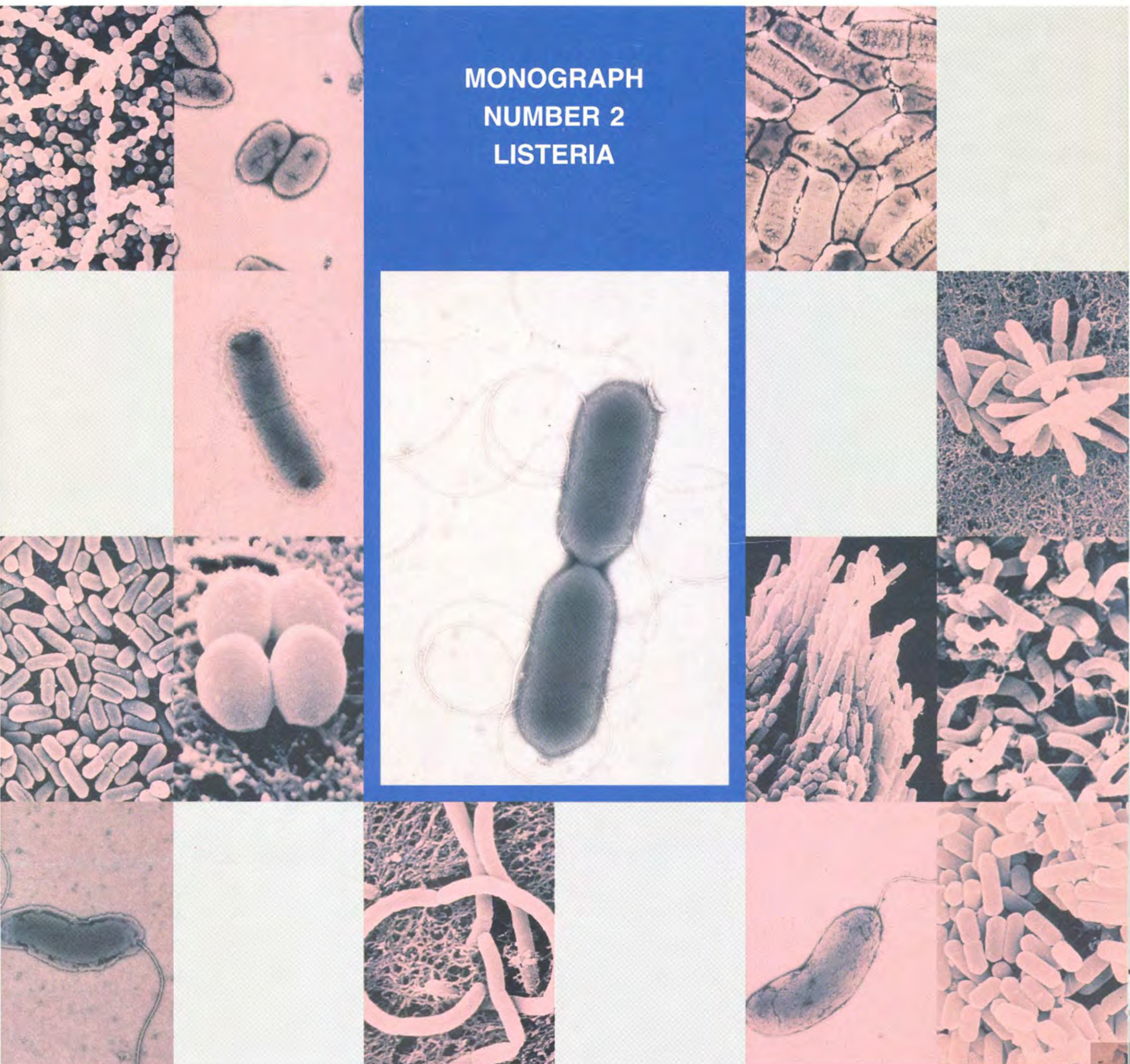
Food-borne Pathogens



OXOID

SETTING STANDARDS

FOOD-BORNE PATHOGENS MONOGRAPH NUMBER 2 LISTERIA

D. E. POST

Technical Support Manager

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Warning

Listeria monocytogenes is contained within Advisory Committee on Dangerous Pathogens (ACDP) Group 2 i.e. "might be a hazard to laboratory workers" and should only be cultured in properly equipped laboratories under the control of a skilled microbiologist.

It is also recommended that pregnant staff should not work with known cultures of listeriae.

Acknowledgements

The electron micrographs were supplied by Dr Peter Hawtin of The Public Health Laboratory Service, Southampton Laboratory, United Kingdom. I am grateful for his expert assistance.

The photographs of *Listeria monocytogenes* growing on plating media were produced by Mr Eric Griffin of the Microbiology Department, Royal Hampshire County Hospital, Winchester, UK. My thanks to him and his colleagues for their hard work and co-operation.

I would also like to thank Mr John Watkins of Yorkshire Environmental Laboratory Services and Dr Jim McLauchlin of Central Public Health Laboratory, Colindale for their permission to reproduce photographs.

Introduction

The Gram-positive bacterium which finally was named *Listeria monocytogenes* was first described in 1926 by Murray and his co-workers at Cambridge. It had been identified in 1924 as the cause of disease amongst laboratory guinea pigs and rabbits kept in the University Department of Pathology.

The authors proposed the name *Bacterium monocytogenes* for the "new" organism because blood of the diseased animals characteristically showed large numbers of mononuclear leucocytes.

In 1925 Pirie isolated a very similar organism from a wild gerbil. He named it *Listerella hepatolytica* in recognition of the involvement of the liver in infection. The similarity of the organisms led to the different workers agreeing the new name of *Listerella monocytogenes*. Subsequently the name *Listerella* was changed to *Listeria* because *Listerella* had already been assigned to a group of protozoa. It is interesting in retrospect that both Murray and Pirie attributed the animal infections to contaminated feed.

Listeria monocytogenes was later shown also to be a cause of disease in cattle and sheep. The feeding of poorly manufactured silage has been implicated.

It was not until 1967 that *L. monocytogenes* was first isolated from a human case, a veterinary surgeon who developed cutaneous listeriosis after examining a cow. This manifestation of the disease is unusual.

Usually infection ranges from a mild influenza-like condition to severe infections of the blood and brain. The consequences of infection are likely to be much more damaging for the very young, the very old, pregnant women and immunodeficient subjects. In maternal cases the mother is generally only mildly effected but the effect on the unborn child is frequently severe. Abortion may occur or the child may be stillborn. Children born alive at full term may suffer from meningitis which can ultimately be fatal.

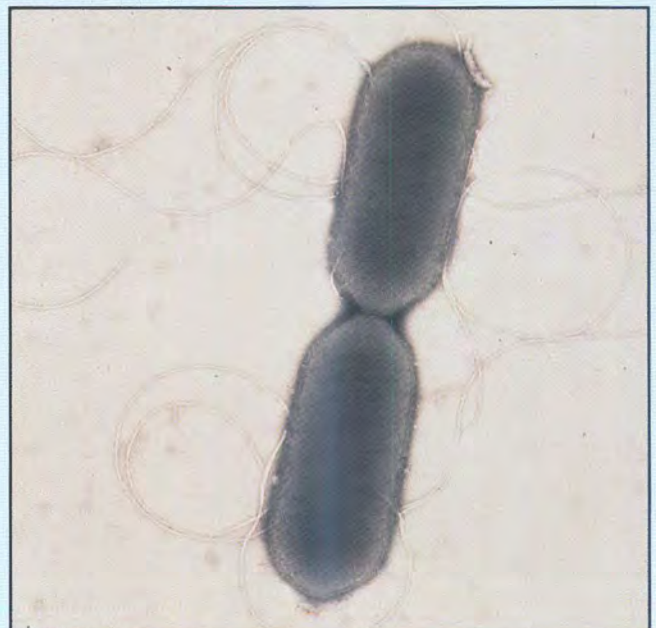
General acceptance that food is the principal vehicle of transmission followed a series of outbreaks in North America and Europe in the 1980s.

L. monocytogenes is widespread in the environment and consequently there is considerable opportunity for foods to become contaminated and for *Listeria* to be disseminated by cross contamination during processing and storage. The organism tolerates widely ranging salt concentrations, pH and temperature. Despite some reports to the contrary, *L. monocytogenes* appears not to be specially resistant to heat although it has been shown to have greater heat resistance than *Salmonella*. It is, however, capable of growing well at ordinary refrigerator temperatures, the low temperatures giving it competitive advantage over the accompanying mesophilic flora.

The recognition that *Listeria* infection can have very serious consequences and the knowledge that refrigeration can actually encourage the growth of *L. monocytogenes* has made it necessary for food manufacturers to make great efforts to exclude the organism from their products. It is not yet known what constitutes an infective dose for humans and for maximum safety it is desirable that *L. monocytogenes* is completely absent.

Much work in recent years has been devoted to formulating improved culture media and great strides have been made in increasing the ability to detect very small numbers of *Listeria*. The intention of this publication is to describe some of the culture media available. An international consensus view is emerging on preferred media and methodology. Where possible, guidance on choice of medium will be offered.

Listeria monocytogenes.



