Journal of Applied Microbiology 2000, **88**, 430–441

Improvement of the detection of *Listeria monocytogenes* by the application of ALOA, a diagnostic, chromogenic isolation medium

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7361/08/99: received 20 August 1999 and accepted 18 October 1999

G. VLAEMYNCK, V. LAFARGE AND S. SCOTTER. 2000. A new selective agar medium, ALOA, for the selective and differential isolation of *Listeria monocytogenes* has been evaluated. All stressed cultures of *L. monocytogenes* serovars tested grew on the medium as bluish colonies surrounded by a distinctive opaque halo and gave a productivity ratio of at least 0·95. Non-pathogenic *Listeria* sp. produced bluish colonies without a halo as was also the case for some enterococci and bacilli. Special attention must be paid to some *Bacillus cereus* strains and *L. ivanovii* since their colony appearance can be misleading. Only some unidentified listeria-like bacteria gave false-positive results. ALOA detected 4·3% more positives from naturally contaminated dairy and meat samples compared with the ISO procedure when used with Genprobe™ or Vidas™ for confirmation of presumptive colonies; 13·9% false negatives were found compared with 38·9% using PALCAM/Oxford. ALOA was also clearly superior to Oxford and PALCAM when samples containing both *L. monocytogenes* and *L. innocua* were examined. The introduction of ALOA in standard isolation procedures as an additional medium would enhance the detection ratio and reduce the time and cost of analysis for *L. monocytogenes.*

INTRODUCTION

Listeria spp. are ubiquitous and almost all kinds of food can be contaminated. However, *Listeria monocytogenes* is the only species of public health concern. This species can cause listeriosis in man, an illness associated at present with the highest lethality (20–30%). Every year people become seriously ill with listeriosis and some of them die. Recently, in Zeeland, Michigan, USA (1998–99) and in France (1999), an outbreak of listeriosis causing several deaths has taken place. Because of the current requirement for the absence of *L. monocytogenes* from foods, specific and sensitive methods are needed. Numerous procedures have been proposed for the detection of *L. monocytogenes* in food (Ryser and Marth 1991). Since efforts to isolate *L. monocytogenes* from food by direct plating are often unsuccessful, procedures consist of an enrichment followed by an isolation on a selective agar. At present, no selective enrichment medium which selects *L. monocytogenes* over other *Listeria* species is available. The various proposed enrichment broths are all based on the same principles and, in all of them, *L. monocytogenes* does not proliferate well when other (often faster growing) *Listeria* spp. are present (Petran and Swanson 1993; Curiale and Lewus 1994; MacDonald and Sutherland 1994; Vlaemynck and Moermans 1996). The specific isolation of *L. monocytogenes* is difficult since none of the selective plating media recommended in official international standard methods can distinguish between *L. monocytogenes* and other *Listeria* spp. Picking at random five presumptive positive colonies from a plate containing low numbers of *L. monocytogenes* and high numbers of other *Listeria* spp. has a high chance of missing *L. monocytogenes.* Therefore, the incidence of *L. monocytogenes* may well be underestimated. An isolation step from the primary enrichment broth (after 24 h), as is now included in official standard methods (International Dairy Federation (International Dairy Federation–IDF; Anon. 1995 and International Standard Organization–ISO; Anon. 1997), can over-

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come some problems during enrichment, especially when the background flora is able to overgrow the *L. monocytogenes* present. Using other confirmation techniques carried out immediately on the enrichment broth or by swabbing a plate can also be useful to reduce the numbers of false negatives.

In this study, a special isolation medium which is not only selective for listeria but which allows the direct differentiation of *L. monocytogenes* in the presence of other Listeriae and other background flora was evaluated. Agar *Listeria* according to Ottaviani and Agosti (ALOA) is both a selective and differential medium for the isolation of *Listeria* spp. and presumptive identification of *L. monocytogenes* (Ottaviani *et al.* 1997a; Ottaviani *et al.* 1997b). The selectivity is obtained by adding a series of antimicrobial compounds comparable to the PALCAM medium. The indicative character is established by the introduction of chromogenic substrates in the selective medium. Chromogenic substrates have proven to be a powerful tool, utilizing specific enzymatic activities of certain micro-organisms (Manafi 1996). Improved accuracy and faster detection of target organisms can be obtained. They have been in use for some years and are at present most commonly used for the detection and enumeration of coliforms, *Escherichia coli* and Enterobacteriaceae (Manafi *et al.* 1991). Some applications have also been described for Salmonella, *Clostridium perfringens, Staphylococcus aureus* and *Lactobacillus acidophilus* (Bulanda *et al.* 1988; Baumgart *et al.* 1990; Kneifel and Pacher 1993; Davies and Wray 1994; Kühn *et al.* 1994). They have recently been introduced for the isolation and/or detection of *L. monocytogenes* (ALOA (Biolife, Milan, Italy) and the Rapid L' mono® medium (Sanofi Diagnostics Pasteur, BioRad, Marnes la Coquette, France)). In the ALOA medium, the chromogenic compound X-glucoside is added as substrate for the detection of β-glucosidase, which is common for all *Listeria* species. The differentiation of *L. monocytogenes* from other *Listeria* spp. is based on the production of a phosphatidylinositol-specific phospholipase C in *L. monocytogenes* strains which can hydrolyse the specific purified substrate added to the medium, resulting in an opaque clear-cut halo surrounding the *L. monocytogenes* colonies.

The objectives of the study were to evaluate the sensitivity, selectivity and electivity of ALOA in comparison with PALCAM and Oxford using pure cultures, inoculated dairy, egg and meat products and naturally contaminated dairy and meat samples.

