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Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media

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Abstract

The performance of four commercial media, polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM), Oxford, Rapid'L.mono (Bio-Rad, Marne la Coquette, France) and Agar *Listeria* according to Ottaviani and Agosti (ALOA: AES Laboratoire, Combourg, France; Biolife, Milan, Italy), used to detect and enumerate 176 Belgian strains of *Listeria monocytogenes* of human and food origin, was evaluated. Four strains showed a low recovery and/or atypical colonies on one or more media. These results showed that a combination of these media, especially alternative media (Rapid'L.mono and/or ALOA) with esculin-containing media (PALCAM and/or Oxford), should therefore be recommended to detect or enumerate atypical strains of *L. monocytogenes*. In outbreak case investigation for example, incubation of plates should be extended to at least 96 h if no colonies are typical or growth does not appear after 48 h. This is a cost/benefit calculation that should be done in the context of recent listeriosis risk assessments.

Keywords: Enumeration; Detection; *Listeria monocytogenes*; Media

1. Introduction

Among the six species of the *Listeria* genus (*L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi*), only *Listeria monocytogenes* has the potential to cause a "flu-like" illness in pregnant women that may ultimately result in abortion, stillbirth, birth of a child with neonatal listeriosis and meningitis or primary bacteremia in nonpregnant adults and in juveniles (Farber and Peterkin, 1991). *L. monocytogenes* is recognized as a food-borne pathogen of major significance (Gahan and Collins, 1991). This species is responsible for sporadic and epidemic cases of listeriosis associated with a variety of foods, including meat products, raw vegetables, coleslaw, and dairy products.

Difficulties in detecting or enumerating *L. monocytogenes* in foods remain challenging insofar as culture media (Vlaemynck et al., 2000) recommended by reference EN ISO 11290 standard methods do not allow the specific distinction of this species

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(Anonymous, 1997, 1998). In these reference standard methods, after selective enrichment or resuscitation steps, the samples or appropriate dilutions are plated on selective agar media (Van Netten et al., 1988). These media, polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) and/or Oxford detect *Listeria* spp. by revealing esculinase (β-glucosidase) activity, a metabolic enzyme common to all *Listeria* species, but does not distinguish *L. monocytogenes* from other species. Gunasinghe et al. (1994) showed that the PALCAM medium is more sensitive and selective than the Oxford medium. A second step to differentiate *L. monocytogenes* strains is carried out by biochemical tests and hemolytic activity. The length (5–6 days) and unreliability of reference standard methods have led to the development of new alternative media: Rapid'L.mono (Bio-Rad, Marne La Coquette) and Agar *Listeria* according to Ottaviani and Agosti (ALOA ready-to-use plate: AES Laboratoire; ALOA media prepared in the laboratory: BioLife).

The ALOA medium (Vlaemynck et al., 2000) is based on the simultaneous detection of specific phosphatidylinositol-specific phospholipase C (PIPLC) and β-glucosidase activities. PIPLC, an enzyme associated with the virulence process (Mengaud et al., 1991), is only present in two pathogenic species of *L. monocytogenes* and *ivanovii* (pathogenic in mice). For the ALOA medium, this activity is detected by degradation of L-alpha-phosphatidylinositol contained in the agar that produces an opaque precipitate forming a halo around the colonies. β-Glucosidase activity is detected by a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucoside, which is hydrolyzed and produces a blue precipitate in all *Listeria* colonies. The Rapid'L.mono medium is based on the chromogenic detection of PIPLC which is demonstrated by hydrolysis of X-inositol phosphate contained in the agar that produces a blue staining of *L. monocytogenes* colonies, and the non-fermentation of xylose, contained in the medium, by *L. monocytogenes*. These two selective media have been validated in France by Association Française de Normalisation (AFNOR, Paris, France) certification: ALOA medium for detection in 24 h and Rapid'L.mono medium for detection and enumeration. None of the previous media can determine the level of virulence of *L. monocytogenes*.

