heipha Dr Müller GmbH, Eppelheim, Germany) T6. PALCAM agar (Solabia Biokar Diagnostics, Pantin Cedex, France) was chosen as standard plating medium for the isolation of *Listeria* spp.

Preparation of test strains, streaking technique and confirmation of *Listeria* spp.

Listeria spp. and non-Listeria test strains stored at −80°C as cryobeads were cultivated overnight in Tryptone Soy broth with 6 g yeast extract (TSB-Y) for 18 ± 2 h. The overnight cultures in TSB-Y corresponded to Mc Farland No. 2–4 (bioMérieux, Marcy l'Etoile, France), depending on the maximum growth achieved by each bacterial organism. *Listeria* spp., non-Listeria strains and articificial and natural contaminated food samples were streaked on chromogenic solid media by the semi-quantitative three-loop technique (Fig. 1, Table 1). After incubation for 24 and 48 h at 37 ± 1°C, plates were observed for qualitative recovery and colony morphology. Bacterial growth was recorded and the intensity of growth assessed. No growth corresponded to designation 0, growth until line 1(>5 colonies) was designated +, and growth until the dilution line 2 and 3 was rated ++ and +++ respectively. *Listeria monocytogenes* colonies recovered in the experimental steps 3–5 were confirmed by PCR detection. Confirmation of *Listeria* spp. colonies isolated from food samples was carried out by multiplex PCR targeting the invasion-associated protein (iap) or cell wall hydrolase A (cwhA) respectively (Bubert *et al.* 1999).

Inclusivity and exclusivity (step 1)

Fifty-three *Listeria*, comprising 34 *L. monocytogenes* and 19 other *Listeria* species, were selected for the inclusivity study (Table 1) while 54 non-Listeria strains were cultured for exclusivity testing. The latter consisted of 12

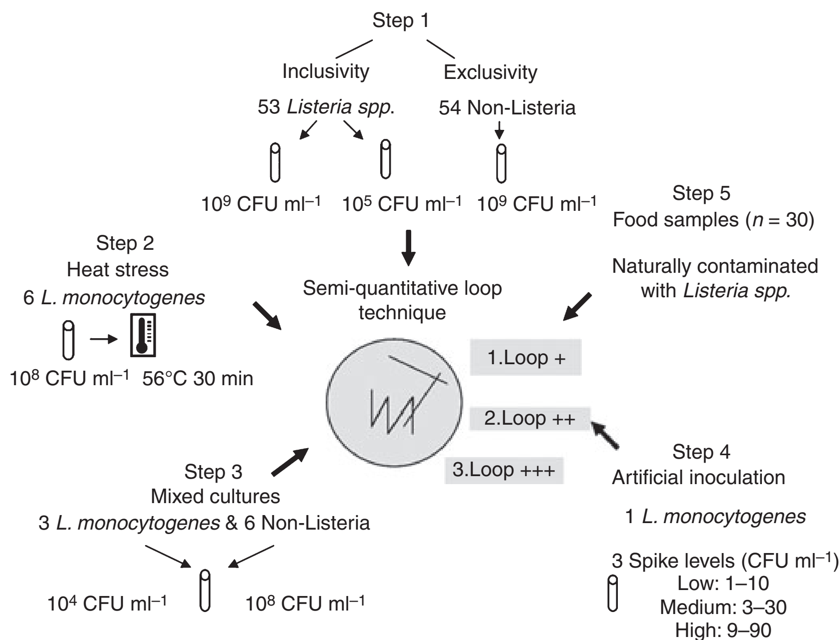

Figure 1 Experimental design (steps 1–5) for examination of six chromogenic Listeria plating media according to Ottaviani and Agosti-type test media (T1–T6).