METHODS AND MATERIALS

Strains

Strains were obtained from a variety of sources and included strains from reference culture collections, from our own culture collection and wild strains isolated from various food samples in our laboratory.

Methods

ALOA was prepared according to the manufacturer's instructions. In the ALOA medium, the chromogenic compound X-glucoside is added as substrate for the detection of β-glucosidase, resulting in blue-coloured listeria colonies. Through the production of a phosphatidylinositol-specific phospholipase C in *L. monocytogenes* strains and the addition of a specific purified substrate for the enzyme to the medium, an opaque halo surrounds the colonies. The selectivity is obtained by the addition of some agents comparable to PALCAM, omitting, however, acriflavine and adding nalidixic acid and cycloheximide. Strains of *Listeria, Bacillus, Enterococcus* and *Staphylococcus* were all cultivated on Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, UK) at 37 °C. In all experiments, viable counts were estimated by surface plate enumeration. Tryptone soy agar (TSA; Oxoid) was used as a non-selective reference agar medium. Plates were incubated at 37 °C for 24 h.

Samples were analysed according to the method described in the International Standard EN ISO 11290-1 (Anon. 1997) using ALOA in parallel with the prescribed PALCAM and Oxford plates (Oxoid). In summary, a primary enrichment was carried out in Fraser broth (Oxoid) containing half concentration of supplements at 30 °C for 24 h followed by a subculture of 0·1 ml in 10 ml full Fraser broth (Oxoid), incubated for 48 h at 37 °C. Subsequently, 10 μl of this second enrichment broth was plated on PALCAM, Oxford and ALOA plates. Both PALCAM and Oxford were incubated for 48 h at 37 °C whereas ALOA plates were incubated at 37 °C for 24 h.

Confirmations were carried out using the Accuprobe™ *Listeria monocytogenes* Culture Identification Reagent Kit (GenProbe, San Diego, CA, USA) for *L. monocytogenes* and the immunological VIDAS™ *Listeria* assay (Biomérieux, Marcy l'Etoile, France) for other *Listeria* spp. (Vlaemynck 1996).

Recovery, elective and selective characteristics of ALOA

At first, the suitability of ALOA agar compared with Oxford and PALCAM agars was assessed. The recovery of cultures (grown for 24 h in BHI with 5% NaCl and stored for 1 week at 4 °C) of 12 different *L. monocytogenes* serovars was investigated on ALOA and quantitatively compared with their growth on the non-selective TSA and the selective media Oxford and PALCAM. The ability of ALOA compared with TSA, PALCAM and Oxford agars for recovering cells from other *Listeria* spp. was investigated in a similar

way. In a second series of experiments, the elective and selective character of the medium was examined. Specific and typical growth of a collection of 50 *L. monocytogenes* strains and 50 other *Listeria* sp., isolates from food as well as from clinical samples, was assessed qualitatively on ALOA by streaking them on the surface of the plates. Selectivity testing of the new diagnostic medium was further extended with another set of 44 strains of possible non-listeria competitors, belonging to different species.

Artificially inoculated samples

A mixture of *L. monocytogenes* and *L. innocua* was spiked at three different ratios (1/10; 10/10 and 1/0·1) into 25 g Gouda-type cheese in 225 ml half Fraser enrichment broth. True spiked levels of listeria ranged from 10 to 1000 cfu $25\,g^{-1}$. Enrichment broths were incubated at 30 °C for 24 h. Subsequently, 0·1 ml of the primary enrichment was transferred to 10 ml full Fraser and the secondary enrichment culture incubated for another 48 h at 37 °C. Isolations on ALOA, Oxford and PALCAM agar were carried out immediately after inoculation of the enrichment broth with the listeria culture and after 24 and 48 h.

International ring trials/collaborative studies

The ALOA medium was evaluated beside PALCAM and/or Oxford in two international ring trials on the detection of *L. monocytogenes* in food according to EN ISO 11290-1:1997 (Anon. 1997) or IDF 143A:1995 (Anon. 1995).

In the first collaborative study organized by MAFF Central Science Laboratory (CSL) (Norwich, UK), 20 samples from each matrix cheese, meat and egg powder inoculated with two different levels of *L. monocytogenes,* equal numbers of *L. innocua* and a background flora typical for the product were distributed. Cheese samples were prepared by CECALAIT (Poligny, France), meat samples by MAFF CSL and egg powder by RIVM-MGB (Bilthoven, the Netherlands). Each series consisted of five blanks (negatives), five samples inoculated with a high level of *L. monocytogenes* and *L. innocua* (50–100 cfu $25\,g^{-1}$) and five samples containing a low level of *L. monocytogenes* (5–10 cfu $25\,g^{-1}$) and *L. innocua* (50–100 cfu $25\,g^{-1}$). All meat samples were additionally inoculated with a simulated autochthonous flora comprising 1×10^4 cfu g^{-1} *B. subtilis,* 1×10^6 cfu g^{-1} *Lactobacillus* sp., 1×10^6 cfu g^{-1} *Micrococcus* sp. and 1×10^4 cfu g^{-1} *Pseudomonas* sp. To all cheese samples a background flora comprising isolates from cheese from the following species was added as non-target flora: *Lactococcus lactis* ssp. *lactis, Enterococcus faecalis, Lactobacillus paracasei* and *Lact. plantarum*. For the egg samples the background flora consisted of the natural flora present in the egg powder.

In a second trial organized by the Community Reference Laboratory for milk and milk products (Paris, France) six samples of pasteurized milk were distributed among the participating laboratories. Two of them contained a high level of *L. monocytogenes* (30–70 cfu $25\,ml^{-1}$) and *L. innocua* (100 cfu $25\,ml^{-1}$), two contained a low level of *L. monocytogenes* (5–10 cfu $25\,ml^{-1}$) and a high level of *L. innocua* (100 cfu $25\,ml^{-1}$) and two samples were not inoculated with *L. monocytogenes* but only with *L. innocua* (100 cfu $25\,ml^{-1}$) (blanks).