The Occurrence of Listeria in Foods

Listeria species are ubiquitous in the environment and consequently they can be present in most foods.

L. monocytogenes has the ability to survive the manufacturing and ripening of many different cheeses and is particularly liable to be found in cottage cheese and other mildly acidic dairy products. *L. monocytogenes* may also be isolated from raw and pasteurised milk, cream, butter and ice cream.

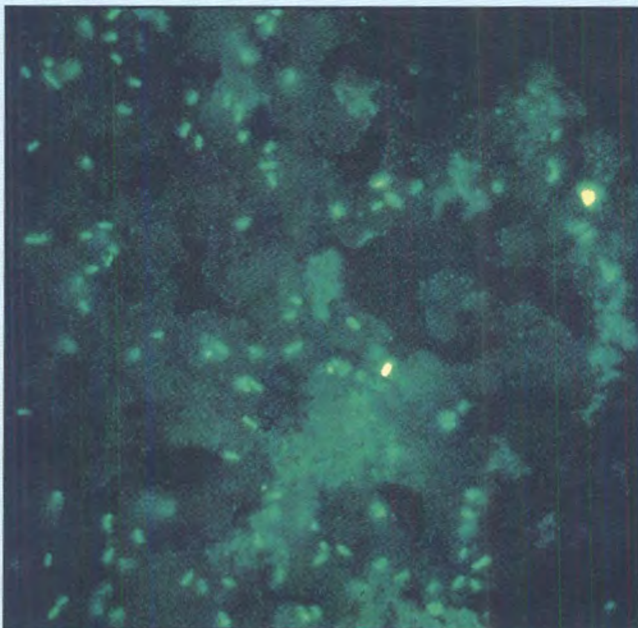
Growth of *L. monocytogenes* on meat and meat products is highly dependent on product type and pH. The organism tends to grow well on meat products that have a pH value near to or above 6.0 whereas it grows poorly or not at all on meats near to or below pH 5.0. Pâté has been shown to be capable of growing *L. monocytogenes* in high numbers. *L. monocytogenes* is relatively resistant to meat curing processes and has been isolated from salami and vacuum-packed ham products.

Chicken also tends to be heavily contaminated with *L. monocytogenes*, different surveys showing contamination rates ranging from 12% to 60%.

L. monocytogenes is able to survive well in refrigerated raw egg and to grow well in cooked eggs.

Many different types of vegetables harbour *Listeria spp*. Potatoes and radishes are reported to be particularly prone to contamination. *L. monocytogenes* can be found in individual salad ingredients and in an even higher proportion of pre-packed mixed salads. Coleslaw has been implicated as a source of *L. monocytogenes* in outbreaks.

Fluorescent antibody – stained cells of L. monocytogenes *in a sample of cheese.*



Photograph reproduced by permission of Dr J McLauchlin, Public Health Laboratory Service, London, England.

Seafoods have also been identified as sources of *L. monocytogenes*. The organism has reportedly been isolated from frozen seafood, smoked salmon and shrimps.

Particular concern has been expressed about the presence of *L. monocytogenes* in cook-chill ready-to-eat meals. Strict controls on manufacture and storage have been introduced to minimise the risk from these foods.

Despite the intensive research efforts of the last few years on *Listeria* and listeriosis, the minimum infective dose is still unknown. Various countries have adopted different policies concerning the presence of *L. monocytogenes* in foods. Tolerance levels are being set and although there is some variation, a complete absence is considered by many to be the most desirable situation.

The culture media to be used in the procedures specified by some National standards and regulatory bodies are given in Tables 1 and 2.

Procedures tend to be broadly similar. Those operated by the United States Food and Drug Administration (FDA) and US Department of Agriculture (USDA) are given in Tables 3 and 4.

Enrichment Media

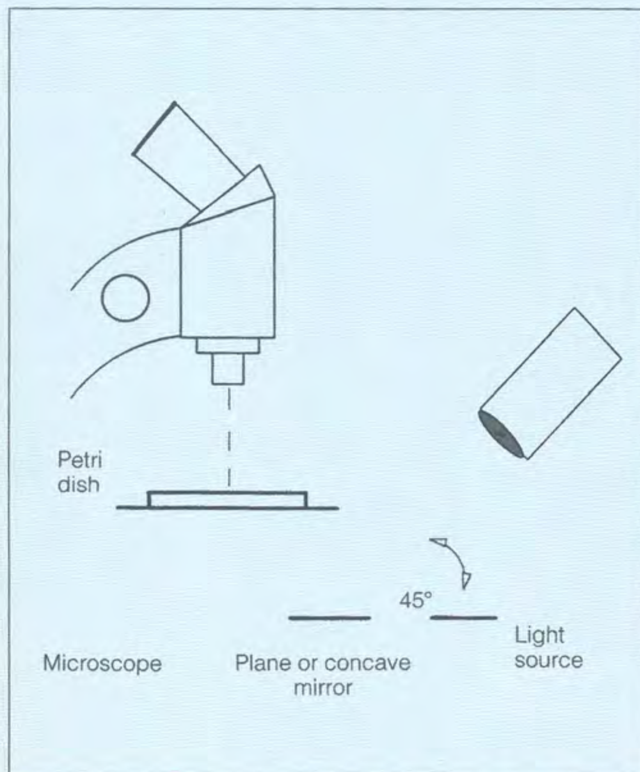
Pre-enrichment in a non-selective medium at incubator temperatures is not commonly employed in *Listeria* isolation procedures because of the interference caused by overgrowth of accompanying organisms which tend to multiply relatively more rapidly.

Cold pre-enrichment culture in which samples are added to a non-selective nutritious medium and refrigerated was the standard procedure until recent years. Cold enrichment takes advantage of the psychrophilic property of *Listeria spp*. *Listeria* will multiply at the low temperature which inhibits multiplication of the accompanying mesophilic flora. However, multiplication of *Listeria* is very slow and several weeks storage at low temperature may be needed before *Listeria* can be detected on sub-culture on plating media.

Detection of *Listeria* colonies is greatly assisted by viewing with a plate microscope with the plate illuminated at an oblique angle as described by Henry[1]. Colonies of *Listeria spp*. are blue or blue/grey making them easily distinguishable to enable suitable colonies to be picked for further testing.

Colonies of most other bacteria do not show this characteristic colour. The probable explanation of the phenomenon is that the cells in a *Listeria* colony tend to lie in the same plane, thus forming a crude diffraction grating which changes the wave length of light transmitted through the colony.

Watkins and Sleath[2], shortened the enrichment process by combining an initial period of cold enrichment in nutrient broth at 4°C with sub-culture to thiocyanate-nalidixic acid broth at 37°C. Subsequent plating was on non-selective tryptose agar. *Listeria* colonies were detected using Henry illumination.



Arrangement of microscope and lamp for Henry's oblique illumination.

The use of enrichment broths containing selective agents has generally replaced cold enrichment. Some enrichment media and the selective agents contained in them are listed in Table 5. Improvements in selective media continue to evolve rapidly. Formulae tend to share the same general composition, but much variation is apparent in the quantities of the selective agents. The effectiveness of the different formulae is likely to be influenced by the type of food, the population of *Listeria spp*. and numbers and types of accompanying organisms. Much of the most recent medium development has been directed towards optimising performance of existing media by varying the concentrations of the selective agents they contain.

Modifications have also been introduced to minimise the inhibitory activity of selective agents on damaged *Listeria* cells by introducing procedures in which samples are first incubated for a period in enrichment broth without antibiotics to enable resuscitation to occur. The selective agents are then added after a suitable period and incubation continued. Modifications of this nature have been made to the protocols specified by the American Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA). Parameters for resuscitation of *L. monocytogenes* have not been fully determined and alternative resuscitation methodology has employed media such as nutrient broth and tryptone soya broth at temperatures of 25°C and 4°C, sometimes with the addition of sodium pyruvate. Tryptone soya broth with added 0.6% yeast extract was reported to repair heat-injured and freeze-injured *Listeria* cells in 6-8 hours[3]. A universal pre-enrichment broth designed to resuscitate both *Listeria* and *Salmonella* species has been developed and patented in America.

The hydrolysis of aesculin incorporated in culture media is now commonly employed as a presumptive test for *Listeria spp*. Fraser and Sperber[4] followed this principle when they modified UVM Broth by altering the selective agents and added aesculin to create Fraser Broth. Fraser Broth itself has now undergone modifications: see Table 5. Care must be taken in interpreting a brownish-black reaction because other organisms such as enterococci can also hydrolyse aesculin and give false positive reactions. Sufficient time must be allowed for colour development in order to minimise false negatives. The early claim for Fraser Broth that it can be used to screen out samples as free of *Listeria spp*. if the medium has failed to blacken after 26 hours is now seen to be unreliable. All Fraser Broth cultures should be sub-cultured to plating media. Continued incubation of Fraser Broth to 48 hours has been shown to increase the number of positive results.

Schiemann, Shope and Brown[5] in noting the inconclusive evidence about pathogenicity of the haemolytic species *L. ivanovii* and *L. seeligeri*, have considered it prudent to devise isolation methods that are capable of recovering all three haemolytic species of *Listeria*[5]. An enrichment broth, designated PPY Broth, containing mixed peptones, yeast extract and sodium pyruvate, when used with selective plating media derived from this basic formula, gave recoveries of *L. monocytogenes* equivalent to those obtained with the single step FDA procedure.