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Table 1 Step 1 – Growth (0/+/++/+++) of 53 *Listeria spp*. test strains‡‡ on ALOA-type media after 48 h incubation

Source	Species	Identification	T1	T2	T3	T4	T5	T6	PALCAM
Clinical	*L. monocytogenes*	CIP 52·118¶	0	0	0	++	0	++	0
		ATCC 7644‡	0	0	+++	0	+++	0	++
		NCTC 7973†	+++	0	++	+++	+++	+++	++
		Ve 1††	++	++	++	+++	+++	++	0
		Ve 3††	++	++	+++	++	+++	+++	0
		Ve 2††	++	++	++	++	++	++	+
		CCM 5577§	++	++	++	++	++	++	++
		SLCC 2482**	++	++	++	++	++	++	++
		NCTC 5105†	++	++	++	++	++	++	++
		NCTC 10888†	++	++	++	++	++	++	++
		ATCC 19118‡	++	++	++	++	++	++	++
		NCTC 10527†	++	++	++	++	++	++	++
		NCTC 5214†	++	++	++	++	++	++	++
		Me 3††	++	++	++	++	++	++	++
		Me 4††	++	++	++	++	++	++	++
		CIP 58·33¶	++	++	++	+++	++	++	++
		ATCC 19116‡	++	++	++	+++	++	++	++
		Re 13‡	++	++	++	+++	++	++	++
		Me 2‡	++	++	++	++	+++	++	++
		SLCC 2540**	++	+++	++	++	+++	++	++
		Me 1††	++	++	+++	+++	++	++	++
		DSM 20600*	++	++	++	+++	+++	++	++
	L. innocua	NCTC 11288†	++	++	++	++	+++	++	+
	L. ivanovii	DSM 20750*	++	+	++	++	++	++	++
	L. seeligeri	CIP 80·12¶	++	++	++	+++	++	++	++
Food	*L. monocytogenes*	Mi 2††	++	++	++	++	++	++	++
		Fi 1††	++	++	++	++	++	++	++
		Fi 4††	++	++	++	++	++	++	++
		Ge 1††	++	++	++	++	++	++	++
		Fe 2††	++	++	++	+++	++	++	++
		U1††	++	++	++	++	++	+++	++
		Fe 1††	++	++	++	+++	+++	++	++
		Ge 2††	++	++	++	+++	+++	++	++
		U2††	+++	++	+++	++	+++	++	++
		NCTC 11994†	++	+++	+++	++	++	+++	++
		Mi 4††	++	+++	++	+++	+++	++	++
		In 2††	++	++	++	++	+++	++	++
	L. innocua	In 18††	++	++	++	+++	+++	++	++
		CCM 4698‡	++	++	0	++	++	++	++
	L. ivanovii	Se 1††	0	0	0	+	+	+	0
		Se 3††	+	+	+	+	+	++	0
		We 3††	+	+	+	+	+	+	+
	L. welshimeri	Gr1††	0	0	0	+	+	+	0
Environmental	*L. innocua*	In 4††	++	++	++	+++	+++	++	++
		In 5††	++	++	++	+++	+++	+++	++
		DSM 20751*	+	0	0	+	+	+	0
	L. welshimeri	DSM 20650*	+	+	+	+	++	+	+
	L. grayi	DSM 20596*	+	++	++	++	++	++	+
Not known	*L. monocytogenes*	SLCC 2479**	++	++	++	+++	+++	++	++
	L. innocua	NCTC 10528†	++	++	++	++	++	++	++
		SLCC 6927**	++	++	++	++	+++	++	++

Bacillus spp., 14 *Staphylococcus* spp., 5 *Enterococcus* spp., 3 *Streptococcus* spp., 2 *Lactobacillus* spp., 1 *Pediococcus* spp., 1 *Brevibacterium* spp., 5 Coryneforme bacteria as *Cellulomonas* spp., 5 Enterobacteriaceae, 1 *Aeromonas* spp., 4 *Pseudomonas* spp. and 1 *Candida* spp. All 107 overnight cultures corresponding to Mc Farland No 2–4 were directly streaked onto the chromogenic media. Additionally, overnight cultures of the 53 *Listeria* strains were

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Table 1 (*Continued*)

Source	Species	Identification	T1	T2	T3	T4	T5	T6	PALCAM
	ATCC 5578‡	+++	++	+++	++	+++	+++	++	
	L. seeligeri	SLCC 5921**	++	+	++	+++	++	++	+