Samples

Different samples were collected from retailers, from factories during the production of cheese or meat and from the environment of a cheese production site over a period of 1 year. A set of 208 food samples expected to be naturally contaminated with listeria was analysed routinely. In total, 25 samples from raw milk, 103 from soft raw milk cheeses, 10 from blue-veined cheeses, 10 from other cheese types, 22 from other dairy products and 38 from poultry products were analysed. Isolations were effected on ALOA, PALCAM and Oxford medium for the detection of *L. monocytogenes.*

RESULTS

Recovery of *L. monocytogenes* and other *Listeria* spp.

The results for *L. monocytogenes* are presented in Fig. 1. Data were evaluated by calculating the productivity ratio (PR), defined as the ratio of the counts on the test medium and the reference medium, both expressed as log 10 (Anon. 1987). The results showed ALOA to have a very good performance in supporting the growth of *L. monocytogenes* cells. None of the strains showed significantly reduced growth on the selective medium compared with the non-selective medium. A PR of at least 0·95 was obtained. Comparing ALOA with PALCAM and Oxford, no significant difference in recovery of broth cultures of *L. monocytogenes* was observed.

The recovery of other *Listeria* spp. on ALOA is shown in Fig. 2. Counts of *L. innocua* and *L. welshimeri* on ALOA reached approximately the same level as those on TSA, PALCAM and Oxford agar. ALOA also performed as well as TSA for the enumeration of *L. ivanovii*, *L. seeligeri* and *L. grayi* ssp. *grayi*. Reduced counts on ALOA were only observed for one *Listeria* strain, *L. grayi* ssp. *murrayi*. Using PALCAM, the number of viable cells recovered for some strains was significantly reduced. A difference in counts was apparent for the *L. grayi* strains and, to a lesser extent, for

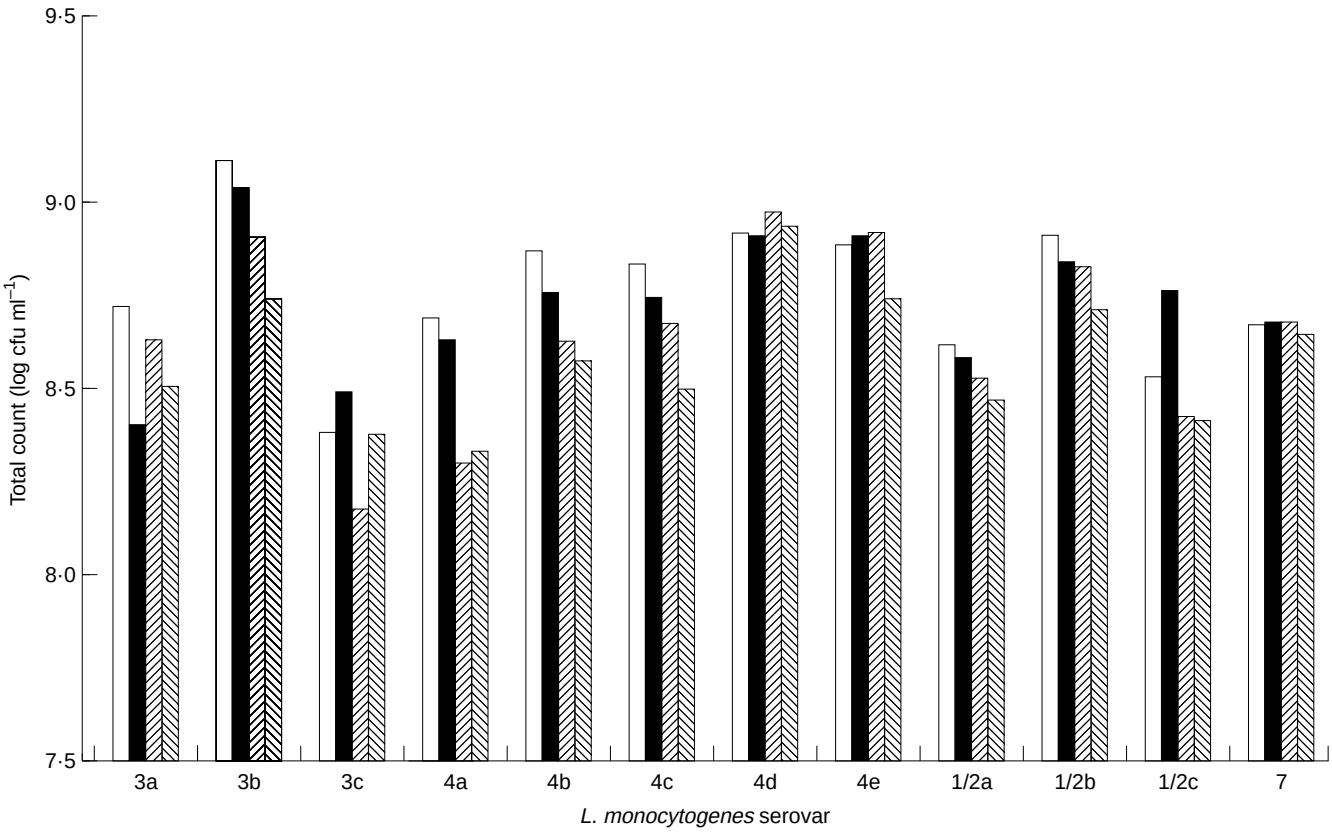

Fig. 1 Recovery of various *Listeria monocytogenes* serovars on ALOA compared with non-selective tryptone soy agar (TSA) and selective Oxford and PALCAM. Log cfu ml^{-1}: □, TSA; ■, ALOA; ▨, Oxford; ▧, PALCAM