Semi-solid modifications of Oxford and PALCAM Agars have been designed to take advantage of the motility of *Listeria spp*. to separate them from non-motile accompanying organisms[6].

Yu and Fung also used the principle of motility enrichment and combined it with the growth-enhancing effect of Oxyrase™ enzyme in the Fung-Yu tube procedure to shorten the time required for detection by colour change in the culture medium[7]. The Fung-Yu procedure gave results as sensitive as the USDA-FSIS procedure in a significantly shorter time.

Van Netten and his co-workers in a development of PALCAM Medium have formulated L-PALCAMY Enrichment Broth[8]. This is based on peptone yeast extract broth with the addition of egg yolk emulsion. The diagnostic agents and inhibitors used in this medium are the same as those in PALCAM Agar but are present in different concentrations. L-PALCAMY Enrichment Broth was reported to possess productivity for *Listeria spp.* similar to that of Columbia Broth which is non-inhibitory.

Enrichment broths currently used in the major isolation procedures (see Table 1) produced by Unipath are described in the following pages.

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Typical appearance of L. monocytogenes *colonies (arrowed) viewed using Henry illumination.*

Photograph reproduced by permission of Mr J Watkins, Yorkshire Environmental Laboratory Services.

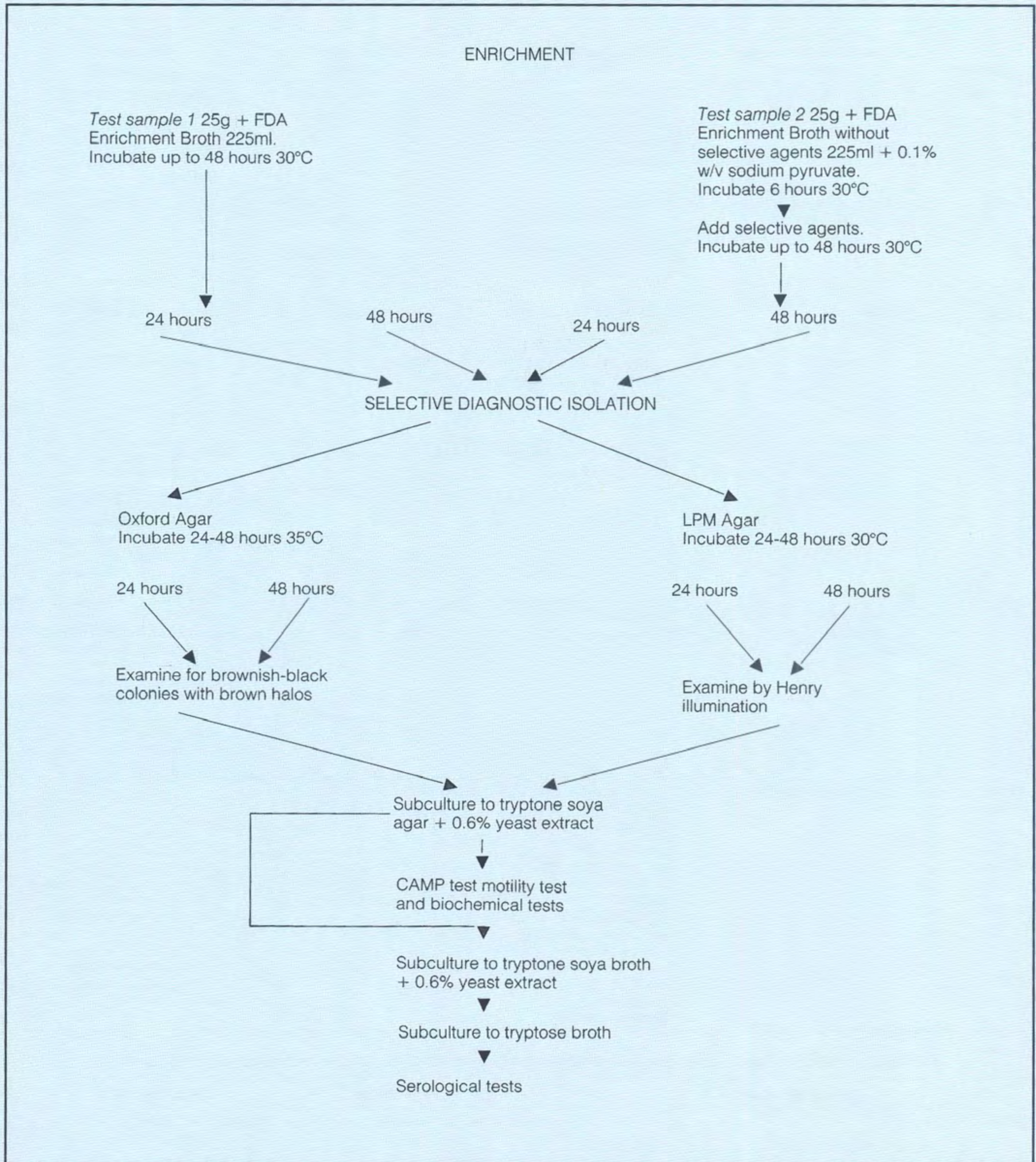
TABLE 1 – Culture media specified by some National standards bodies for detection of *Listeria monocytogenes*. The codes for the Oxoid dehydrated culture media available from Unipath are in parentheses.

Country	Organisation responsible for the procedure	Culture media: enrichment	Culture media: plating	Other media specified in procedure
Australia	Standards Australia Committee on Food Microbiology	Listeria Enrichment Broth (*CM862 + SR141). (*The formula has been modified: CM862 is the closest equivalent)	Oxford Agar (CM856 + SR140)	Blood Agar Base No. 2 (CM271) Tryptone Soya Agar (CM131) Tryptone Soya Broth (CM129)
Canada	Health and Welfare Canada MFHPB-30 (Sept 93)	(1) Fraser Broth CM895 (2) UVM 1 Broth (CM863 + SR142) (Add 1g aesculin to 1 litre before sterilisation)	(1) Oxford Agar (CM856 + SR140), MOX Agar (CM856 + SR157) (2) PALCAM Agar (CM877 + SR150) (3) LPM Agar	Tryptone Soya Agar (CM131). MRVP Medium (CM143). Triple Sugar Iron Agar (CM277). Tryptone Soya Broth (CM129)
France	AFNOR DGAL/SDHA/N93/8105 June 1993	(1) AFNOR half strength Fraser Broth (CM895 + SR156) *1 vial per litre of medium*. (Add 0.25g of ferric ammonium citrate to 1 litre of medium.) (2) Fraser Broth (CM895 + SR156)	(1) Oxford Agar (CM856 + SR140) (2) PALCAM Agar (CM877 + SR150)	Blood Agar Base No. 2 (CM271)
Germany	BGA Official Collection of Methods 35 LMBG Part L 00 00-22 December 1991	Listeria Enrichment Broth (CM862 + SR149)	(1) Oxford Agar (CM856 + SR140) (2) PALCAM Agar (CM877 + SR150)	Columbia Agar (CM331). Tryptone Soya Yeast Extract Broth; use Listeria Enrichment Broth Base (CM862). Tryptone Soya Yeast Extract Agar; use CM862 + 10-15 grams Bacteriological Agar (L11) per litre
Italy	Institute Superiore di Sanità with the Italian Health Ministry	Fraser Broth (CM895 + SR156)	Oxford Agar (CM856 + SR140)	Buffered Peptone Water (CM509). Tryptone Soya Yeast Extract Broth: use Listeria Enrichment Broth Base (CM862). Tryptone Soya Yeast Extract Agar; use Listeria Enrichment Broth Base (CM862) + 10-15 grams Bacteriological Agar (L11) per litre
New Zealand	New Zealand Meat Board	(1) UVM 1 Broth (CM863 + SR142) (2) Fraser Broth (CM895 + SR156)	Modified Oxford Agar (MOX) (CM856 + SR157)	
Spain	Instituto de Salud Carlos III Centro Nacional de Alimentación	Pre-enrichment: Listeria Enrichment Broth Base (CM862) DO NOT ADD SUPPLEMENT SR141. Enrichment: Listeria Enrichment Broth (CM862 + SR141). Listeria UVM 1 Broth (CM863 + SR142). Listeria UVM 2 Broth (CM863 + SR143)	(1) Oxford Agar (CM856 + SR140) (2) Modified McBride Agar	Brain Heart Infusion (CM225). Blood Agar Base (CM55). Columbia Agar (CM331). Tryptone Soya Agar (CM131). Urea Agar (CM53). SIM Medium (CM435). MRVP Medium (CM43). Triple Sugar Iron Agar (CM277)

TABLE 2 – Culture media recommended by some regulatory bodies for detection of *Listeria monocytogenes*.