T1 CHROMagar™ Listeria agar (CHROMagar).
T2 OCLA – Oxoid Listeria agar (Oxoid Ltd.).
T3 Compass L. mono agar (Biokar Diagnostics).
T4 OAA – Ottaviani Agosti agar (bioMérieux).
T5 Chromoplate® Listeria agar (Merck KGaA).
T6 Listeria agar (Heipha Dr Müller GmbH).
*DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
†NCTC National Collection of Type Cultures, London, UK.
‡ATCC American Type Culture Collection, Wesel, Germany.
$CCM Collection of Czech Microorganisms, Brno, Czech Republic.
¶CIP Biological Resource Center of the Pasteur Institute, Paris, France.
**SLCC Special *Listeria* Culture Collection, Würzburg, Germany.
††Isolate Collection of Institute of Milk Hygiene and Milk Technology, UVM, Vienna, Austria.
‡‡Cell concentration ml^{-1}: *Listeria* spp. 10^5.

decimally diluted. A loopful of dilution 10^{-4} was streaked onto each test medium (Fig. 1).

Growth ability under heat stress conditions (step 2)

Listeria monocytogenes strains (ATCC 7644, CIP 52·118, Ve 1, Me 1, Mi 2 and Fi 4) from different sources and with a low recovery rate (Table 1) were tested. Overnight cultures were adjusted to Mc Farland Standard 0.5 and thermal stress was applied for 30 min at 56°C in an agitated water bath with an integrated electronic thermometer (Julabo 36B, Seelbach, Germany). *Listeria monocytogenes* strains were heated to produce a minimum three-log reduction of viable cells. After thermal stress, cultures were streaked onto chromogenic media and observed after 24 and 48 h.

Mixed culture studies (step 3)

In the study, three *L. monocytogenes* strains were mixed with six competitive non-Listeria strains to determine the recovery rate and ease of recognition of *L. monocytogenes* on ALOA-type media (T1–T6). Each of 18 mixed cultures consisted of one *L. monocytogenes* (Ve 1, Me 4 and CIP 52.18) and one non-*Listeria* strain (1 *Bacillus* spp., 1 *Brevibacillus* spp., 2 *Staphylococcus* spp., 1 *Enterococcus* spp. and 1 *Cellulomonas/Microbacterium* spp.). The mixed suspensions contained 10^4 CFU ml^{-1} *L. monocytogenes* and 10^8 CFU ml^{-1} non-Listeria cells.

Artificial inoculation of soft cheese (step 4)

The soft cheese (surface-ripened red smear cheese, Schlierbacher, purchased from a local retail store) used in this study tested negatively for the presence of *Listeria* spp. before inoculation. Sub-samples of 25 g each were diluted with 225 ml half-Fraser (bioMérieux, Marcy l'Etoile, France), homogenized for 180 s in a Stomacher 400 and inoculated with three different spike levels of *L. monocytogenes* Mi 4. Target inoculation of each batch was (i) 1–10 CFU ml^{-1}(low level), (ii) 3–30 CFU ml^{-1} (medium level) and (iii) 9–90 CFU ml^{-1}(high level). After incubation for 24 h at 30°C, 0·1 ml of the primary enrichments were transferred to 10 ml full strength-Fraser (bioMérieux, Marcy l'Etoile, France) and incubated for 48 h at 37°C. Semi-quantitative loop inoculation was carried out after 24 h of half-Fraser and 48 h of full strength-Fraser enrichment.

Naturally contaminated samples (step 5)

Thirty confirmed positive naturally contaminated food samples (EN ISO 11290-1, Anon 1996) were provided by a local food analytical laboratory. Samples including meat, meat products, fish, vegetables and ready-to-eat food were stored at −80°C. Twenty-five grams of each sample was enriched according to EN ISO11290-1,-2 (Anon 1996, 1998). Test media were evaluated by the spatula method and by the semi-quantitative loop technique (Fig. 1).