L. ivanovii. Counts on PALCAM were at least 3 log lower than those on TSA. A small reduction in counts on PALCAM and also on Oxford compared with ALOA and TSA was observed for *L. seeligeri*. The recovery of five more strains of *L. ivanovii* and *L. seeligeri* on the various selective media was assessed (results not shown). The recovery on ALOA was relatively good for all strains of *L. ivanovii* (PR 0·88–0·98) and for four of five strains of *L. seeligeri* (PR 0·91–0·99 and 0·4). Using PALCAM, of five strains of *L. ivanovii* tested, the growth of three seemed to be significantly reduced while almost all strains of *L. seeligeri*, except one, were inhibited to a lesser extent. Using Oxford, one strain of *L. ivanovii* and two strains of *L. seeligeri* showed reduced growth.

Evaluation of the elective and selective character of the ALOA medium

All *Listeria* spp. tested grew with a typical appearance as light blue-coloured smooth colonies, 1·0–2·0 mm diameter after 24 h, while colonies of all selected serovars of *L. monocytogenes* were surrounded by a distinctive opaque halo. *Listeria ivanovii* strains also showed a vague halo after 24 h incubation which was not as distinctive as for *L. monocytogenes*. However, after 48 h, the halo was clearly visible and *L. ivanovii* sp. showed an appearance which could, in some cases, be confused with *L. monocytogenes* strains.

The degree of inhibition of unwanted strains of other genera on this selective test medium was examined quantitatively by plating dilutions of various cultures on ALOA and non-selective TSA and is expressed as the selectivity factor (SF) of the medium (Anon. 1998). Bacilli, lactobacilli, enterococci, staphylococci and some unidentified listeria-like species previously isolated in our laboratory (Vlaemynck 1996; Rijpens *et al*. 1998) were included. For all strains tested, except the unidentified listeria-like species, a calculated SF value of at least 6 was achieved. Counts of the listeria-like strains on ALOA were comparable with those on TSA and a PR value of at least 0·97 was calculated.

The growth of some *B. cereus* strains, enterococci and staphylococci was evaluated qualitatively since Ottaviani *et al*.

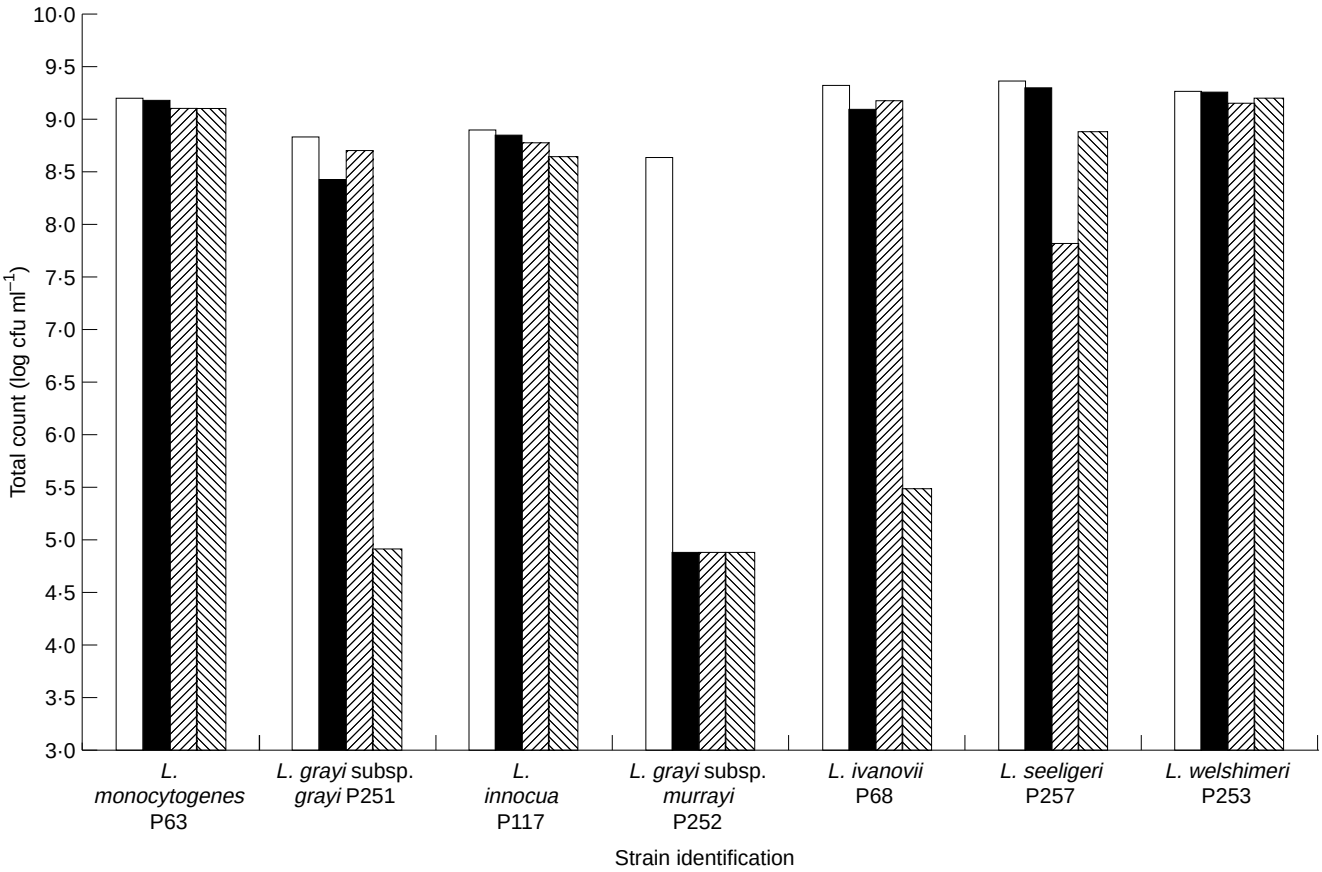

Fig. 2 Recovery of various *Listeria* spp. on ALOA compared with non-selective tryptone soy agar (TSA) and selective Oxford and PALCAM. Log cfu ml^{-1}: □, TSA; ■, ALOA; ▨, Oxford; ▧, PALCAM

