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Evaluation of chromogenic media for the detection of *Listeria* species in food

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ABSTRACT

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Aims: The aim of this study was to evaluate the performance of chromogenic agars, Agar Listeria according to Ottaviani and Agosti (ALOA) and Rapid L. mono agar, compared with Oxford agar for the enumeration and detection of *Listeria* species in food.

Methods and Results: A total of 170 food samples were examined using the three plating media. *Listeria* species were isolated from 63 samples. In contrast to Oxford agar, detection of *Listeria* colonies on chromogenic media was as good after 24 h of incubation of plates as after 48 h. While there was no significant difference in recovery of *Listeria monocytogenes* on the three media, recovery of other *Listeria* species was significantly poorer on Rapid L. mono agar compared with Oxford and ALOA agars. Recovery of species other than *L. monocytogenes* was significantly improved by including a secondary enrichment stage in the detection method.

Conclusions: Using chromogenic agars, presumptive identification of *L. monocytogenes* is possible after 24 h, compared with 3–4 days using Oxford agar. However, the poor detection of species other than *L. monocytogenes* on Rapid L. mono agar is a disadvantage of this medium.

Significance and Impact of the Study: This study provides new information regarding the isolation of *Listeria* species other than *L. monocytogenes* from food using chromogenic plating media. This is important, as non-pathogenic *Listeria* species act as markers for the likelihood of presence of *L. monocytogenes* and allow preventive action to be taken to avoid its presence.

Keywords: chromogenic agar, detection, enumeration, food, *Listeria*, media.

INTRODUCTION

Although cases of listeriosis are relatively rare, with approximately 100–200 cases per year being reported to the Health Protection Agency in England and Wales (Health Protection Agency 2004), the presence of *Listeria monocytogenes* in food is of concern because of the severe symptoms it can cause. These include meningitis, septicaemia and, in pregnant women, miscarriage (Rocourt 1996).

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The ability of *Listeria* species to grow and multiply at refrigeration temperatures is a further reason for concern, particularly for products with an extended shelf-life such as vacuum-packed meats and pâté. For example, pâté was identified as the likely cause of the increase in listeriosis cases in England and Wales between 1985 and 1989 (McLauchlin *et al.* 1991), and there was a further report of pâté-related illness in 1991 (Cumber *et al.* 1991).

A number of recent studies and outbreaks have shown that *Listeria* is still a cause for concern in many food products. A survey of ready-to-eat foods from retail and catering premises in the South of England highlighted a number of incidents in which hygiene problems during production led

to significant contamination rates of sliced meats and pâté with *Listeria* species (Willis and Greenwood 2003). Moreover, an outbreak of listeriosis occurred in France in 1999/2000 that was linked to consumption of pork tongue (Public Health Laboratory Service 2000). Recently an increase in the number of cases in northern England was linked with the consumption of contaminated butter (Health Protection Agency 2004).

While *L. monocytogenes* is the most pathogenic species of this genus, *Listeria ivanovii* and *Listeria seeligeri* have also been known to cause infection on rare occasions (Lessing *et al.* 1994). Although the presence of other *Listeria* species (e.g. *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi*) in food is not of direct pathogenic significance, these organisms can be considered as useful indicators of a deterioration in hygiene or process conditions, leading to an increased risk of contamination with pathogenic *Listeria* species. Therefore, the use of microbiological methods that can detect all *Listeria* species is advantageous when testing food samples. Current standard methods for detecting and enumerating *L. monocytogenes* have poor specificity for the species, although good specificity for the genus. This is partly because of the poor discrimination of plating media between *L. monocytogenes* and other species (Vlaemynck *et al.* 2000), and also due to the ability of species such as *L. innocua* to outgrow *L. monocytogenes* during enrichment, such that the low proportion of *L. monocytogenes* colonies compared with the proportion of other species leads to failure to detect the pathogenic species (Petran and Swanson 1993; Curiale and Lewus 1994). Therefore, when only non-pathogenic species are isolated, the possibility must be considered that *L. monocytogenes* is also present, but undetected.