The aim of this study was to compare the performance (inclusive selectivity and recovery) of solid media used in reference standard methods with that of alternative solid media in detecting and enumerating isolated *L. monocytogenes* strains of human and food origin, and detect atypical colonial morphologies and low recoveries of *L. monocytogenes* strains on these solid media.

2. Materials and methods

2.1. Strains

One hundred and seventy-six previously characterized strains (Tables 1–3) referred to the Belgian Reference Center over the period 1990 to 1992 (Gilot et al., 1996) were kindly provided by Dr. Gilot (Institut Louis Pasteur, Brussels, Belgium). Ninety-five of these strains were isolated from sporadic cases of human listeriosis and 81 strains were isolated from cheese. The 95 strains isolated from patients were distributed among four serovars, namely 4b (45.2%), 1/2a (32.6%), 1/2b (19%) and 1/2c (3.2%). Most strains (79.8%) isolated from cheese were serotyped as 1/2a, the other strains being distributed between serovars 4b (11.8%), 1/2b (5%), 1/2c (1.7%) and 3a (1.7%).

The strains, stored on cryobeads (AES Laboratoire) at −80 °C, were cultivated on Casein and Soya peptone broth (Caso: Merck, Darmstadt, Germany) supplemented with 0.6% yeast extract (Merck) at 30 ± 1 °C for 18 h. One milliliter of this preculture was transferred to 9 ml of Caso broth supplemented with 0.6% yeast extract and incubated for 18 h at 37 ± 1 °C.

2.2. Inclusive selectivity and recovery testing

From the Caso-supplemented cultures of strains incubated 18 h at 37 ± 1 °C, decimal dilutions were performed in Caso broth to inoculate media plates with an inoculum comprising 15 to 300 colonies. Caso-supplemented cultures were streaked on four media, PALCAM (Merck), Oxford (Merck), Rapid'L.mono and ALOA to determine the inclusive selectivity of each medium. The inclusive selectivity of medium (Anonymous, 2003a) was the capacity of a

Table 1
Recovery results obtained on media used in reference standard methods (PALCAM and Oxford) and alternative media (Rapid'L.mono and ALOA), incubated at 37 ± 1 °C with strains of *L. monocytogenes* giving typical colony morphologies and normal recoveries

Serovar	Origin	Number tested	PALCAM		Oxford		Rapid'L.mono		ALOA	
			Colony aspect[a]	Median time (h) to obtain recovery of 95%[b]	Colony aspect[a]	Median time (h) to obtain recovery of 95%[b]	Colony aspect[a]	Median time (h) to obtain recovery of 95%[b]	Colony aspect[a]	Median time (h) to obtain recovery of 95%[b]
1/2a	Food	62	Typical (24 h) One strain, Typical (48 h)	24	Typical (24 h) One strain, Typical (48 h)	24	Typical (24 h) Two strains, Typical (48 h)	24	Typical (24 h) Two strains, Typical (48h)	24
	Clinical	30	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24
1/2b	Food	4	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24
	Clinical	18	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24
1/2c	Clinical	3	Typical (24 h)	24	Typical (24 h) One strain, Typical (48 h)	24	Typical (24 h) One strain, Typical (48h)	24	Typical (24 h) One strain, Typical (48h)	24
3a	Food	1	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24
4b	Food	11	Typical (24 h) One strain Typical (48 h)	24	Typical (24 h) One strain, Typical (48 h)	24	Typical (24 h) One strain, Typical (48h)	24	Typical (24 h)	24
	Clinical	43	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24	Typical (24 h)	24

[a] Typical colony on PALCAM: green colony, 1.5–2 mm in diameter, with greenish reflection and sometimes black center surrounded by black halo after 24 h and green colony, 1.5–2 mm in diameter, with sunken center surrounded by black halo; typical colony on Oxford: black colony, 1 mm in diameter, surrounded by black halo after 24 h and black colony, 2–3 mm in diameter, with black halo and sunken center after 48 h; typical colony on Rapid'L.mono: blue colonies not surrounded by a distinctive yellow halo; typical colony on ALOA: bluish colonies surrounded by a distinctive opaque halo.