Definition of terms and statistical analysis

Technical terms were defined according to EN ISO 16140 (Anon 2003). Concerning inclusivity and exclusivity of test strains, inclusivity was the proportion of *Listeria*-positive strains correctly found to be positive. Exclusivity was defined as the proportion of negative strains which

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were inhibited from growing. The relative accuracy was defined as true positives and true negatives divided by the total number of test samples. For heat-stressed strains, mixed cultures and artificial inoculation recovery rates were calculated.

Results

Inclusivity and exclusivity (step 1)

Six chromogenic plating media (T1-T6) and PALCAM agar were evaluated with a compilation of 53 *Listeria* spp. and 54 non-Listeria strains isolated from food or those of clinical origin. With the exception of one *L. grayi* test strain (Gr1) which could not grow on PALCAM Agar and T2 (Oxoid), all evaluated media achieved satisfactory growth (++, +++) and the typical colony morphology for *Listeria* spp. overnight cultures after 48 h of incubation (data not shown). A significant difference was observed when *Listeria* spp. cultures were diluted to 10^{-4}, which corresponds to 10^5 cfu ml^{-1}, resulting in 10/53 strains which could not grow on each test medium (Table 1). The performance data of the tested media, illustrating their suitability to recover target strains at a lower inoculum, are presented in Table 2. The best inclusivity (98.1%) was observed on T4 (bioMérieux), T5 (Merck) and T6 (Heipha), whereas 7/53 *Listeria* spp. test strains did not grow on PALCAM Agar, resulting in 86·8% inclusivity (Tables 1 and 2). The recovery rate of non-Listeria strains was generally high. Twelve of 54 to 36/54 non-Listeria strains were able to grow on PALCAM and T4 medium respectively. Only strains with β-D-glucosidase production were deemed false positive and were included in the calculation of exclusivity. Apart from that, selective supplements of PALCAM agar suppressed 49/54 non-*Listeria* strains and achieved an exclusivity of 90·7% (Table 2). The latter medium only failed to inhibit the growth of some tenacious *Bacillus* spp. (*Bacilus licheniformis*, *Bacillus pumilus*) and Coryneforme bacteria (*Cellulomonas/Microbacterium* spp.). The best exclusivity accomplished by chromogenic media (83.3% were achieved equally by T2 (Oxoid) and T3 (Biokar Diagnostics/Solabia).

Growth ability under heat stress conditions (step 2)

All six *L. monocytogenes* strains were recovered after heat treatment and achieved a mean log reduction of 3.6–4.4 on T1 (Chromagar), T2 (Oxoid), T4 (bioMérieux), T5 (Merck) and T6 (Heipha). The generally typical colony character disappeared after heat treatment. Pinpoint growth and a pale shade of turquoise were achieved.

Mixed culture studies (step 3)

In experimental step 3, T4 (bioMérieux) and T5 (Merck) were the best performers concerning qualitative recovery from 18 mixed cultures (83.3% presumptive colonies). T2

Table 2 Step 1 – Performance Data of ALOA-type media after 48 h incubation

	T1	T2	T3	T4	T5	T6	PALCAM
§*Listeria* spp.(n = 53)							
True positive	49	47	48	52	52	52	46
False negative	4	6	5	1	1	1	7
†Non-Listeria (n = 54)							
Recovery	29	25	19	36	32	33	12
False positive (typical *Listeria* spp. morphology)	14	9	9	14	14	14	5
True negative	40	45	45	40	40	40	49
*Accuracy	83·2	85·9	86·9	85·9	85·9	85·9	88·9
†Exclusivity	74·1	83·3	83·3	74·1	74·1	74·1	90·7
‡Inclusivity	92·5	88·7	90·6	98·1	98·1	98·1	86·8

T1 CHROMagar™ Listeria agar (CHROMagar).
T2 OCLA – Oxoid Listeria agar (Oxoid Ltd.).
T3 Compass L. mono agar (Biokar Diagnostics).
T4 OAA – Ottaviani Agosti agar (bioMérieux).
T5 Chromoplate® Listeria agar (Merck KGaA).
T6 Listeria agar (Heipha Dr Müller GmbH).
ALOA, Agar Listeria according to Ottaviani and Agosti.
*Accuracy (%) = (true pos + true neg) *100/total.
†Exclusivity (%) = true neg *100/(true neg + false pos).
‡Inclusivity (%) = true pos *100/(true pos + false neg).
§Cell concentration ml^{-1}: *Listeria* spp. 10^5, non -Listeria 10^9.