(1997a,b) mentioned them as giving possible false positive results. The unidentified strains were isolated from Oxford or PALCAM plates as background flora during analysis of food samples for the presence of listeria. These strains were resistant to the selective compounds present in the enrichment broths and in the selective plates used to isolate listeria after enrichment according to ISO (Anon. 1997). All strains used are given in Table 1 together with their growth characteristics on ALOA.

All *Staphylococcus* strains were completely inhibited on the ALOA medium. *Bacillus cereus*, on the other hand, gave rise to distinctive blue colonies with an irregular margin and, in some cases, surrounded by a small, rather vague halo. However, these colonies could (with some experience) be readily differentiated by their visual appearance from listeria colonies. Other bacilli tested could not grow. ALOA also supported the growth of eight of 10 strains of *E. faecium* tested while none of the *E. faecalis* strains could grow. Two of 10 bacterial strains isolated from the selective Oxford and PALCAM agars (yellow colonies) could grow on ALOA as bluish colonies without halo and as such gave no problem for differentiation from *L. monocytogenes*.

Recovery of *Listeria monocytogenes* from mixed listeria populations

The results are summarized in Table 2. On the countable plates of the diagnostic medium it was shown that the presence of *L. innocua* inoculated at a ratio 10 times higher than *L. monocytogenes* resulted in very small numbers of *L. monocytogenes* in the primary enrichment as well as in the secondary enrichment. Indeed, taking those plates with a maximum of 150 colonies into account, 110 and two colonies of *L. innocua* and *L. monocytogenes*, respectively, could be detected after primary enrichment on ALOA. On PALCAM and Oxford, the total counts for listeria were 83 and 71, respectively. After secondary enrichment, 121 and four colonies of *L. monocytogenes* and *L. innocua*, respectively, were counted. Following the prescriptions of the ISO/IDF Standards and picking five colonies for confirmation from Oxford/

Table 1 Qualitative evaluation of the specificity of ALOA with various genera of Gram-positive bacteria

Bacterial species	No. of strains	No growth on ALOA	Growth on ALOA (bluish)	Growth on ALOA (opaque halo)
Enterococcus spp.	20	12	8	0
Staphylococcus spp	3	3	0	0
Bacillus spp.	7	2	5	1
Unidentified isolates from Oxford/ PALCAM plates	10	8	2	0
Listeria-like bacteria	4	0	4	0

Table 2 Differentiation of *Listeria monocytogenes* on ALOA after primary and secondary enrichment of samples inoculated with various ratios of *L. monocytogenes* and *L. innocua*

	ALOA		PALCAM	Oxford
Ratio *L.m./L.i.*	Suspected *L.m.*	Suspected *L.i.*	Suspected *Listeria* spp.	Suspected *Listeria* spp.
Counts after primary enrichment (24 h, 30 °C)				
1/0·1	27×10^4	$<1 \times 10^4$	16×10^4	20×10^4
1/10	2×10^4	110×10^4	83×10^4	71×10^4
10/10	92×10^4	35×10^4	205×10^4	109×10^4
Counts after secondary enrichment (48 h, 37 °C)				
1/0·1	18×10^7	$<1 \times 10^7$	25×10^7	28×10^7
1/10	4×10^6	121×10^6	131×10^6	127×10^6
10/10	45×10^7	6×10^7	32×10^7	34×10^7

* Inoculation of almost 100 cfu *L.m.* or *L.i.* ml^{-1} in 25 ml enrichment broth.
L.m., Listeria monocytogenes; L.i., L. innocua.

PALCAM plates significantly reduces the chance of detecting the presence of *L. monocytogenes*. When the inoculation ratio of *L. monocytogenes* was higher than or equal to that of *L. innocua*, counts of *L. monocytogenes* compared with *L. innocua* were higher as was shown on ALOA, enhancing their detection by standard procedures.

Subsequently, our laboratory participated in two ring trials analysing different food samples, e.g. milk, cheese, meat and egg powder, inoculated with a mixture of *L. monocytogenes* and *L. innocua*. ALOA was compared with PALCAM and/or Oxford as the selective isolation medium. The results obtained for cheeses are given in Table 3.

Using ALOA, no false positives or false negatives were detected. For all cheese samples analysed, the negative samples on the ALOA plates yielded suspected bluish listeria colonies without a halo. Samples inoculated with a high level of *L. monocytogenes* and *L. innocua* showed high numbers of colonies with an opaque halo on the ALOA plates. However, when high numbers of colonies were present it was often not possible to distinguish blue colonies without a halo. When low levels of *L. monocytogenes* were inoculated, in some cases (two of five) it was also possible to show the presence of *L. innocua* on the ALOA plate.