The current international standard method for the detection of *Listeria* in food (ISO 11290-1; British Standards Institution 1997) involves a two-step enrichment process with plating to selective media that use aesculin hydrolysis as the basis for detection of *Listeria* species. This method requires a minimum of 5 days to obtain a result. Enumeration uses the same plating media (British Standards Institution 1998). As all *Listeria* species have a similar colonial morphology on selective agars such as Oxford agar and PALCAM, subculture of multiple colonies for confirmatory testing is required to identify the species. This takes at least a further 2 days. It is therefore very difficult, with the current method, to be proactive in preventing the consumption of contaminated food, as many short shelf-life ready-to-eat foods would have been consumed before microbiological results were available.

A variety of chromogenic agars have recently become commercially available, with the aim of shortening the time taken to obtain a result, and distinguishing between pathogenic and non-pathogenic *Listeria* species. For example, the recognition of *Listeria* colonies on Agar Listeria according to Ottaviani and Agosti (ALOA) (Ottaviani *et al.* 1997) is based on β-glucosidase activity of the bacteria. This enzyme cleaves a chromagen resulting in blue/green colouration of the colonies. There are a number of other organisms with β-glucosidase activity, such as enterococci, but selective agents incorporated into the agar are aimed at inhibiting the growth of these species. In addition, lecithin present in the agar is hydrolysed by the phospholipase enzyme produced by *L. monocytogenes* and *L. ivanovii* but not other *Listeria* species, resulting in the production of an opaque halo around the colony. Thus, the producers claim that *L. monocytogenes* can be distinguished from other *Listeria* species after 24 h of incubation. The same principles are used in a similar agar produced by Oxoid Ltd (Basingstoke, UK), known as Oxoid Chromogenic Listeria Agar.

On a second type of chromogenic agar, Rapid L. mono agar, *L. monocytogenes* and *L. ivanovii* produce blue colonies because of phospholipase C activity, while other *Listeria* species are seen as white colonies. Furthermore, *L. monocytogenes* is distinguished from *L. ivanovii* by its inability to ferment xylose. Thus, fermentation of this substrate by *L. ivanovii* results in the formation of a yellow halo around the colony, while *L. monocytogenes* colonies have no halo.

Evaluations of chromogenic media for the isolation of *L. monocytogenes* have been reported previously (Jinneman *et al.* 2003; Sacchetti *et al.* 2003). However, the efficiency of these novel media at isolating other *Listeria* species has not been previously determined. The aim of this multi-laboratory study was to evaluate the performance of chromogenic agars, ALOA and Rapid L. mono agar, compared with Oxford agar for the detection and enumeration of both *L. monocytogenes* and other *Listeria* species.

MATERIALS AND METHODS

Food samples

Foods (including raw and cooked meat, dairy products, pâté, smoked fish and salad) were purchased from retail outlets by laboratory staff at Wessex Environmental Microbiology Services, Preston Public Health Laboratory and London Food Water and Environmental Laboratory, or were submitted to the laboratories by Environmental Health Officers as part of their routine sampling programmes.

Comparison of selective agars

The procedure described in ISO 11290-1 (British Standards Institution 1997) was used for detection of *L. monocytogenes* and other *Listeria* species. Volumes of 0·5 ml of the initial food suspension before incubation, and 10 μl volumes of the primary and secondary enrichment cultures after incubation,

were used to inoculate Oxford agar (Oxoid Ltd), ALOA (AES Laboratoire, Combourg, France) and Rapid L. mono agar (Bio-Rad, Marne-la-Coquette, France). Oxford agar plates were incubated at 30°C, while ALOA and Rapid L. mono agars were incubated at 37°C. All plates were examined after 24 and 48 h.

Recognition and confirmation of *Listeria* species

Five presumptive *Listeria* colonies of each morphological type (see Table 1) from each agar plate were subcultured to Colombia blood agar plates containing 5% horse blood (or all colonies if <5 were present). Blood agar was incubated for 24 h at 37°C and cultures examined for presence or absence of haemolysis. The identity of presumptive *Listeria* species was confirmed using API Listeria biochemical strips (Biomerieux, Craponne, France) according to the manufacturer's instructions.

Statistical analyses

Colony numbers on agar plates used for enumeration (i.e. those spread with 0·5 ml of primary suspension) were compared using the Students *t*-test. Recovery of *Listeria* species on plates after primary and secondary enrichment was compared using the chi-squared test.

RESULTS

In total, 170 food samples were examined. These consisted of cooked meat (78), raw meat (27), soft cheese (10), smoked fish (24), sandwiches (2), cream (3), pâté (14) and salad (12). Of these, *Listeria* species (including *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. grayi* and *L. seeligeri*) were isolated from 63 samples.