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(Oxoid), T3 (Biokar) and PALCAM agar suppressed more non-Listeria strains than did the other tested plating media. Results are shown in Table 3. Non-Listeria strains overgrew *L. monocytogenes*. Furthermore, as the morphology of the target colony turned atypical (Fig. 2c), PCR identification was required (Bubert *et al.* 1999).

Artificial inoculation of soft cheese (step 4)

Samples were spiked with 3·38 CFU ml^{-1}(spike level, 1–10 CFU ml^{-1}), 16·9 CFU ml^{-1} (spike level, 3–30 CFU ml^{-1}) and 84·5 CFU ml^{-1} (spike level, 9–90 CFU ml^{-1}). The evaluated test media (T1–T6) achieved 100% recovery after 24 h of half-Fraser and 48 h of full-strength Fraser in combination with a confirmatory PCR step (Bubert *et al.* 1999). After 24 h half-Fraser, presumptive colonies were difficult to identify. The background flora was not entirely suppressed by selective antibiotics and hampered detection of target strains.

Naturally contaminated samples (step 5)

Thirty food samples were provided by a food analytical laboratory after positive confirmation of *L. monocytogenes*. Representative sub-samples were stored at −80°C and were found to be *Listeria spp*. positive after half-Fraser and full-strength Fraser enrichment by the PCR method. Direct enumeration according to EN ISO 11290-2 (Anon 1998) indicated that the samples were initially not highly contaminated (<100 cfu *Listeria* spp. g^{-1}). After primary and secondary enrichment 15 samples were confirmed to be *L. monocytogenes* positive, 13 samples contained *L.monocytogenes* mixed with other *Listeria* spp. and two samples *L.innocua*. Relative specificity detected by the semiquantitative loop technique was highest on PALCAM agar (94·4%) and T2 (Oxoid) (88·8%), and lowest on T4 (bioMérieux) (50%) after 24 h half-Fraser enrichment. Relative sensitivity was highest on T2 (Oxoid) (96·7%) and PALCAM agar (86·7%) (Table 4). There was no

Table 3 Step 3 – Recovery rate of test strains applied in challenge experiments after 48 h incubation

Recovery of 3 *L. monocytogenes* in mixed cultures with 6 non-Listeria strains†							
	T1	T2	T3	T4	T5	T6	PALCAM
18 mixed samples							
R* (%) presumptive colonies	77·8	55·6	50	83·3	83·3	66·6	72·2
R* (%) after PCR identification	83·3	55·6	56·6	88·9	94·4	83·3	83·3
R*(%)	100	61·1	83·3	100	100	100	33·3

*R Recovery rate of target and non-target strains.
†Cell concentration ml^{-1}: *Listeria* spp. 10^4, non-Listeria 10^8.