Comparing plates after primary and secondary enrichment, a higher number of presumptive *L. monocytogenes* colonies, which were also confirmed as *L. monocytogenes*, were found after primary enrichment. Due to the high numbers of *L. monocytogenes* it was almost impossible to distinguish colonies of *L. innocua*. On ALOA plates after secondary enrichment, a few *L. monocytogenes* colonies (one to five) were detected together with many suspected listeria colonies (bluish colonies without halo) especially for samples inoculated with the lower level *L. monocytogenes*/*L. innocua*. Using Oxford agar as a selective plating medium, one sample (low inoculation

Table 3 Isolation of *Listeria monocytogenes* from cheese samples artificially inoculated with various levels of *L. monocytogenes* and *L. innocua*

Sample code	Inoculation level	Primary enrichment		Secondary enrichment	
		ALOA type of suspected colonies	Oxford	ALOA type of suspected colonies	Oxford
1867	Blank	Bluish	−	Bluish	−
1868	Blank	Bluish	−	Bluish	−
1869	Blank	Bluish	−	Bluish	−
1870	Low	*L.m.*+bluish	+	Bluish+*L.m.* (1)	−
1871	Low	*L.m.*+bluish	++	Bluish+*L.m.* (5)	+
1872	Low	*L.m.*	++	Bluish+*L.m.* (2)	+
1873	Low	*L.m.*	++	Bluish+*L.m.* (2)	−
1874	High	*L.m.*	++	*L.m.*+bluish	+
1875	High	*L.m.*	++	*L.m.*+bluish	+
1876	High	*L.m.*	++	*L.m.*+bluish	+
1877	High	*L.m.*	++	*L.m.*+bluish	−
1878	Blank	Bluish	−	Bluish	−
1879	Blank	Bluish	−	Bluish	−
1880	Low	*L.m.*	−	Bluish+*L.m.*	−
1881	High	*L.m.*	++	*L.m.*+bluish	++

−, None of the colonies picked for confirmation was confirmed as *L. monocytogenes*; ++, at least three of five colonies were confirmed as *L. monocytogenes*; +, less than three of five colonies were confirmed as *L. monocytogenes.*
L.m., Listeria monocytogenes; between ( ) number of colonies

level) was found to be false negative after both primary and secondary enrichment. Three samples, found to be positive on Oxford after the first enrichment, became negative after the secondary enrichment. Specifically for the cheese samples, a higher percentage of colonies picked from Oxford and confirmed as *L. monocytogenes* were obtained after 24 h compared with 48 h of incubation. Using the Oxford plate the detection rate was clearly different depending on the enrichment stage. This is in agreement with the numbers of *L. monocytogenes* colonies obtained with ALOA.

For the meat samples (Table 4), all samples were correctly identified with ALOA and Oxford. Using ALOA, all positives were detected after both primary and secondary enrichment. However, the number of *L. monocytogenes* colonies after primary enrichment was smaller compared with that after secondary enrichment. Using Oxford, of 10 samples inoculated with *L. monocytogenes*, four (three inoculated at a low level and one at a high level) were observed as negative after primary enrichment. Secondary enrichment was necessary to detect *L. monocytogenes*.

Detection of *L. monocytogenes* in the egg samples as distributed was rather difficult (Table 4). Two false negatives were obtained on ALOA and Oxford. Using Oxford, two additional samples were detected as false negatives. No agreement could be detected between the inoculation level and the numbers of *L. monocytogenes* colonies on the plates. The detection rate also seemed not to be influenced by the enrichment stage.

In 12 of 56 (21·4%) isolations on Oxford from primary and secondary enrichment no *L. monocytogenes* were detected, whereas *L. monocytogenes* were detected on ALOA. Using ALOA the importance of isolations after primary and secondary enrichment and the differences between them in counts of *L. monocytogenes* could be demonstrated. A higher score was obtained after primary enrichment for cheese samples whilst the opposite was true for meat samples.

In the second collaborative study six samples of pasteurised milk were examined. Two were only inoculated with *L. innocua* (blanks) and the others were spiked with *L. monocytogenes* and *L. innocua*. Two levels of contamination were introduced. Following the ISO Standard none of the samples was found positive for *L. monocytogenes*. The picking of only five colonies from the Oxford/PALCAM plates resulted in four false negative results. Isolations from the spiked samples on ALOA revealed two to be weakly positive since few colonies (two to five) were detected on the agar plate. For two others a higher

Table 4 Isolation of *Listeria monocytogenes* from meat and egg samples artificially inoculated with various levels of *L. monocytogenes* and *L. innocua*

Sample code	Meat samples inoculum level	ALOA colony type	Oxford first/second enrichment	Sample code	Egg samples inoculum level	ALOA colony type	Oxford first/second enrichment
1582	Blank	Bluish	−/−	1567	High	Bluish+*L.m.* (3)	−/−
1583	Blank	Bluish	−/−	1568	Blank	−	−/−
1584	High	*L.m.*	+/++	1569	Low	Bluish+*L.m.* (5)	−/+
1585	Low	*L.m.*	−/++	1570	High	*L.m.*	+/++
1586	Low	*L.m.*	++/++	1571	Low	*L.m.*+bluish	+/+
1587	Low	*L.m.*	−/++	1572	High	*L.m.*	+/++
1588	Blank	Bluish	−/−	1573	High	*L.m.*	++/++
1589	Low	*L.m.*	−/−−	1574	Low	*L.m.*	−/−
1590	High	*L.m.*	+/++	1575	Blank	−	−/−
1591	Blank	Bluish	−/−	1576	Low	Bluish	−/−
1592	High	*L.m.*	−/++	1577	High	*L.m.*+bluish	+/+
1593	Blank	Bluish	−/−	1578	Blank	Bluish	−/−
1594	Low	*L.m.*	−/++	1579	Low	Bluish	−/−
1595	High	*L.m.*	+/++	1580	Blank	−	−/−
1596	High	*L.m.*	+/++	1581	Blank	−	−/−

−, None of the colonies picked for confirmation was confirmed as *L. monocytogenes*; ++, at least three of five colonies were confirmed as *L. monocytogenes*; +, less than three of five colonies were confirmed as *L. monocytogenes*.
L.m., *Listeria monocytogenes*.

number of *L. monocytogenes* could be detected on ALOA. Two samples only inoculated with *L. innocua* were correctly identified on both ALOA and Oxford/PALCAM.