Regulatory body	Enrichment media	Plating media
FDA	Listeria Enrichment Broth (LEB)	Modified McBride Agar (MMA) Oxford Agar. LPM Agar
USDA	UVM 1 Broth Fraser Broth	Modified Oxford Agar (MOX)
BSI/ISO/IDF/AOAC for milk and milk products	Listeria Enrichment Broth (LEB)	Oxford Agar
BSI/ISO Provisional proposal for foods	Half strength Fraser Broth Fraser Broth	Oxford Agar
AFNOR to be confirmed. Derived from Ministry of Agriculture National Standard Method	Half strength Fraser Broth Fraser Broth	Oxford Agar PALCAM Agar

TABLE 3 – FDA BAM *Listeria* isolation procedure.*



*No well-characterised pre-enrichment culture procedure for repairing stress damage has been developed.

It is recommended that test sample 2 and the procedure described be included as an additional enrichment broth culture when examining samples suspected of containing heat- or freeze-damaged *Listeria* cells.

TABLE 4 – USDA procedure for isolation of *Listeria monocytogenes*.

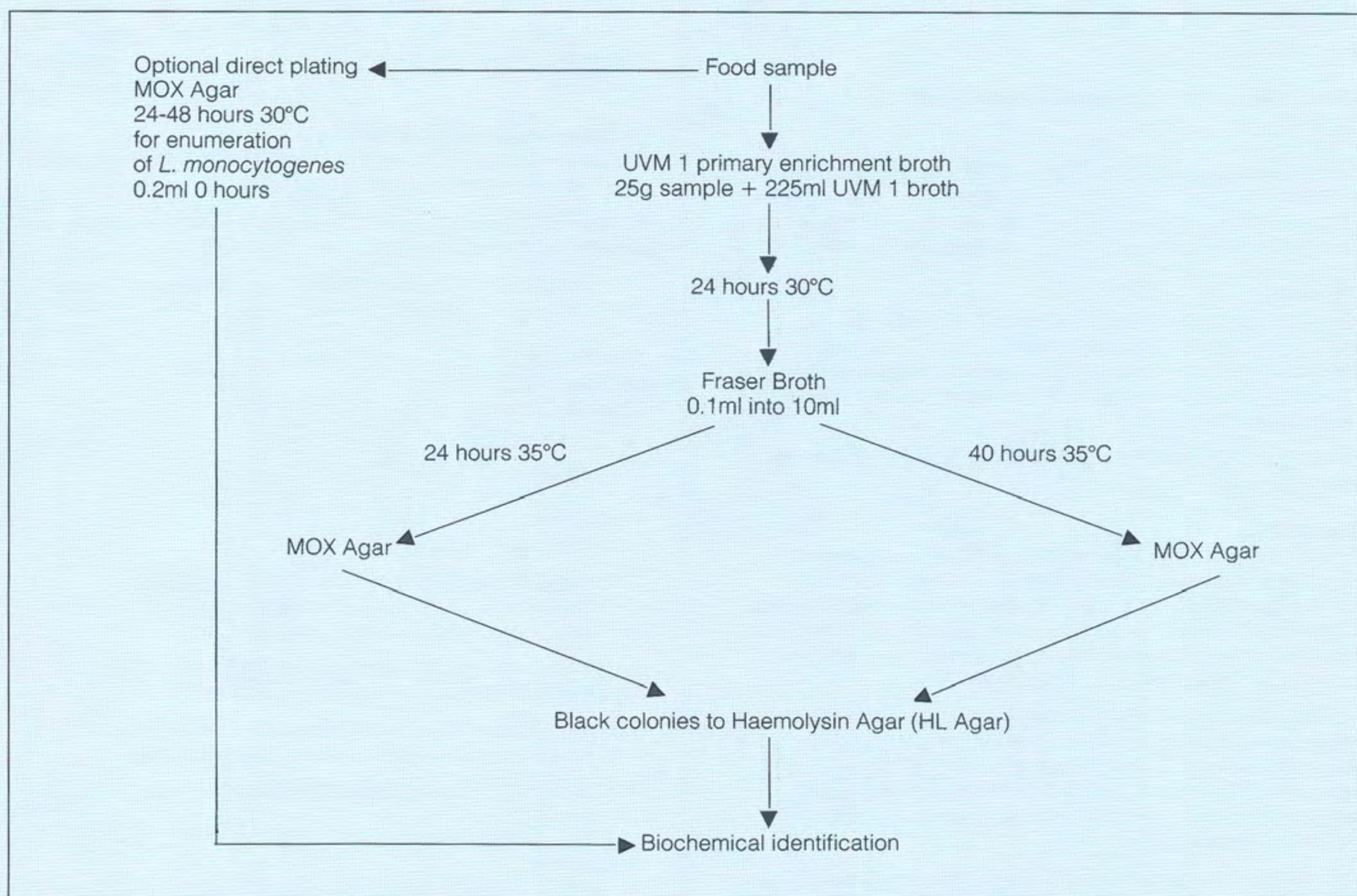


TABLE 5 – *Listeria* Selective Enrichment Broths. Concentration of selective agents in mg/litre unless stated grams/litre.

Selective agent	FDA Listeria Enrichment Broth	Buffered FDA Enrichment Broth	Modified FDA Listeria Enrichment Broth	UVM 1	UVM 2	Fraser	Half strength Fraser	Organon-teknika Fraser
Acriflavine HCl	15	15	10	12	25	25	12.5	12.5
Nalidixic acid	40	40	40	20	20	20	10	20
Cycloheximide	50	50	50	–	–	–	–	–
Lithium chloride	–	–	–	–	–	3 grams	3 grams	3 grams

FDA Listeria Enrichment Broth (LEB)

Listeria Enrichment Broth Base

Code: CM862

Formula (grams per litre)

Tryptone soya broth	*30*
Yeast extract	*6.0*
pH 7.3 ± 0.2	

Listeria Selective Enrichment Supplement
Code: SR141

Vial contents (each vial is sufficient for 500ml of medium)

Nalidixic acid	*20.0mg (equivalent to 40mg/l)*
Cycloheximide	*25.0mg (equivalent to 50mg/l)*
Acriflavine hydrochloride	*7.5mg (equivalent to 15mg/l)*

Directions

Suspend 18g in 500ml of distilled water. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of Listeria Selective Enrichment Supplement code SR141 reconstituted with 2ml of sterile distilled water.

Mix well and distribute into sterile containers in volumes as required.

Description

Listeria Selective Enrichment Broth (LEB) is based on the formulation described by Lovett et al.[1] It is the enrichment medium specified in the AOAC/FDA Bacteriological Analytical Manual for the selective enrichment of *Listeria spp.* from food. The enrichment procedure has been shown to recover an inoculum of less than 10 cfu/ml from raw milk.

In order to achieve a higher isolation rate it is recommended that the enrichment broth is subcultured onto *Listeria* selective agar plates after 1, 2 and 7 days. Agello et al.[2] showed that extending the incubation period to 7 days allowed better recovery of environmentally stressed *Listeria spp.* from milk and milk products.

Continuing improvements in the FDA procedure, including a period for resuscitation in medium not containing selective agents and with added sodium pyruvate, have enabled the incubation period to be shortened to 2 days. Details of the FDA BAM procedure are given in Table 3.

The treatment of enrichment culture with potassium hydroxide to improve selectivity is no longer considered necessary because of the improvements in selective plating media.

Improved performance of Listeria Enrichment Broth by reduction of the acriflavine content to 10mg/litre has been reported[3]. This modification to Oxoid Listeria Enrichment Broth can be made by using Listeria Enrichment Supplement code SR149 instead of Listeria Enrichment Supplement code SR141 for addition to the broth base CM862.

Note

In addition to LPM Agar suitable *Listeria* selective agars are:

Oxford Agar	(CM856 and SR140)
Modified Oxford Agar (MOX)	(CM856 and SR157)
PALCAM Agar	(CM877 and SR150)

Storage conditions and Shelf life

Store the dehydrated medium below 25°C in a dark dry place and use before the expiry date on the label.

Store the supplement at 2-8°C away from light and use before the expiry date on the label.

Store the prepared medium at 2-8°C, tightly capped, in the dark and use as soon as possible.

Quality Control

Positive control:
Listeria monocytogenes ATCC® 19117

Negative control:
Staphylococcus aureus ATCC® 25923

Precautions

For in vitro diagnostic use

Listeria Selective Enrichment Supplement contains cycloheximide and is toxic if swallowed, inhaled or by skin contact. When handling, wear gloves and eye/face protection.

Do not use beyond the expiry date or if the product is caked, discoloured or shows any signs of deterioration.

Store prepared medium away from light. Acriflavine can photo-oxidise to form compounds inhibitory to *Listeria spp.*

Broth cultures are more dangerous than colonies on agar plates.

References

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3 Hammer, P., Hahn, G. and Heeschen, W. (1988) *Deutsche Molkerei-Zeitung D.M.Z.* **50,** 1700-1706.

Buffered Listeria Enrichment Broth (BLEB)

A selective enrichment medium for the detection of *Listeria monocytogenes* when prepared from Buffered Listeria Enrichment Broth Base code CM897 and Listeria Selective Supplement code SR141.