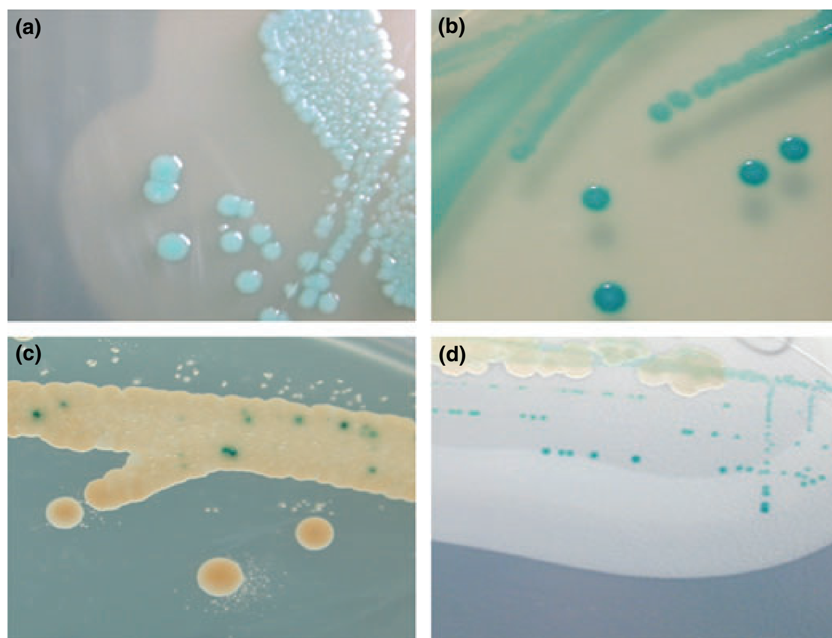

Figure 2 Examples of the morphology of *Listeria* spp. colonies and non-Listeria colonies on chromogenic media (a–d). (a) *Listeria monocytogenes*, pure culture, blue turquoise colonies, distinctive halo, step 1 (T2); (b) *Bacillus circulans*, pure culture, blue turquoise colony, step 1 (T4); (c) *L. monocytogenes* – atypical blue particles and *Staphylococcus* spp. (yellow), mixed culture step 3 (T3); (d) *Bacillus* spp. (cream-white, irregular margin, halo formation) and *Enterococcus* spp. (blue-turqouise), natural contaminated samples, step 5 (T1).

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Table 4 Step 5 – Performance data of six chromogenic media and PALCAM from 30 *Listeria* spp. food isolates tested by the semi-quantitative loop technique after 24 h half-Fraser

	T1	T2	T3	T4	T5	T6	PALCAM
True positive	26	29	25	27	25	25	26
True negative	13	16	15	9	12	12	17
False positive	4	1	5	9	6	6	1
False negative	5	2	3	3	5	5	4
*Accuracy	81·1	93·8	83·3	87·5	77·1	77·1	89·6
†Specificity	72·2	88·9	83·3	50	66·7	66·7	94·4
‡Sensitivity	86·6	96·7	83·3	90	83·3	83·3	86·7

*Accuracy (%) = (true pos + true neg) *100/total.
†Specificity (%) = true neg *100/(true neg + false pos).
‡Sensitivity (%) = true pos *100/(true pos + false neg).

significant difference regarding isolation from each medium after full-strength Fraser enrichment, therefore all samples were confirmed to be positive (Table 5). Some non-Listeria organisms produced colonies similar to *Listeria* spp.: *Bacillus circulans, B. pumilus, Enterococcus* spp., *Staphylococcus xylosus* and Coryneforme bacteria such as *Cellulomonas/Microbacterium* spp. Only these bacteria were counted as false positive, although many bacterial organisms grew on chromogenic media, especially after half-Fraser enrichment (Fig. 2d).