Comparison of ALOA and standard selective isolation plates using naturally contaminated food samples

The results are summarized in Table 5. A comparison between the results obtained on ALOA and those from standard plating media indicated that 31 samples were positive for *L. monocytogenes* on ALOA while 22 samples were positive for *L. monocytogenes* using PALCAM and Oxford, with confirmation of the presumptive positive colonies with Genprobe™ and/or Vidas™. For 17 samples (8·2%) the results for the presence of *L. monocytogenes* were in accordance for both methods. This was also the case for 39 samples (18·8%) positive for non-pathogenic *Listeria* spp. and for 126 samples (60·6%) where no listeria could be found. Five false-negative results were obtained for *L. monocytogenes* using ALOA. In two of these other *Listeria* spp. were found while three samples were completely negative for *Listeria* spp. Of the false negatives obtained on PALCAM/Oxford, eight samples contained non-pathogenic Listeriae whereas with ALOA *L. monocytogenes* was detected in these samples and for six samples no Listeriae could be detected. Using ALOA, five samples containing a mixed population of both pathogenic and non-pathogenic *Listeria* strains could be determined while only one was detected as positive for *L. monocytogenes* using PALCAM/Oxford. Two of these were found to be completely negative and for two others only non-pathogenic listeria were detected. Since presumptive colonies on Oxford/PALCAM were identified by Genprobe™ and Vidas™, it was not possible to determine exactly the presence of both *L. monocytogenes* and other *Listeria* spp. in one sample. In general, ALOA was superior to PALCAM/Oxford for the detection of *L. monocytogenes* with an overall sensitivity of 31 of 36 (86·1%) and 22 of 36 (61·1%), respectively. Total numbers of false-negative results were five of 36 (13·9%) using ALOA and 14 of 36 (38·9%) using Oxford/PALCAM.

DISCUSSION

Listeria monocytogenes was not considered an important foodborne pathogen until some large listeriosis outbreaks occurred at the end of the 1980s, which were due to the consumption of contaminated food. Hence, few selective media existed for the isolation of *L. monocytogenes* from food. McBride and Girard (1960) developed a selective medium for the isolation of *L. monocytogenes* from clinical samples since problems were encountered with contaminating flora such as streptococci, micrococci, some strains of Enterobacteriaceae and pseudo-

Table 5 Comparison of the performance of ALOA, Oxford and PALCAM for the detection of *Listeria monocytogenes* (number of samples found positive for either *L. monocytogenes* or other *Listeria* spp. or both)

		Isolation medium ALOA				
		L.m. pos. *L.* spp. pos	*L.m.* pos. *L.* spp. neg.	*L.m.* neg. *L.* spp. neg.	*L.m.* neg. *L.* spp. pos.	Total
Isolation medium Oxford/ PALCAM	*L.m.* pos*	1	16	3	2	22
	L.m. neg. *L.* spp. neg.	2	4	126	7	139
	L.m. neg. *L.* spp. pos.	2	6	2	37	47
	Total	5	26	131	46	199

* Using PALCAM or Oxford, the presence of other *Listeria* spp. besides *L.m.* was not checked.
L.m., Listeria monocytogenes; *L.* spp., *Listeria* spp.

monads. Many modifications of their medium were subsequently developed and used to isolate *L. monocytogenes* from food expected to contain *Listeria* spp. (Modified McBride's agar (FDA-MMLA); Lovett *et al*. 1987). However, the selectivity was insufficient. Many researchers sought to improve the selectivity and proposed improved formulas. The development of lithium chloride phenylethanol moxalactam agar (LPM) by Lee and McClain (1986) was a significant improvement for the isolation of *L. monocytogenes*. They took advantage of the general resistance of *Listeria* spp. to lithium chloride and to cephalosporins such as moxalactam and ceftazidime. These principles were the basis for other selective media. The indicative character in this medium was still based on the development of bluish-grey colonies when using Henry's illumination.