[b] Medium recovery obtained by comparing enumeration on studied medium with that of tryptic soya agar.

medium to detect a target organism within a wide range of target or non-target strains. PALCAM and Oxford agar were prepared according to the manufacturer's instructions, and standard requirements for quality assurance (Anonymous, 2003b). Ready-to-use Rapid'L.mono and ALOA plates were used according to the manufacturer's instructions but ALOA was also prepared according to Biolife's instructions.

An aliquot (0.1 ml) of the appropriate decimal dilutions in Caso broth of the last Caso-supplemented cultures of strains was plated on each medium and on Tryptic Soya Agar (TSA: AES Laboratoire) to determine the medium recovery. Inoculated media were incubated at 37 ± 1 °C for 24 h and observed every 24 h for 6 days. The medium recovery, or productivity ratio of each medium (Anonymous, 2003b), was obtained by comparing enumeration on the tested culture medium with that on the reference medium, TSA.

At the same time, from the Caso-supplemented cultures of strains incubated for 18 h at 37 ± 1 °C, appropriate decimal dilutions were used to perform Mossel's econometric method (Mossel et al., 1980) on four media, PALCAM, Oxford, Rapid'L.mono and ALOA and on TSA. Plates were pre-dried for 18 h at 37 ± 1 °C which also allowed them to be tested for sterility. After thorough shaking, this culture and appropriate decimal dilutions were streaked with a standardized nichrome loop of 1 μl in series of five parallel lines over the four quadrants of a 7 cm circle painted at the bottom of 9 cm Petri dishes. Finally, one 4-cm-long straight line was drawn through the center. After inoculation, the plates were incubated at 37 ± 1 °C, the temperature at which both inoculated cultures had been grown, and observed every 24 h for 6 days. An absolute growth index (AGI) n was assigned, when all streaks in quadrant numbers $n-1$ and n showed full growth, while virtually no growth was observed on any of the streaks in quadrant number $n+1$. When approximately half of the streaks of a quadrant with number n showed growth, number $n+1$ no growth and $n-1$ full growth, an AGI $= n + 0.5$ was awarded. Fully grown plates were assigned AGI = 5.

Interference from the enrichment step during *L. monocytogenes* detection was investigated: Caso-supplemented broth culture (1 ml) of each strain was mixed with 9 ml of "Half-Fraser" (Merck) pre-enrichment broth (Fraser and Sperber, 1988) and incubated at 30 ± 1 °C for 24 h. An aliquot (0.1 ml) of incubated "Half-Fraser" pre-enrichment broth was added to 10 ml of Fraser enrichment broth (Merck) and incubated at 37 ± 1 °C for 48 h (Anonymous, 1997). "Half-Fraser" and Fraser broth were prepared according to the manufacturer's instructions and standard requirements for quality assurance (Anonymous, 2003b). From incubated "Half-Fraser" broth and Fraser broth, four media, PALCAM, Oxford, Rapid'L.mono and ALOA were streaked and tested according to Mossel's ecometric method and recovery, as described previously. The inoculated plates were incubated at 37 ± 1 °C and observed every 24 h for 6 days.

In each case, two colonies were tested using Columbia agar with 5% sheep's blood (bioMérieux, Marcy l'Etoile, France) for haemolysis, API *Listeria* (bioMérieux), L.Mono disk (AES Laboratoire), according to the manufacturer's instructions.

Minimal inhibitory concentration (MIC) determinations of antibiotics or antimicrobial agents containing in each studied media were performed for each strain of *L. monocytogenes* which gave atypical colonies or poor recovery on all of the studied media. MIC determinations were conducted with a PDM epsilometer (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. PDM epsilometer comprises a thin impervious test carrier (5×50 mm) with a continuous exponential gradient of antibiotics or antimicrobial agents immobilized on one side and a reading and interpretative scale on the other. Plates of Mueller–Hinton agar with or without blood supplement (AES Laboratoire) were swabbed with a suspension of microorganisms adjusted to 0.5 McFarland standard of a Caso broth culture supplemented with 0.6% yeast extract incubated for 18 h at 37 ± 1 °C. Test carriers were then applied in an optimal pattern with the nearest concentration maxima (position 10) nearest to the circumference of the Petri dish. The plates were immediately incubated for 18 h at 37 ± 1 °C. After incubation, MIC was directly read from the graded test carriers. Results were reported as a quantitative value (in. $\times$ μg·ml^{-1}) and/or a susceptibility group as defined by specific MIC/inhibitory concentration correlated break points.