Comparison of *Listeria* enumeration on Oxford, ALOA and Rapid L. mono agars

Strains of *Listeria* were detected in 19 samples by direct enumeration (Table 2). Colony numbers on Rapid L. mono were lower than those on Oxford agar, at a borderline level of significance ($P = 0{\cdot}050$; Student's *t*-test); differences in bacterial numbers between Oxford and ALOA agars, and between ALOA and Rapid L. mono were not significant.

In four samples (samples 3, 4, 17 and 18), *Listeria* strains (both *L. monocytogenes* and other *Listeria* species) were detected using Oxford agar, that were not detected using ALOA or Rapid L. mono agar. Similarly, *L. monocytogenes* was detected in sample 9 using ALOA, but not using Oxford or Rapid L. mono agars. *Listeria monocytogenes* was also

Table 2 Bacterial counts on the three selective media for each sample from which *Listeria* species were detected by direct enumeration

	Bacterial count (CFU per g)		
	Oxford	Rapid L. mono	ALOA
L. monocytogenes			
Sample 1	2.6×10^2	2.4×10^2	6.6×10^2
Sample 2	2.1×10^3	1.4×10^3	1.8×10^3
Sample 3	1.0×10^2	<20	<20
Sample 4	2.2×10^2	<20	<20
Sample 5	<20	20	<20
Sample 6	1.6×10^2	40	60
Sample 7	$>3.0 \times 10^3$	$>3.0 \times 10^3$	$>3.0 \times 10^3$
Sample 8	<20	20	<20
Sample 9	<20	<20	40
Sample 10	1.2×10^3	9.6×10^2	$4.8 \times \times 10^2$
Sample 11	1.6×10^3	1.8×10^3	1.3×10^3
Sample 12	1.4×10^3	7.8×10^2	6.4×10^2
Sample 13	$>3.0 \times 10^3$	$>3.0 \times 10^3$	$>3.0 \times 10^3$
Sample 14	40	20	20
Sample 15	40	<20	<20
Other *Listeria* species			
Sample 15	<20	20	40
Sample 16	2.0×10^2	80	1.8×10^2
Sample 17	20	<20	<20
Sample 18	20	<20	<20
L. mono + other *Listeria* species			
Sample 19	2.2×10^2	3.0×10^2	2.0×10^2
Total number of samples from which *Listeria* detected by enumeration	16	14	13

Table 1 Typical colonial morphology of *Listeria* species on selective agars

Agar	*L. monocytogenes*	*L. ivanovii*	Other *Listeria* species
Oxford	Grey, concave colony surrounded by black/brown zone	Grey, concave colony surrounded by black/brown zone	Grey, concave colony surrounded by black/brown zone
ALOA	Blue/green colony with opaque halo appearing after 24 h	Blue/green colony with opaque halo appearing after 48 h	Blue/green colony without halo
Rapid L. mono	Blue/green colony with no coloured halo	Blue/green colony surrounded by yellow halo	White colonies with or without yellow halo

detected in two samples (samples 5 and 8) using Rapid L. mono but not the other two agars. In sample 15, *L. monocytogenes* was detected by Oxford agar and a different *Listeria* species by ALOA and Rapid L. mono agar. However, differences in frequency of detection between the three agars were not significant.

Comparison of *Listeria* detection on Oxford, ALOA and Rapid L. mono agars after enrichment

After primary enrichment, strains of *Listeria* were detected in 51 samples (Table 3). *Listeria monocytogenes* was detected in 36 samples and other *Listeria* species in 25 samples. Ten samples contained both *L. monocytogenes* and another strain of *Listeria*; eight of these were detected using ALOA agar and seven using Rapid L. mono, but only four using Oxford agar. The use of Oxford agar resulted in the detection of 25 samples contaminated with *L. monocytogenes* compared with 34 samples using ALOA and 30 using Rapid L. mono medium. However, no differences between media recovery were significant.

Listeria strains were detected in a total of 60 samples after secondary enrichment. The total number of samples in which *Listeria* was detected on Oxford agar increased from 41 after the primary enrichment to 60 after secondary subculture. However, compared with the chromogenic media, the number of samples containing a combination of *L. monocytogenes* and other species after secondary enrichment was underestimated using Oxford agar. Meanwhile, the number containing either *L. monocytogenes* or other species alone was overestimated by the use of Oxford agar.