Buffered Listeria Enrichment Broth
Code: CM897

Formula (grams per litre)

Buffered Listeria Enrichment Broth Base code CM897	
Tryptone soya broth	*30.0*
Yeast extract	*6.0*
Potassium di-hydrogen orthophosphate	*1.35*
Disodium hydrogen orthophosphate	*9.60*

Final pH 7.3 ± 0.2

Listeria Selective Enrichment Supplement
Code: SR141

Vial contents

Nalidixic acid	*20.0mg (equivalent to 40mg/l)*
Cycloheximide	*25.0mg (equivalent to 50mg/l)*
Acriflavine hydrochloride	*7.5mg (equivalent to 15mg/l)*

Each vial is sufficient to supplement 500mls of Buffered Listeria Enrichment Broth code CM897.

Directions

Add 23.5g to 500ml of distilled water and mix well to dissolve. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of 1 vial of Listeria Selective Enrichment Supplement code SR141 reconstituted with 2ml of sterile distilled water. Mix well and aseptically distribute into sterile containers in volumes as required.

Description

Buffered Listeria Enrichment Broth is a selective enrichment medium prepared from Buffered Listeria Enrichment Broth Base code CM897 and Listeria Selective Supplement code SR141 for the detection of *L. monocytogenes*. The medium is intended for use with samples of fermented products, and as a method for the enrichment of environmental samples.

Buffered Listeria Enrichment Broth is a development of the Listeria Enrichment Broth described by Lovett et al.[1] Subsequent work concluded that the enrichment properties of Listeria Enrichment Broth can be improved by increasing the buffering capacity of the medium by the addition of potassium di-hydrogen orthophosphate and disodium hydrogen orthophosphate. Buffered Listeria Enrichment Broth code CM897 is therefore a modification of the original medium which is marketed as Oxoid Listeria Selective Enrichment Medium code CM862.

Tryptone soya broth and yeast extract are rich sources of the biochemical components and trace nutrients required for bacterial metabolism. The addition of the buffer enhances the enrichment of *Listeria spp.* when used with fermented products.

Selectivity is achieved by the addition of nalidixic acid, cycloheximide and acriflavine hydrochloride.

Improved performance of Listeria Enrichment Broth by reduction of the acriflavine content to 10mg/litre has been reported[3]. This modification to Oxoid Buffered Listeria Enrichment Broth can be made by using Listeria Selective Enrichment Supplement code SR149 instead of Listeria Selective Enrichment Supplement code SR141 for addition to the broth base code CM897.

Technique

1 Add 25g or 25ml samples to 225ml of Buffered Listeria Enrichment Broth. Homogenise if required. Alternatively, incubate for a period in enrichment broth not containing selective agents for resuscitation of damaged cells to occur. Then add the selective supplement.
2 Incubate at 30°C for 48 hours.
3 Subculture from the Buffered Listeria Enrichment Broth onto Listeria Selective Agar plates (see Note) after 24 and 48 hours.

Note

Suitable *Listeria* selective agar media are:

1 Listeria Selective Medium (Oxford formulation) (Oxoid CM856 and Oxoid SR140).
2 Listeria Selective Medium (MOX) (Oxoid CM856 and Oxoid SR157).
3 PALCAM Medium (Oxoid CM877 and Oxoid SR150).

Quality Control

Positive Control:
Listeria monocytogenes ATCC® 19117

Negative Control:
Staphylococcus aureus ATCC® 25923

Precautions

For in vitro diagnostic use

Listeria Selective Enrichment Supplement contains cycloheximide and is toxic if swallowed, inhaled or by skin contact. As a precaution when handling wear gloves and eye/face protection.

Do not use beyond the expiry date or if the product is caked, discoloured or shows any signs of deterioration.

Store prepared medium away from light. Acriflavine can photo-oxidise to form compounds inhibitory to *Listeria spp.*

Broth cultures are more dangerous than colonies on agar plates.

References

1 Lovett, J., Francis, D.W. and Hunt, J.M. (1987) *J. Food Prot.* **50,** 188-192.
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UVM Primary and Secondary Listeria Enrichment Broths

Listeria Enrichment Broth Base (UVM formulation)

Code: CM863

Formula (grams per litre)

Proteose peptone	*5.0*
Tryptone	*5.0*
"Lab-Lemco" powder	*5.0*
Yeast extract	*5.0*
Sodium chloride	*20.0*
Disodium hydrogen phosphate	*12.0*
Potassium di-hydrogen phosphate	*1.35*
Aesculin	*1.0*
pH 7.4 ± 0.2	

Listeria Primary Selective Enrichment Supplement (UVM 1)
Code: SR142

Vial contents (each vial is sufficient for 500ml of medium)

Nalidixic acid	*10.0mg (equivalent to 20mg/l)*
Acriflavine hydrochloride	*6.0mg (equivalent to 12mg/l)*

Listeria Secondary Selective Enrichment Supplement (UVM 2) Code: SR143

Vial contents (each vial is sufficient for 500ml of medium)

Nalidixic acid	*10.0mg (equivalent to 20mg/l)*
Acriflavine hydrochloride	*12.5mg (equivalent to 25mg/l)*

Directions

Suspend 27.2g in 500ml of distilled water. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C.

To prepare Listeria Primary Selective Enrichment Medium (UVM 1)

Aseptically add 2ml of sterile distilled water to a vial of Listeria Primary Selective Enrichment Supplement (UVM 1) Code SR142. Invert gently to dissolve. Aseptically add the vial contents to 500ml of sterile Listeria Enrichment Broth Base (UVM formulation) Code CM863, cooled to 50°C. Mix well and distribute into sterile containers.

To prepare Listeria Secondary Selective Enrichment Medium (UVM 2)

Aseptically add 2ml of sterile distilled water to a vial of Listeria Secondary Selective Enrichment Supplement (UVM 2) Code SR143. Invert gently to dissolve. Aseptically add the vial contents to 500ml of sterile Listeria Enrichment Broth Base (UVM formulation) Code CM863, cooled to 50°C. Mix well and distribute into sterile containers.

Description

The Listeria Selective Enrichment Media (UVM formulations) are based on the original formulation described by Donnelly and Baigent[1], and its subsequent modification[2] which reduced the nalidixic acid concentration in both the primary and secondary selective enrichment media and increased the concentration of acriflavine hydrochloride in the secondary selective enrichment medium.

This modification, and the two step selective enrichment method developed (USDA-FSIS method), results in a higher detection rate of *L. monocytogenes* from meat products and has the added advantage of taking only 3-4 days.

Technique

Primary Enrichment

1 Add 25g or 25ml samples of Listeria Primary Selective Enrichment Medium (UVM 1). Homogenise in a Stomacher for 2 minutes.
2 Incubate the prepared sample in the Stomacher bag at 30°C.
3 From this bag, carry out the following procedures:

After 4 hours incubation, spread 0.2ml on Listeria Selective Agar plates (see Note).

After 24 hours incubation (i) transfer 0.1ml to 10ml of Listeria Secondary Enrichment Medium (UVM 2), and (ii) transfer 1ml to 4.5ml KOH solution. Vortex mix and within one minute subculture onto Listeria Selective Agar plates. For details of KOH preparation see below.

Secondary Enrichment

4 Incubate the inoculated Listeria Secondary Selective Enrichment Medium (UVM 2) at 30°C. See 3 (i).
5 After 24 hours incubation, (i) spread 0.2ml onto Listeria Selective Agar plates; (ii) transfer 1ml to 4.5ml potassium hydroxide (KOH) solution. Vortex mix and within one minute subculture this mixture onto Listeria Selective Agar plates.

Preparation of KOH solution

Dissolve 2.5g of KOH and 20g of NaCl in 1000ml of distilled water. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 18-25°C and ensure that the pH is above 12.0 before use.

Note

The Listeria selective agar recommended for use in the USDA method[2] is LPM plating medium[3]. However, Oxoid laboratory studies[4] have shown that comparable results can be achieved with Listeria Selective Medium (Oxford formulation) CM856 and SR140.

Following revision of the USDA procedure Fraser Broth is now specified as the secondary enrichment broth and modified Oxford Agar (MOX) as the plating medium. Treatment of the secondary enrichment broth with KOH is no longer necessary. Full details may be found in Table 2.

Storage conditions and Shelf life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2-8°C.

Quality Control

Positive control:
Listeria monocytogenes ATCC® 19117

Negative control:
Staphylococcus aureus ATCC® 25923

Precautions

For in vitro diagnostic use

Do not use beyond the expiry date or if the product is caked, discoloured or shows any signs of deterioration.

Store prepared medium away from light. Acriflavine can photo-oxidise to form compounds inhibitory to *Listeria spp.*

Broth cultures are more dangerous than colonies on agar plates.

References

1 Donnelly, C.W. and Baigent, G.J. (1986) *Appl. Environ. Microbiol.* **52,** 689-695.
2 McClain, D. and Lee, W.H. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 660-664.
3 Lee, W.K. and McClain, D. (1986) *App. Environ. Microbiol.* **52,** 1215-1217.
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Fraser Broth

A secondary selective and diagnostic enrichment medium for the isolation of *Listeria spp*. from food and environmental specimens.