2.3. Statistical methods

To ensure data normality, the colony counts (CFU) were transformed to log counts (log CFU). Counts reported as <1 or <0.1 were set to 0 during statistical analysis. The sign test, performed with SAS software (SAS Institute, 1987), was used to compare results.

3. Results

3.1. Inclusive selectivity testing

On PALCAM, of the 176 inoculated strains tested, six strains (MBLA 58, 75, 78, 83, 149, 154) of human origin and from cheese, serotyped 1/2a and 4b, gave only typical colonies after 48 h, as shown in Tables 1 and 2.

On Oxford, seven strains (MBLA 58, 75, 78, 92, 131, 149, 168), of human origin and from cheese, serotyped 1/2a, 1/2c and 4b, also gave only typical colonies after 48 h, as shown in Tables 1 and 2.

On Rapid'L.mono, as shown in Tables 1 and 3, three strains gave atypical results after 48 h. They are *L. monocytogenes* serovars 1/2a (MBLA 58) and 1/2c (MBLA 78) isolated from cheese linked with sporadic human cases and one *L. monocytogenes* serovar 1/2a (MBLA 149) of human origin. All these strains were identified and confirmed as *L. monocytogenes*. Strains MBLA 58 and 149 on Rapid'L.mono developed a white color after 24 h but a light blue color after 48 h. Strain MBLA 78 developed a light blue color after 96 h.

On ALOA, as shown in Tables 1 and 3, one strain (MBLA 149), serotyped 1/2a, gave typical colonies with a distinctive opaque halo after 96 h, even when a high inoculum content was used (around 10^8 CFU/ml).

Results obtained after the enrichment step in "Half Fraser" and Fraser broths were not significantly different ($p>0.05$) according to the sign test (SAS Institute, 1987; Snedecor and Cochran, 1980).

3.2. Recovery testing

Three strains (MBLA 75, 78, 149) of the 176 inoculated strains tested showed a poor recovery on one or more media (Tables 1–3), particularly strain MBLA 149, which gave the smallest colonies. Results obtained from Mossel's ecometric methods and recoveries were not significantly different ($p>0.05$) according to the sign test (SAS Institute, 1987; Snedecor and Cochran, 1980). Strain MBLA 75 of *L. monocytogenes* serovar 1/2a isolated from cheese linked with sporadic human cases, showed a low recovery after 48 h on Oxford ($<0.1\%$) and on PALCAM (32%). Strain MBLA 78 showed a low density of colonies (recovery $<0.1\%$) on Oxford or Rapid'L.mono within 48 h and had a low recovery on ALOA (8%) and PALCAM (0.3%). Strain MBLA 149 grew with a low density of colonies (recovery $<0.1\%$)

Table 2
Recovery results obtained on media used in reference standard methods (PALCAM and Oxford), incubated at 37 ± 1 °C with strains of *L. monocytogenes* giving atypical colony morphologies and low recoveries

Strain no.	Serovar	Origin	PALCAM			Oxford		
			Colony aspect[a]	Recovery (%) after 48 h[b]	Time (h) to obtain recovery of 95%	Colony aspect[a]	Recovery (%) after 48 h[b]	Time (h) to obtain recovery of 95%
MBLA 58	1/2a	Cheese	Typical (48 h)	111	24	Typical (48 h)	106	24
MBLA 75	1/2a	Cheese	Typical (48 h)	32	96	Typical (48 h)[c]	<0.1	144
MBLA 78	1/2c	Cheese	Typical (48 h)[c]	0.3	96	Typical (48 h)[c]	<0.1	>144
MBLA 149	1/2a	Human	Typical (48 h)[c]	<0.1	96	Typical (48 h)	27	144

[a] Typical colony on PALCAM: green colony, 1.5–2 mm in diameter, with greenish reflection and sometimes black center surrounded by black halo after 24 h and green colony, 1.5–2 mm in diameter, with sunken center surrounded by black halo; typical colony on Oxford: black colony, 1 mm in diameter, surrounded by black halo after 24 h and black colony, 2–3 mm in diameter, with black halo and sunken center after 48 h.