A more pronounced colony appearance, which was easier to interpret, was effected by adding esculin to the medium, resulting in the formation of greyish–green to black colonies and, in some cases, black media as well. These elements have been exploited in most of the media currently used, such as Oxford agar (Curtis *et al*. 1989), modified Oxford agar (McClain and Lee 1988) and PALCAM agar (van Netten *et al*. 1989). However, at present there is still an apparent need for a medium which can distinguish between *L. monocytogenes* and other non-pathogenic *Listeria* spp. Furthermore, it is well known that, during enrichment in a selective broth, *L. monocytogenes* can be overgrown by the faster growing *L. innocua* (Due and Schaffner 1993; Petran and Swanson 1993; Barbosa *et al*. 1994; Curiale and Lewus 1994; MacDonald and Sutherland 1994) and, as a consequence, may be missed when picking only five colonies from the isolation media for confirmation. The haemolytic ceftazidime lithium chloride agar (HCLA; Blanco *et al*. 1989; Dominguez *et al*. 1990; Poysky *et al*. 1993), enhanced haemolysis agar (EHA; Cox *et al*. 1991ab) and some modifications of it (Beumer *et al*. 1992; Heisick *et al*. 1995; Beumer *et al*. 1997), *L. monocytogenes* blood agar (LMBA; Johansson and Kankare 1996; Johansson 1998), the recently developed Rapid L' mono® (Foret and Dorey 1997; Ghafir *et al*. 1998) and the ALOA medium (Ottaviani *et al*. 1997ab) are examples of such media enabling differentiation of *L. monocytogenes* from other *Listeria* spp. The ALOA medium studied here utilizes the basic principles of existing media by adding selective agents including lithium chloride, ceftazidime and polymyxin to reduce the growth of competitors. Esculin is omitted and substituted by a chromogenic substrate and a lipase substrate resulting in a typical bluish colony appearance for all Listeriae and the possibility of differentiating between *L. monocytogenes* and other *Listeria* spp. by the production of a clear-cut opaque halo surrounding the colonies. Recovery experiments with various serovars of *L. monocytogenes* strains on the medium showed that ALOA performed as well as TSA when enumerating broth cultures of *L. monocytogenes* while all of them grew as typical bluish colonies with an opaque halo. In food samples containing low numbers of *L. monocytogenes* in the presence of higher numbers of *L. innocua*, the superior performance of ALOA in detecting *L. monocytogenes* was clearly demonstrated. The picking of colonies for confirmation was much easier and small numbers could be detected. When high numbers of *L. monocytogenes* were present, the detection of other listeria was sometimes difficult due to the often large opaque zones surrounding the *L. monocytogenes* colonies. ALOA also supported good growth of various non-pathogenic *Listeria* spp. at a level similar to the non-selective medium TSA. Only the growth of a *L. grayi* ssp. *murrayi* strain was clearly reduced on ALOA. However, a comparable inhibition of this strain was observed on Oxford and PALCAM agars. The growth of most *Listeria* spp. on ALOA was

comparable to their growth on Oxford but superior to that on PALCAM agar when enumerating some *L. grayi* ssp. *grayi* and *L. ivanovii* strains. This could be due partly to the elimination of acriflavine in ALOA since it is well known that acriflavine is toxic to some *Listeria* strains (Smith and Archer 1988). The greater selectivity of PALCAM compared with Oxford and thus the reduced growth of some listeria, especially sublethally injured cells, has been reported by Gunasinghe *et al*. (1994). PALCAM is often proposed as the medium when a high background flora is expected whereas Oxford performs better when low numbers of stressed listeria are expected.

The advantage of ALOA is the possibility of differentiating between *L. monocytogenes* and other *Listeria* spp. All non-pathogenic listeria formed colonies with a typical bluish appearance. Attention has to be paid to the appearance of some *L. ivanovii* strains since some of them can also produce a rather vague halo especially after 48 h of incubation.

Concerning the performance of ALOA for the isolation of *L. monocytogenes* with reference to non-listeria background flora, only growth of some *B. cereus* strains could result in suspect presumptive positives (bluish colonies with a vague opaque halo). The growth of bacilli on ALOA may be due to the reduction in the lithium chloride concentration of the medium. Since the lithium chloride concentration in ALOA ($10\,g\ l^{-1}$) is reduced compared with that in Oxford/PALCAM ($15\,g\ l^{-1}$) not all *Bacillus* species are inhibited and some can produce bluish colonies on ALOA. A higher concentration of lithium chloride would probably reduce the numbers of competitive micro-organisms and could overcome the potential problem of false-positive colonies which have to be confirmed. The growth of some unidentified listeria-like bacteria (Rijpens *et al*. 1998) on the medium was also investigated. The four strains grew well, giving the typical appearance of *L. monocytogenes* strains. Some other genera, especially enterococci and other bacilli, could produce light-blue colonies on ALOA without, however, a clear opaque halo. Indeed, these genera are not completely inhibited during enrichment and they form an important background flora in some cheeses and meat products.

The studies on samples inoculated with *L. innocua* and *L. monocytogenes* at various ratios clearly indicated the superiority of ALOA. The picking of five colonies according to standard protocols resulted in false negatives especially when the number of colonies of *L. monocytogenes* was small in comparison to the numbers of *L. innocua*. Picking higher numbers of colonies could reduce the false negative proportions. However, it is difficult to define how many extra colonies must be picked to enhance the detection rate significantly and this will vary according to food type. Furthermore, this would result in a higher work load and increased analysis time and cost.

ALOA also performed generally well in the study on naturally contaminated food samples. Only five of 36 false negatives were detected while 14 of 36 false negatives were found using PALCAM/Oxford plates; 4·3% more samples were positive for *L. monocytogenes* on ALOA. It must be taken into account that this percentage would probably have been higher if only five colonies were confirmed. In summary, ALOA is a valuable additional medium for the detection of *L. monocytogenes* in food samples. Furthermore, since the presence of presumptive *L. monocytogenes* can be easily detected on ALOA after 24 h incubation, a definitive result can be obtained after 48 h which, in the case of a positive sample, negates the need for a secondary isolation reducing work load, time of analysis and cost. Furthermore, counting the cost for the preparation of 1 l ALOA from the dehydrated base and adding the supplements needed, ALOA is less expensive than Oxford and costs almost 20% more than PALCAM.

In conclusion, it can be stated that rapid methods which will reliably detect *L. monocytogenes* in a background of potentially healthy competing bacteria are required by the food industry and public health microbiologists. Therefore, it is advantageous to introduce a diagnostic isolation medium such as ALOA which allows identification of *L. monocytogenes* in the presence of high numbers of non-pathogenic *Listeria* spp. and other competitors. In this study, ALOA has proven to be a valuable addition to the media capable of differentiating *L. monocytogenes* from non-pathogenic *Listeria* spp. No significant differences in the number of cells recovered from non-selective media and ALOA were detected.

ACKNOWLEDGEMENTS

The authors wish to thank A. Van de Walle and J. Baert for their technical assistance.

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