Fraser Broth Base
Code: CM895

Formula (grams per litre)

Proteose peptone	*5.0*
Tryptone	*5.0*
"Lab-Lemco" powder	*5.0*
Yeast extract	*5.0*
Sodium chloride	*20.0*
Disodium hydrogen phosphate	*12.0*
Potassium di-hydrogen phosphate	*1.35*
Aesculin	*1.0*
Lithium chloride	*3.0*
pH 7.2 ± 0.2	

Fraser Supplement
Code: SR156

Vial contents (each vial is sufficient for 500ml of medium)

Ferric ammonium citrate	*0.25g*	*(equivalent to 0.5mg/l)*
Nalidixic acid	*10.0mg*	*(equivalent to 20mg/l)*
Acriflavine hydrochloride	*12.5mg*	*(equivalent to 25mg/l)*

Directions
Suspend 28.7g in 500ml of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of Fraser Selective Supplement code SR156 reconstituted with 5ml of ethanol/sterile water (1:1). Mix well and distribute into sterile containers.

Description
Fraser Medium is a modification of the USDA-FSIS (United States Department of Agriculture-Food Safety Inspection Service) UVM Secondary Enrichment Broth and is based on the formula described by Fraser and Sperber[1]. It contains ferric ammonium citrate and lithium chloride. Blackening of the medium is presumptive evidence of the presence of *Listeria spp*.

The early assumption that cultures which do not blacken after 48 hours incubation are *Listeria*-free is now recognised as unreliable. All cultures in Fraser Enrichment Broth should be subcultured to selective agar media.

Note
Suitable *Listeria* selective agar media are:

1 Listeria Selective Medium (Oxford) CM856 and SR140.
2 Listeria Selective Medium (Modified Oxford [MOX]) CM856 and SR157.
3 PALCAM Medium CM877 and SR150.

Fraser Medium is intended for the isolation of *Listeria spp*. from food and environmental samples when used as the secondary enrichment medium in the USDA-FSIS methodology for *Listeria* isolation.

All *Listeria spp*. hydrolyse aesculin to aesculetin. Aesculetin reacts with ferric ions which results in blackening. Another possible advantage to the addition of ferric ammonium citrate is that it has been shown that ferric ions enhance the growth of *L. monocytogenes*[3].

Variants of Fraser Broth showing improved performance have been developed. "Half-strength Fraser Broth" contains half the concentrations of acriflavine and nalidixic acid. Organon-Teknika have devised a variant which halves the concentration only of acriflavine for enrichment culture prior to use of ELISA detection methods. Lithium chloride is included in the media to inhibit growth of enterococci which can also hydrolyse aesculin. Full details of the selective agents contained in the various Fraser Broths are given in Table 5.

It is generally accepted that the USDA-FSIS two stage enrichment method employing UVM Primary and Secondary Enrichment Broths is the most suitable for the examination of meat products. Fraser Broth has proven to be remarkably accurate in detecting *Listeria spp*. in food and environmental samples[1,2].

Technique

1 Inoculate 10ml of Fraser Broth with 0.1ml of the primary enrichment broth (i.e. FDA or UVM 1 Enrichment Broth) which has been incubated for 20 to 24 hours.
2 Incubate at 35°C for 26 ± 2 hours in air.
3 Compare each inoculated tube to an inoculated control against a white background. Tubes that darken or turn black should be subcultured onto Oxford Medium, Modified Oxford Medium (MOX) or PALCAM Medium. Tubes that retain the original yellow colour should be inoculated on plating media and confirmed as free from *Listeria spp*. before discarding.

The incubation period must be controlled. Fraser Medium should be incubated for 26 ± 2 hours to ensure at least a 24 hour incubation period to permit the development of the black colour.

Note
When testing food samples suspected of being involved in disease, subculture the tubes regardless of colour development and continue incubation of the tubes for a further 24 hours.

Storage conditions and Shelf life
Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the selective supplement in the dark at 2°C to 8°C and use before the expiry date on the label.

The prepared medium may be stored for up to 2 weeks at 2°C to 8°C.

Quality Control
Positive control:
Listeria monocytogenes ATCC® 19117

Negative control:
Enterococcus faecalis ATCC® 29212

Precautions
For in vitro diagnostic use

Do not use beyond the expiry date or if the product is caked, discoloured or shows any signs of deterioration.

Store prepared medium away from light. Acriflavine can photo-oxidise to form compounds inhibitory to *Listeria spp*.

Broth cultures are more dangerous than colonies on agar plates.

References

1 Fraser, J.A. and Sperber, W.H. (1988) *J. Food Protect.* **51** (10), 762-765.
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Fraser Broth. Blackening resulting from hydrolysis of aesculin by Listeria spp. *is seen in the culture on the right.*

Plating Media

The recognition that foods can be a vehicle for infection by *Listeria monocytogenes* has brought with it the necessity for isolation procedures more appropriate to food microbiology. In the past the time-consuming approach of cold enrichment followed by culture on non-selective or weakly-selective plating media has served clinical microbiology purposes by providing retrospective confirmation of the infecting organism. However, the procedure is neither rapid nor sufficiently sensitive when used for detection of the very small numbers of *Listeria* cells generally present in foods amongst considerable numbers of accompanying organisms.

Plating media have consequently undergone considerable refinement in recent years to increase their productivity and selectivity. As usual this has occurred by a process of evolution, many formulae being improved later by other workers.

Almost all of the methods developed for isolation of *L. monocytogenes* use one or both distinct characteristics of the bacterium. The first characteristic, the ability to grow at refrigerator temperatures, has been exploited in cold enrichment broth culture, but is not applied to plating media. The second, resistance to many antibiotics, is exploited in recent plating medium formulations. Antibiotics, including nalidixic acid, moxalactam and cefotetan are used in conjunction with other selective agents. The most frequently used are phenylethanol, lithium chloride, glycine anhydride, potassium tellurite and acriflavine. The selective characteristics of phenylethanol agar and Baird-Parker Agar have been used as a basis for some formulations.

Early work on improving the performance of plating media was carried out by Gray, McBride and Girard and Ralovich[1,2,3]. Gray utilised potassium tellurite tryptone agar. Micrococci and streptococci also grew at concentrations that did not inhibit *Listeria spp.* but *Listeria spp.* could readily be distinguished by the characteristic green colour at the periphery of the colonies when viewed by a dissecting microscope. McBride Medium contains 2-phenylethanol to inhibit Gram-negative organisms and prevent the swarming of *Proteus spp.* Blood was added to detect haemolytic strains of *Listeria*. Glycine and lithium chloride were also included to enhance selectivity. McBride Agar was later improved by other workers who added cycloheximide to inhibit fungi, but the medium has now largely been superseded because of its relatively poor selectivity.

Blanco et al. further developed methodology for detecting haemolytic *Listeria* strains by adding an overlay of blood agar to growth on selective medium[4].

Ralovich and his co-workers recognised the value of trypaflavine (acriflavine) for inhibiting Gram-positive cocci and combined it with nalidixic acid. The consequent reduction in growth from fluid enrichment cultures considerably assisted detection of *Listeria* colonies by Henry illumination.

The recognition that *Listeria*-contaminated food can be the cause of listeriosis stimulated intense activity in development of selective media during the decade 1980-1990. A number of these continued to rely on microscopy using Henry illumination for detection of *Listeria* colonies. Substitution of gum for agar as a gelling agent in GBNA Medium is reported to improve the visualisation of *Listeria* colonies.

The introduction of formulae containing aesculin and ferric citrate was a further significant advance. It is now possible to detect colonies of *Listeria spp.* by the colour of hydrolysed aesculin. A further refinement, designed to differentiate enterococci, which also hydrolyse aesculin, from *Listeria spp.* was the incorporation of mannitol in PALCAM Agar so that the enterococci could be differentiated by mannitol fermentation[5].

Some of the more commonly used plating media and the systems they use for presumptive identification are shown in Table 6.

Oxford[6] and PALCAM Agars[4] have emerged as highly effective plating media which are widely specified in official methodology. Detailed descriptions of both media are given in the following pages.

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TABLE 6 – The selective agents and diagnostic systems of some *Listeria* plating media. Concentration in mg/litre unless otherwise stated as grams/litre.

Medium	Acriflavine hydro-chloride	Glycine anhydride	Lithium chloride	Phenyl-ethanol	Potassium tellurite	Bacitracin	Cefotetan	Ceftazi-dime	Colistin	Cyclo-heximide	Fosfo-mycin	Moxal-actam	Nalidixic acid	Polymixin B	Diagnostic system
		(grams)	(grams)	(grams)	(grams)										
AC Agar[1]	10							50							Henry microscopy
Despierres' Agar[2]													40	3	Henry microscopy Rhamnose fermentation
Dominguez-Rodriguez Agar[3]	12												40		Aesculin hydrolysis
GBNA Agar[4]													50		Henry microscopy
LPM Agar[5]		10	5									20			Henry microscopy
McBride Agar[6]		10	0.5	2.5											Haemolysis
Modified McBride Agar[7]		10	0.5	2.5						200					Henry microscopy
ARS Modified McBride Agar (ARS MMA)[8]		10	0.5	2.5	2	20						5	50		Henry microscopy
Oxford Agar[9]	5		15				2		20	400	10				Aesculin hydrolysis
Modified Oxford Agar (MOX)[10]			15						10			15			Aesculin hydrolysis
PALCAM Agar[11]	5		15					20						10	Aesculin hydrolysis Mannitol fermentation
Modified VJ Agar[12]		10	5	2.5	2	20						5	50		Tellurite reduction

Refer to page 17 opposite for references to table 6

References

1 **AC Agar**
Bannerman, E.S. and Bille, J. (1988) *Appl. Env. Microbiol.* **54,** 165-167.