[b] Medium recovery obtained by comparing enumeration on studied medium with that of tryptic soya agar.

[c] Low density of colonies (semiquantitative appreciation). Incubation time needed to obtain first typical colonies shown in brackets.

Table 3
Recovery results obtained on alternative media (Rapid'L.mono and ALOA), incubated at 37 ± 1 °C with strains of *L. monocytogenes* giving atypical colony morphologies and/or low recoveries

Strain no.	Serovar	Origin	Rapid'L.mono			ALOA		
			Colony aspect[a]	Recovery (%) after 48 h[b]	Time (h) to obtain recovery of 95%	Colony aspect[a]	Recovery (%) after 48 h[b]	Time (h) to obtain recovery of 95%
MBLA 58	1/2a	Cheese	Atypical-white colony with blue center (48 h)	96	48	Typical (24 h)	112	24
MBLA 75	1/2a	Cheese	Typical (24 h)	120	24	Typical (24 h)	90	48
MBLA 78	1/2c	Cheese	Atypical-white colony (until 96 h)[c]	<0.1	96	Typical (24 h)[c]	8	72
MBLA 149	1/2a	Human	Atypical-white colony with blue center (48 h)[c]	11	96	Typical but small halo (48 h)[c]	20	96

[a] Typical colony on Rapid'L.mono: blue colonies not surrounded by a distinctive yellow halo; typical colony on ALOA: bluish colonies surrounded by a distinctive opaque halo.
[b] Recovery was obtained by comparing enumeration on studied medium with that of tryptic soya agar.
[c] Low density of colonies (semiquantitative appreciation). Incubation time need to obtain first typical colonies shown in brackets.

on PALCAM within 48 h and showed a low recovery on Rapid'L.mono (11%), as well as ALOA (20%) and Oxford (27%).

4. Discussion

In this study, we examined the performance of media (PALCAM/Oxford) used in reference standard methods and alternative media (Rapid'L.mono and ALOA) to detect and recover *L. monocytogenes* strains. No medium has the capacity to detect and enumerate all the strains tested within the incubation time described in reference standard methods or in the manufacturer's instructions. All these media have a poor inclusive selectivity for some strains even if injured cells or naturally and artificially inoculated foods were not tested.

This casts some doubt on the monitoring capabilities for the presence of *L. monocytogenes* in food. Appropriate monitoring capabilities should lead to development of improved strategies to control this organism.

ALOA agar appeared to be the best medium, although one strain grew slowly on this medium and on other media (Table 3). One explanation for these late growth colonies and development of typical colonies on new selective media was that ALOA uses a natural substrate of PIPLC in contrast with Rapid'L.mono, which uses a chromogenic synthetic substrate cleaved by PIPLC. The growth and expression of enzymatic activity on selective agar media seems to differ according to the *L. monocytogenes* strains. It could also be related to repression of expression of the virulence gene or to inhibition of the enzymatic activity by the compounds of selective media (Beumer et al., 1997). Inclusive selectivity of PALCAM and Oxford is based on several antibiotics or antimicrobial agents (PALCAM: polymyxin B, acriflavine hydrochloride, ceftazidime/moxalactam; Oxford: cycloheximide, colistin sulphate, acriflavine, cefofetan, fosfomycin). Inclusive selectivity of Rapid'L.mono is patented. ALOA contains several antibiotics and antimicrobial agents [nalidixic acid, ceftazidime, polymyxin B, cycloheximide (Biolife) or amphotericin B (AES Laboratoire)]. MIC of strains (MBLA 58, 75, 78, 149) with antibiotics or antimicrobial agents found on the media were investigated using PDM epsilometers. No particular MIC according to media concentration were obtained but the growth of strains, latent period before colony observation and expression of enzymatic activity, could be related to the combination of several antibiotics and antimicrobial agents contained in the media and the multidrug resistance of strains. Test results were affected by the poor growth of some strains, such as MBLA 78 and, in particular, MBLA 149 (data not shown). This last strain seems to require special compounds or a lower incubation temperature for optimal growth. Based on the work of Camilli et al. (1991), *L. monocytogenes* strains that give atypical colonies on chromogenic media