Discussion

Chromogenic media are important tools in microbiological detection of pathogenic microorganisms. Comparative studies have shown the advantages of chromogenic solid media (Reissbrodt 2004; Beumer and Hazeleger 2007). The aim of the present study was to examine a broad range of ALOA-type chromogenic media according to standard evaluation criteria (inclusivity, exclusivity), in challenging experiments (heat stress, mixed cultures) and in naturally contaminated samples. To date, this is the most comprehensive evaluation of ALOA-type media. The results of inclusivity testing showed that high-inoculated *Listeria* spp. test cultures achieved typical and adequate colonies on all test media after 48 h of incubation. These findings are similar to other studies (Vlaemynck *et al.* 2000; Willis *et al.* 2006). Producers of chromogenic media ensure differentiation of *L. monocytogenes* even in the presence of a mixed flora and after incubation for 24 ± 2 h. In the present analysis, several *L. monocytogenes* strains required more than 24 ± 2 h to develop a distinctive halo. Prolonged incubation for a further 24 h is therefore advised to prevent misinterpretation and underestimation of *L. monocytogenes*-contaminated samples. Research on atypical colonial morphology and low recoveries showed that some of *L. monocytogenes* strains even need 96 h of incubation (Leclercq 2004). In experiments with chromogenic media, *Listeria* spp. overnight cultures were diluted to 10^{-4} in order to detect differences in the recovery of lower inocula on each test medium. The results indicate that PALCAM agar, T2 and T3 produce more negative results for *Listeria* spp. strains than do other test media. Regarding exclusivity, several non-Listeria strains were not effectively suppressed by selective agents such as lithium chloride, nalidixic acid and polymyxin B. The most selective media were PALCAM, T2 and T3. This might have been related to the higher lithium chloride concentration (15 g l^{-1}) in the agar formulation than in the original ALOA formulation and in other ALOA-type test media (10 g l^{-1}) (Vlaemynck *et al.* 2000; Bauwens *et al.* 2003). Furthermore, PALCAM contains acriflavine, which is another suppressor of the competitive flora and is discussed to inhibit the growth of *L. monocytogenes* (Bauwens *et al.* 2003). Subsequent investigations on chromogenic media report that non-Listeria organisms are suppressed or can be easily distinguished from *Listeria* spp. by their colony morphology. This fact may be applicable in experiments using pure cultures, but the growth ability of non-*Listeria* organisms in mixed cultures often impeded the isolation of *L. monocytogenes*. Experiments with mixed cultures pointed out that competitive flora overgrew target strains at lower inocula (Fig. 2c). The results of heat stress experiments also demonstrated the loss of typical colony morphology. This would signify the grave circumstance of *L. monocytogenes* positive samples being underestimated in routine or outbreak investigations. As a consequence, PCR confirmation of presumptive *Listeria* spp. colonies should be mandatory (Bubert *et al.* 1999). The results of naturally contaminated samples indicate the limitations of *L. monocytogenes* detection in a routine analysis. The previously recommended extension of incubation for a further span of 24 h is a crucial step in the detection of *L. monocytogenes* in food samples. Furthermore, investigators must consider the fact that a competitive background flora also possesses the ability to utilize either one or both enzymes systems (β-D glucosidase and phospholipase C). It has been shown, in previous studies, that *Enterococcus* spp. and *Bacillus* spp. are β-D glucosidase positive (Vlaemynck *et al.* 2000). The results of naturally contaminated samples indicated that these bacterial organisms could not be sufficiently suppressed by enrichment broths or plating media. *Bacillus circulans* impeded the interpretation of presumptive colonies, particularly those isolated from half-Fraser enrichment. In several samples, non-Listeria isolates limited *Listeria* spp. growth. The tested chromogenic media were either more sensitive or more specific, both conditions were not found and are probably difficult to achieve. The advantage of the new

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Table 5 Step 5 – Enumeration of *Listeria monocytogenes* isolated from naturally contaminated food on six ALOA-type media and PALCAM