Using ALOA agar, *L. monocytogenes* was detected in 34 samples after primary enrichment, but only 33 after secondary subculture. In contrast, the number of other *Listeria* species detected increased from 22 to 33 after the second enrichment.

There was no significant difference in recovery of *L. monocytogenes* on the three agars after secondary enrichment. However, other *Listeria* species were detected significantly less frequently on Rapid L. mono agar compared with Oxford agar ($P = 0{\cdot}011$; chi-squared test) and ALOA ($P = 0{\cdot}003$; chi-squared test).

Evaluation of primary compared with secondary enrichment for the detection of *Listeria*

The total number of samples in which *Listeria* was detected increased from 51 after primary enrichment to 60 after the secondary enrichment stage. Two of these additional samples were found to contain *L. monocytogenes* after the secondary enrichment, while the total number of samples in which other *Listeria* species were detected increased from 25 to 34 after the second enrichment stage. Although this overall difference in recovery was not significant, the recovery of other *Listeria* species on Oxford agar was significantly greater after the secondary enrichment ($P = 0{\cdot}030$; chi-squared test). On ALOA agar, the difference between recovery of *Listeria* species from the primary and secondary enrichments was of borderline statistical significance ($P = 0{\cdot}048$; chi-squared test).

Comparison of 24-h and 48-h incubation of selective agar plates

From the primary enrichment, *Listeria* was detected significantly less frequently on Oxford agar after 24 h of incubation than after 48 h ($P = 0{\cdot}013$; chi-squared test; data not shown). There was no significant difference in detection between 24 and 48 h of incubation for the two chromogenic media. Differences in detection after 1 or 2 days incubation were not significant for any of the three agars after secondary enrichment.

On ALOA and Rapid L. mono agars, *Listeria* colonies could be presumptively identified as *L. monocytogenes* or other *Listeria* species after only 24 h of incubation. No significant difference was observed between the number of samples containing presumptive *L. monocytogenes* after 24 h and the number from which a confirmed identity was obtained after 48 h. In contrast, no distinction could be made between *Listeria* strains on Oxford agar without further confirmatory tests.

Table 3 Number of samples from which *Listeria* species were detected by enrichment using the three different selective media

	Primary enrichment				Secondary enrichment			
	Oxford	ALOA	Rapid L. mono	Total	Oxford	ALOA	Rapid L. mono	Total
L. monocytogenes	21	26	23	26	28	21	22	26
Other *Listeria* species	16	14	8	15	28	21	16	22
L. mono + other *Listeria* species	4	8	7	10	4	12	8	12
Total	41	48	38	51	60	54	46	60

Reliability of colonial appearance on chromogenic agars for detection of *Listeria*

ALOA. With two exceptions, all blue colonies exhibiting a halo on ALOA were confirmed as *L. monocytogenes* by API Listeria. The first exception was a strain isolated through both primary and secondary enrichment broths that showed a small halo after 24 h, that was approximately 1 mm wide after 48 h. This strain was identified as *L. seeligeri*/*L. ivanovii* by API Listeria. The second occurred with a sample that yielded a heavy growth of blue colonies with halo through the secondary enrichment. Subculture after 48 h yielded *L. monocytogenes* and Gram-positive cocci. In this case, the Gram-positive cocci may have had a false positive appearance because of the close proximity of large zones produced by neighbouring *L. monocytogenes* colonies.

Strains yielding blue colonies without a halo that subsequently did not confirm as *Listeria* species were found in 20 samples. These organisms included staphylococci, *Carnobacterium* and streptococci.

Rapid L. mono agar. *Listeria* strains had the expected appearance on Rapid L. mono agar with the exception of four samples. In two of these, white colonies were identified as *L. monocytogenes*, and one also yielded blue colonies that confirmed as *L. innocua*. In a further two samples, *L. seeligeri* colonies were described as yellow rather than white, but this may have been affected by the extensive yellow haloes produced by these strains.

Four samples yielded blue colonies without haloes that did not confirm as *Listeria* species. Three of these were *Bacillus* species and one was a presumptive *Staphylococcus*. While many samples yielded white colonies that did not have the same morphology as *Listeria* species, 43 samples produced white colonies on which further confirmatory work was carried out before concluding that they were not *Listeria* species. These were largely staphylococci, with a small number of *Bacillus* species and haemolytic streptococci also being detected. Increasing familiarity with the medium led to a reduction in confirmatory work on non-*Listeria* colonies over the course of the study.