could be PIPLC defective mutants which are less virulent than normal strains. Failure to detect such strains on these media should not give rise to public health concerns. In our study, these strains were virulent because of their origin, as shown in Tables 2 and 3. Another explanation for these late growth and development of typical colonies is that, for esculin-containing agar media such as PALCAM and Oxford, Siragusa et al. (1990) found that *Listeria* species, in particular *L. monocytogenes*, formed petite-sized colonies from parent stock cultures when grown on esculin-containing media. A possible role of the esculin hydrolysis product, esculetin, in causing petite colony formation and an inhibitory effect on colony formation could be established. Nevertheless, it is possible that ferric iron contained in media and complexing this inhibitory effector molecule could lessen the amount of free uncomplexed esculetin available to inhibit cell growth and explain the variability in colony sizes. Siragusa et al. (1990) also showed that cells forming petite colonies are β-glucosidase (esculinase) constitutive variants, with more rapid formation of esculetin, within the parent population while cells that form normal-sized colonies are inducible for β-glucosidase activity that could explain also these late growth colonies on media (PALCAM, Oxford and ALOA) which reveal esculinase (β-glucosidase) activity and development of atypical colonies on ALOA agar.

Inclusive selectivity and recovery results were not significantly different ($p>0.05$) in the sign test results between ready-to-use ALOA (AES Laboratoire) and ALOA produced in the laboratory from base powder and supplement (BioLife). These results were obtained even if cycloheximide was initially replaced by amphotericin B in these last media and, for the detection of *L. monocytogenes*, the enrichment step in ''Half Fraser'' and Fraser broths were performed before streaking on agar plates (data not shown).

This study highlights difficulties in obtaining typical colonies and recovering initial concentration on reference standard methods or alternative media with respect to some *L. monocytogenes* strains. Strains (MBLA 58, 75, 78 and 149) have a low recovery and one strain (MBLA 149) needed 144 h to obtain a recovery of 95% on ALOA agar. So far, in outbreak case investigation for example, observations after 24 h or 48 h, as described in International Standardization Organization standards (Anonymous, 1997, 1998), should be extended to at least 96 h if no typical colonies or no growth appear after 48 h. The use of several media, at least two, is recommended. If an approximately atypical strain behavior rate of about 3 or less per 176 (about 1.7% or less) is critical then the use of two media would reduce it to about 0.5% or less. This is a cost/benefit calculation that should be done in the context of recent listeriosis risk assessments. Karpiskova et al. (2000) proposed the replacement of PALCAM and Oxford media used in the EN ISO 11290-1 standard method with Rapid'L.mono. Our findings showed that the introduction of Agar *Listeria* according to Ottaviani and Agosti (Vlaemynck et al., 2000), ALOA, instead of or with PALCAM and/or Oxford in actual detection or enumeration standards should enhance detection and enumeration of atypical strains of *L. monocytogenes*.

Using established qualitative and quantitative criteria for *L. monocytogenes* for regulation and economical impact generated by *L. monocytogenes* detection, atypical strains can give rise to false-negative results. Molecular methods based on amplification of *L. monocytogenes* virulence genes should be able to detect atypical strains and/or enumerate them with methods such as real-time quantitative PCR (Karpiskova et al., 2000). To control this pathogen in the food industry, false-negative and false-positive results should be more thoroughly examined in order to improve the classical detection or enumeration of *L. monocytogenes*.

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