2 **Despierres' Agar**
Despierres, M. (1971) *Ann. Inst. Pasteur.* **121,** 493-501.

3 **Dominguez-Rodriguez Agar**
Dominguez-Rodriguez, L., Fernandez Garayzabal, J.F., Vazuez-Roland, J.A., Rodriguez Ferri, E. and Suarez Fernandez, G. (1985) *Can. J. Microbiol.* **31,** 938-941.

4 **GBNA Agar**
Martin, R.S., Sumarah, R.K. and MacDonald, M.A. (1984) *Clin. Invest. Med.* **7,** 233-237.

5 **LPM Agar**
Lee, W.H. and McClain, D. (1986) *Appl. Env. Microbiol.* **52,** 1215-1217.

6 **McBride Agar**
McBride, M.E. and Girard, K.F. (1960) *J. Lab. Clin. Med.* **55,** 153-157.

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Lovett, J., Francis, D.W. and Hunt, J.M. (1987) *J. Food Prot.* **50,** 188-192.

8 **ARS Modified McBride Agar**
Buchanan, R.L., Stahl, H.G. and Archer, D.L. (1987) *Food Microbiology* **4,** 269-275.

9 **Oxford Agar**
Curtis, G.D.W., Mitchell, R.G., King, A.F. and Griffin, E.J. (1989) *Lett. in Appl. Microbiol.* **8,** 95-98.

10 **Modified Oxford Agar (MOX)**
McClain, D. and Lee, W.M. (1989) FSIS method for the isolation and identification of Listeria monocytogenes from processed meat and poultry products. USDA Laboratory communication number 57.

11 **PALCAM Agar**
van Netten, P., Perales, I., van de Moosdijk, A., Curtis, G.D.W. and Mossel, D.A.A. (1989) *Int. J. Food Microbiol.* **8,** 299-316.

12 **Modified VJ Agar**
Buchanan, R.L., Smith, J.L., Stahl, H.G. and Archer, D.L. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 651-654.

TABLE 7 – Advantages and disadvantages of some *Listeria* plating media.

Medium	Advantages	Disadvantages
McBride	Possesses some selective activity	Poor selectivity
Modified McBride	Inhibitory to fungi	Poor selectivity
ARS-MMA	Detects a wide range of *Listeria spp.* More productive than McBride Inhibitory to fungi More selective than Modified VJ	Comparatively poor sensitivity and specificity KOH pre-treatment necessary Poor inhibition of enterococci, pseudomonads and *Bacillus spp.*
LPM	More selective than McBride	Poor inhibition of enterococci and *Bacillus spp.*
Modified VJ	*Listeria spp.* colonies have a characteristic black appearance	Poor inhibition of enterococci, micrococci and staphylococci
AC	Greater selectivity and sensitivity than McBride	Poor inhibition of enterococci, pseudomonads and *Bacillus spp.*
Modified Oxford (MOX)*	Inhibits enterococci and *Bacillus spp.* Improved inhibition of antibiotic resistant staphylococci	Slower growth of *Listeria spp.* Colonies comparatively small
Oxford*	Inhibits enterococci and staphylococci Presumptive identification of *Listeria spp.*	Identification system does not differentiate between aesculin positive genera Some *Listeria spp.* inhibited at 35-37°C Lactobacilli may not be inhibited
PALCAM*	Inhibits enterococci and staphylococci Microaerophilic incubation atmosphere inhibits pseudomonads Good productivity of *Listeria spp.* Differential identification system distinguishes *Listeria spp.* and enterococci	Poor inhibition of *Bacillus spp.* Comparatively inhibitory to stressed *Listeria spp.*

*Available in the Oxoid product range.

Oxford Agar

A selective and diagnostic medium for the detection of *Listeria spp.*

Listeria Selective Agar Base
Code: CM856

Formula (grams per litre)

Columbia blood agar base	*39.0*
Aesculin	*1.0*
Ferric ammonium citrate	*0.5*
Lithium chloride	*15.0*
pH 7.0 ± 0.2	

Listeria Selective Supplement (Oxford Formulation)
Code: SR140

Vial contents (each vial is sufficient for 500ml of medium)

Cycloheximide	*200.00mg (equivalent to 400mg/l)*
Colistin sulphate	*10.00mg (equivalent to 20mg/l)*
Acriflavine	*2.5mg (equivalent to 5mg/l)*
Cefotetan	*1.0mg (equivalent to 2mg/l)*
Fosfomycin	*5.0mg (equivalent to 10mg/l)*

Directions

Suspend 27.75 of the Listeria Selective Agar Base (Oxford Formulation) CM856 in 500ml of distilled water. Bring gently to the boil to dissolve. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of Listeria Selective Supplement (Oxford Formulation) SR140 reconstituted with 5ml of ethanol/sterile distilled water (1:1). Mix well and pour into sterile petri dishes.

Description

Outbreaks of infection by *Listeria monocytogenes* have prompted increased concern for detecting this organism in foods, in the environment and in pathological specimens from both human and animal subjects.

Most infections in adult humans are symptomless and result in intestinal, vaginal and cervical carriage. Infection during pregnancy may cause abortion, premature delivery and neonatal infection. The possibility of listeriosis should be considered in any woman with unexplained recurrent miscarriage, premature labour or foetal death. The organism should be sought in blood cultures and genital-tract swabs[1].

The most common clinical manifestation in both adults and neonates is meningitis. Widely disseminated infection, abscesses, sub-acute bacterial endocarditis and opportunistic infections in immuno-suppressed patients occur less frequently.

Birds, fish and other animals are all susceptible to infection with *L. monocytogenes*. It is of particular importance in domestic farm animals. In the Federal Republic of Germany reporting of listeriosis in animals is compulsory and meat inspection law in the same country requires examination for *L. monocytogenes* because of its significance in meat hygiene.

L. monocytogenes is very widespread in the environment. Isolation has been reported from milk[2,3], cheese[4], sewage and river water[5] and silage[6]. Because *L. monocytogenes* is so widespread, sources of infections are numerous. Uncooked vegetable foods have been implicated; an episode associated with consumption of coleslaw[7] was linked with cabbage from a farm using sewage fertiliser. In outbreaks caused by dairy products, cattle with mastitis may be the source of the organism. Of great importance to veterinarians is the considerable increase amongst sheep of infection manifesting as abortion or encephalitis due largely to changing practices in silage manufacture[8].

The ability to isolate the organism has been impeded in the past by lack of an effective selective medium, as *L. monocytogenes* can be easily and completely overgrown by competing flora.

Listeria Selective Medium (Oxford Formulation) is based on the formulation described by Curtis et al.[9] and is recommended for the detection of *L. monocytogenes* from clinical and food specimens.

The medium utilises the selective inhibitory components lithium chloride, acriflavine, colistin sulphate, cefotetan, cycloheximide and fosfomycin and the indicator system aesculin and ferrous iron for the isolation and differentiation of *L. monocytogenes*.

L. monocytogenes hydrolyses aesculin, producing black zones around the colonies due to the formation of black iron phenolic compounds derived from the aglucon. Gram-negative bacteria are completely inhibited. Most unwanted Gram-positive species are suppressed, but some strains of enterococci grow poorly and exhibit a weak aesculin reaction, usually after 40 hours incubation. Some staphylococci may grow as aesculin-negative colonies.

Typical *L. monocytogenes* colonies are almost always visible after 24 hours, but incubation should be continued for a further 24 hours to detect slow-growing strains.

Techniques for isolation vary with the author and the material under examination[10,11]. For all specimens selective enrichment and cold enrichment have been shown to increase isolation rates significantly[12,13,14]. The efficacy of Listeria Selective Medium (Oxford Formulation) has been confirmed for various foods[15,16] following the methodology and using selective enrichment media described in the literature[17,18,19,21,22,23,24].

Oxford Agar is a specified plating medium in the FDA/BAM isolation procedure.

Differences in susceptibility of *L. monocytogenes*, *L. seeligeri* and *L. ivanovii*, to β-lactam antibiotics and fosfomycin have been observed dependent on whether incubation is at 30°C or 35-37°C[20].

Incubation at 30°C is now recommended for Oxford Listeria Selective Agar.

Colony appearance of Listeria spp. *on Oxford Agar.*

Technique

Faecal and Biological Specimens

The sample is homogenised in 0.1% peptone water CM9 (1 part to 9 parts peptone water).

Direct Surface Plate Method

1 Inoculate 0.1ml of the homogenised specimen onto the Oxford Agar plates.
2 Incubate at 30°C for up to 48 hours.
3 Examine for typical colonies of *Listeria spp.* after 24 hours and 48 hours incubation.