		Bacterial counts after 24 half-Fraser enrichment (Log CFU ml$^{-1)}$							Bacterial counts after 48 h full-strength Fraser enrichment (Log CFU ml^{-1})						
Origin	Sample no.	PALCAM	T1	T2	T3	T4	T5	T6	PALCAM	T1	T2	T3	T4	T5	T6
Fish															
Cod	2	5·8	6·0	6·0	6·0	6·0	6·0	6·0	8·0	8·0	8·1	8·1	8·2	8·1	7·9
Smoked salmon	11	3·4	3·9	3·7	3·5	3·5	3·2	3·7	8·3	7·8	7·8	7·8	7·8	7·8	7·8
Smoked salmon	12	4·4	4·2	4·4	4·4	4·5	4·4	4·5	8·1	8·0	8·1	8·3	8·3	8·2	8·1
Smoked salmon	21	7·3	6·8	6·8	6·8	6·7	7·0	6·8	8·0	8·0	8·0	8·1	8·1	8·1	8·0
Smoked salmon	22	3·5	3·7	3·7	3·7	3·6	3·6	3·8	6·9	6·9	7·1	7·1	7·1	7·0	7·0
Smoked salmon	24	4·1	4·2	4·2	4·1	4·2	4·0	4·2	7·9	8·0	8·0	8·2	8·2	8·0	7·9
Smoked salmon	26	3·3	4·0	3·7	3·7	3·6	3·7	3·6	8·5	8·5	8·4	8·6	8·7	8·3	8·6
Smoked salmon	27	3·2	2·8	3·4*	3·2*	3·2	3·3	3·2	7·4	7·3	7·4	7·4	7·5	7·4	7·4
Smoked salmon	28	4·5	4·4	4·5	4·3	4·5	4·3	4·0	7·2	7·0	7·4	7·5	7·5	7·4	7·2
Smoked salmon	31	2·5	2·7	2·5	2·6	2·7	2·7	2·8	6·3	6·2	6·4	6·3	6·4	6·3	6·3
Zander	36	7·8	6·0	6·0	6·0	6·0	6·0	6·0	6·4	6·4	6·4	6·5	6·7	6·3	6·4
Smoked salmon	41	2·8	2·8	0	2·7	2·8	2·5	2·8	6·7	6·5	6·5	7·3	7·0	7·3	6·6
Smoked salmon	46	6·5	6·5	6·6	6·7	6·5	6·5	6·7	7·3	7·3	7·5	7·7	8·0	7·9	7·3
Meat products															
Bratwurst	5	4·3	4·3	4·4	4·4	4·3	4·5	4·4	7·5	7·7	7·5	7·8	7·0	7·7	7·5
Liver dumpling	6	4·8	5·1	5·5	5·0	5·5	5·5	5·5	6·3	6·4	6·2	6·5	6·1	6·4	5·9
Liver sausage	7	4·0	3·3	3·5	4·1	4·0	4·0	4·0	7·5	7·8	8·0	8·0	8·0	7·8	7·8
Risolets (turkey)	8	5·2	5·4	5·3	5·5	5·3	5·4	5·3	7·0	6·9	6·8	7·3	7·1	7·1	7·1
Liver loaf	9	5·4	5·4	5·1	5·3	5·2	5·1	5·1	6·7	6·3	6·9	7·1	7·2	7·2	6·8
Liver dumpling	19	4·9	4·7	4·7	4·8	4·7	4·8	4·6	8·5	8·5	8·5	8·5	8·5	8·5	8·5
Bratwurst	37	5·7	5·6	5·7	5·8	5·7	5·8	5·8	7·2	7·5	7·7	7·5	7·7	7·6	7·6
Kebab (chicken)	39	5·9	5·6	5·6	5·7	5·7	5·8	5·6	7·3	7·3	7·1	7·3	7·3	7·4	7·1
Liver loaf	42	4·9	4·8	4·8	4·9	5·0	5	4·8	7·6	7·3	7·5	7·9	7·8	7·8	7·5
Ham	43	2·7	3·0	2·9	2·8	3·0	2·8	2·8	7·2	7·3	7·4	7·6	7·4	7·6	7·3
Other															
Salad	16	5·1	5·2	5·2	5·2	5·5	5·1	5·2	8·3	8·1	8·3	7·8	8·2	8·0	8·0
Pasta	34	6·2	6·0	6·2	6·2	6·4	6·6	6·0	8·3	8·1	8·4	8·3	8·3	8·2	8·1
Pasta	35	7·5	6·0	6·0	6·0	6·0	6·0	6·0	7·3	7·5	7·8	8·2	7·6	7·7	7·8
Risolets (vegetarian)	40	7	5·0	5·0	5·0	5·0	5·0	5·0	7·7	7·3	7·6	8·0	7·8	7·8	7·8
Risolets (vegetarian)	47	5·2	5·2	5·3	5·2	5·3	5·3	5·2	9·0	8·8	9·0	9·8	9·0	8·9	8·7

ALOA, Agar Listeria according to Ottaviani and Agosti.
***No presumptive halo.

chromogenic media is the fact that they permit better recognition of presumptive colonies than do PALCAM agar. However, a more critical validation of chromogenic media is warranted. Traditional performance studies might well be completed by investigation of *L. monocytogenes* test strains with lower inocula, as this would correlate with the incidence of this microorganism in food samples. Furthermore, challenge testing of *L. monocytogenes* should be a crucial checkpoint for validation studies as this simulates their behaviour and survival during and after food processing.

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