DISCUSSION

Chromogenic media have become increasingly common in food microbiology over the last few years, and the British Standards Institution has recently published a method for the detection of *Escherichia coli* in food which is based on the β-glucuronidase activity of *E. coli* at 44°C cleaving a chromogen to produce blue/green coloured colonies (British Standards Institution 2001a,b). The use of this chromogenic agar under the conditions specified in the standard (incubation at 44°C) has the advantage over previous media that no further confirmation of *E. coli* colonies is required.

The chromogenic media evaluated in this study were able to give a presumptive identification of *L. monocytogenes* before confirmatory tests were performed; the ability to presumptively detect pathogenic *Listeria* species after only 24-h incubation, rather than 3 or 4 days with the current method, is important in reacting promptly to food contamination problems.

ALOA agar, in particular, appeared to perform at least as well as Oxford agar in the enumeration and detection of both *L. monocytogenes* and other *Listeria* species, with no significant difference in recovery or colony counts being observed. Moreover, in contrast to Oxford agar, results read after only 24-h incubation were not significantly different to those after 48 h. Previous evaluations of ALOA agar found that use of the chromogenic medium resulted in a marked improvement in sensitivity for *L. monocytogenes* compared with Oxford and PALCAM agars (Vlaemynck *et al.* 2000; Sacchetti *et al.* 2003). While a small increase in recovery of *L. monocytogenes* was observed in this study when ALOA rather than Oxford agar was used, this difference was not found to be statistically significant.

Using the enumeration method, numbers of *Listeria* colonies on Rapid L. mono agar were slightly reduced compared with Oxford agar. Using the enrichment method, Rapid L. mono showed similar recovery of *L. monocytogenes* strains to ALOA and Oxford agars and, like ALOA, this strain was detected as frequently after 24 h of incubation as after 48 h. However, recovery of other *Listeria* species was significantly reduced on this medium. This relatively poor performance may have been at least partly the result of the difficulty of recognizing the white colonies of *Listeria* species among background growth. The similarity in appearance between *Listeria* species and other organisms such as staphylococci also led to an increased amount of confirmatory work compared with ALOA and Oxford agar, in order to confirm the presence of *Listeria* species. The manufacturers have since modified the formulation, which has led to a reduction in background flora.

Both types of chromogenic medium tested increased the chance of detecting mixed populations of *L. monocytogenes* and other *Listeria* species compared with Oxford agar, because of the distinct appearance of *L. monocytogenes* colonies. On Oxford agar, all strains of *Listeria* have a similar colonial appearance, and identification of more than one strain from a single agar plate is therefore reliant on the chance picking of both strains for confirmatory tests.

The Association Française de normalisation (AFNOR) has developed a protocol in which only a primary enrichment stage is required for the detection of *Listeria* using ALOA. Results from our study confirm that there was no significant increase in recovery of *L. monocytogenes* by using

a secondary enrichment. However, it is clear from our results that a secondary enrichment is necessary if other *Listeria* species are to be detected. ALOA detected a greater number of nonpathogenic *Listeria* species after secondary compared with primary enrichment but one less *L. monocytogenes* strain. This may have been due to the overgrowth of *L. monocytogenes* by other species, as described by Curiale and Lewus (1994). This suggests that subculturing to agar plates after both the primary and secondary enrichments is necessary in order to improve the chances of detecting all *Listeria* strains present.

Studies of pâté samples (Willis and Greenwood 2003) have recovered *L. monocytogenes* at the point of sale although this species was not detected in tests performed by the manufacturer immediately after production. This may have been the result of low level contamination below the sensitivity of the test, with multiplication during extended storage in the food chain to reach detectable levels at the retail point. If other *Listeria* species were also sought immediately after manufacture and action taken if they were found, this might help to prevent the occurrence of *L. monocytogenes* contamination.

In conclusion, the use of chromogenic agars facilitates the detection of *L. monocytogenes* in mixed cultures of *Listeria* species. Presumptive identification of *L. monocytogenes* is possible after 24 h, compared with 3–4 days using Oxford agar. Secondary enrichment significantly improves recovery of species other than *L. monocytogenes*; this is advantageous as they act as markers for the likelihood of presence of *L. monocytogenes* and allow preventive action to be taken to avoid its presence.

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