Selective Enrichment Method

1 Add the homogenised specimen to the selective enrichment broth and incubate at 30°C for up to 7 days.
2 Inoculate 0.1ml of the selective enrichment broth, after 24 hours, 48 hours and 7 days, onto the Oxford Agar plates.
3 Incubate the plates at 30°C for up to 48 hours.
4 Examine for typical colonies of *Listeria spp.* after 24 and 48 hours incubation.

Food and Environmental Samples

For detection of *L. monocytogenes* when present in small numbers, the test samples must be inoculated into an enrichment broth to allow multiplication before isolation and identification. Depending on the type of sample under test, an appropriate method and selective enrichment broth should be chosen prior to inoculation onto the Oxford Agar plates.

1 Inoculate 0.1ml of the selective enrichment broth onto the Oxford Agar.
2 Incubate at 30°C for up to 48 hours.
3 Examine for typical colonies after 24 and 48 hours incubation.

Colonies presumptively identified as *L. monocytogenes* must be confirmed by biochemical and serological testing[21].

Storage and conditions of Shelf life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared plates at 2-8°C. Use as soon as possible.

Quality Control

Positive control:
Listeria monocytogenes ATCC® 19117

Negative control:
Staphylococcus aureus ATCC® 25923

Precautions

For in vitro diagnostic use

Listeria media containing acriflavine should be protected from light because photo-oxidation makes them inhibitory to *Listeria spp.*

Supplement SR140 used in this medium contains a toxic concentration of cycloheximide and is toxic if swallowed, inhaled or by skin contact. When handling wear gloves and eye/face protection.

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Modified Oxford Agar (MOX)

A selective and diagnostic medium for the isolation of *Listeria monocytogenes* from foods containing a mixed bacterial flora.

Listeria Selective Agar Base (Oxford Formulation)
Code: CM856

Formula (grams per litre)

Columbia blood agar base	*39.0*
Aesculin	*1.0*
Ferric ammonium citrate	*0.5*
Lithium chloride	*15.0*
pH 7.0 ± 0.2	

Listeria Selective Supplement (MOX)
Code: CM157

Vial contents (each vial is sufficient for 500ml of medium)

Colistin	*5.0mg (equivalent to 10mg/l)*
Moxalactam	*7.5mg (equivalent to 15mg/l)*

Note
Listeria Selective Supplement (MOX) must not be used in addition to Listeria Selective Supplement (Oxford) SR140.

Directions
Aseptically add 2ml of sterile distilled water to a vial and mix well to dissolve.

Add the contents to 500ml of sterile Listeria Selective Agar Base (Oxford) CM856 at 50°C. Mix well and pour into sterile petri dishes.

Description
Listeria Selective Medium (MOX) is a modification of the Listeria selective medium described by Curtis et al.[1] and is based on the formulation described by McClain and Lee[2] which forms part of the USDA-FSIS (United States Department of Agriculture-Food Safety Inspection Service) recommended method for the isolation and identification of *Listeria monocytogenes* from processed meat and poultry products. This formulation has been modified to incorporate a reduction in the level of moxalactam as the higher level has been shown to be inhibitory to some strains of *Listeria*[3].

The medium consists of Oxford Listeria Agar Base (Oxford) CM856 to which is added Listeria Selective Supplement (MOX) SR157 which contains the antibiotics moxalactam and colistin.

Colony appearance of Listeria spp. *on modified Oxford Agar (MOX).*

Listeria Selective Agar Base (Oxford) contains aesculin and ferric ammonium citrate. *Listeria spp.* will hydrolyse the aesculin in the medium to form aesculetin which reacts with the ferric ions to form a black/brown complex. This appears as blackening in and around colonies of *Listeria spp.*

Lithium chloride is contained in the medium to inhibit the growth of enterococci which also hydrolyse aesculin. Moxalactam and colistin suppress the growth of other micro-organisms that may form part of the normal flora of the specimen e.g. *Staphylococcus*, *Proteus* and *Pseudomonas*. Moxalactam is considered more effective than cefotetan (incorporated in Listeria Selective Supplement [Oxford] SR140) against methicillin-resistant *Staph. aureus* which presents a problem in some countries.

Listeria Selective Supplement (MOX) may be used as an alternative for laboratories not wishing to have acriflavine in the medium.

Technique
The sample should be pre-enriched by following the laboratory's normal enrichment procedures. After secondary enrichment, inoculate the specimen on to Listeria Selective Medium (MOX) plates by streaking. Incubate the plates at 35°C for 24 and 48 hours aerobically.

Note
When testing food samples suspected of causing disease, streak MOX plates from the secondary enrichment broth regardless of any colour reaction. Incubate the secondary enrichment broth for an additional 24 hours then restreak to MOX agar plate. Incubate the secondary enrichment broth for an additional 24 hours then restreak to a MOX agar plate, incubate this plate at 35°C and read at both 24 and 48 hours.

Listeria spp. will produce brown/black colonies with a halo. Typical *L. monocytogenes* colonies are almost always visible after 24 hours, but incubation should be continued for an additional 24 hours to detect slow growing strains.

Colonies presumptively identified as *L. monocytogenes* must be confirmed by biochemical and serological testing[4].

Storage conditions and Shelf life
Store the selective supplement at 2 to 8°C. Store the prepared medium plates for up to 2 weeks at 2 to 8°C.

Quality Control
Positive control:
Listeria monocytogenes ATCC® 19117

Negative control:
Staphylococcus aureus ATCC® 25923

References

1 Curtis, G.D.W., Mitchell, M.G., King, A.F. and Griffin, E.J. (1989) *Lett. Appl. Microbiol.* **8,** 95-98.
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PALCAM Agar

A selective and differential diagnostic medium for the detection of *Listeria monocytogenes*.

PALCAM Agar Base
Code: CM877

Formula (grams per litre)

Columbia blood agar base	*39.0*
Yeast extract	*3.0*
Glucose	*0.5*
Aesculin	*0.8*
Ferric ammonium citrate	*0.5*
Mannitol	*10.0*
Phenol red	*0.08*
Lithium chloride	*15.0*
ph 7.2 ± 0.2	

PALCAM Selective Supplement
Code: SR150

Vial contents

	Code: SR150E To supplement 500ml	Code: SR150B To supplement 2.5 litres	
Polymixin B	*5mg*	*25mg*	*(equivalent to 10mg/l)*
Acriflavine hydrochloride	*2.5mg*	*12.5mg*	*(equivalent to 5mg/l)*
Ceftazidime	*10mg*	*50mg*	*(equivalent to 20mg/l)*

Directions

Suspend 34.5g in 500ml of distilled water. Bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of PALCAM Selective Supplement SR150E, reconstituted with 2ml of sterile distilled water. Mix well and pour into sterile petri dishes.

To prepare 2.5 litres of medium, suspend 172.5g in 2.5 litres of distilled water. Sterilise and cool as above and add the contents of one vial of SR150B, reconstituted with 10ml of sterile distilled water.

The addition of 2.5% (v/v) Egg Yolk Emulsion (Oxoid code SR47) to the medium may aid the recovery of damaged *Listeria spp.*

Description

PALCAM Medium is based on the formulation described by van Netten et al.[1] and is recommended for the isolation of *Listeria monocytogenes* from foods.

The heightened awareness and concern surrounding the presence of *L. monocytogenes* in food has resulted in the development of many media for its isolation[2-9]. However, Cassiday and Brackett[10] conclude that no single method currently available is suitable for use with all types of food.

PALCAM Medium is highly selective due to the presence of lithium chloride, ceftazidime, polymixin B and acriflavine hydrochloride. It allows the easier differential diagnosis of *L. monocytogenes* by using the double indicator system:

1 Aesculin and ferrous iron
2 Mannitol and phenol red.

L. monocytogenes hydrolyses aesculin resulting in the formation of a black halo around colonies. *L. monocytogenes* does not ferment mannitol so easy differentiation from contaminants such as enterococci and staphylococci can be made as these will ferment mannitol and produce a change from red to yellow in the pH indicator phenol red.

Incubation under micro-aerophilic conditions serves to inhibit strict aerobes such as *Bacillus spp.* and *Pseudomonas spp.* that might otherwise appear on the medium.

Technique

Techniques for the isolation of *L. monocytogenes* will depend on the material under test[10]. It is usual for the test sample to be inoculated into an enrichment broth to allow multiplication before isolation and identification on plating media. Depending on the type of sample used, the appropriate method of selective enrichment broth should be used prior to inoculation onto PALCAM Medium plates. As a general rule use Listeria Selective Enrichment Medium (Oxoid codes CM862 and SR141 or SR149) for dairy products and Listeria Selective Enrichment Media UVM (Oxoid codes CM863, SR142 and SR143 or Fraser Broth CM895 and SR156) for meats and poultry.

1 Inoculate one loopful of the selective enrichment broth on the PALCAM Medium plates.
2 Incubate at 37°C for 48 hours under micro-aerophilic conditions. The micro-aerophilic atmosphere can best be achieved by using Oxoid Campylobacter Gas Generating Kit (BR56) in conjunction with the Oxoid Anaerobic Jar and an active Catalyst (BR42). For jars of smaller capacity (2.5 litres) use the Oxoid Campylobacter Gas Generating Kit (BR60).
3 Examine for typical colonies of *Listeria spp.* after 48 hours incubation.
4 Colonies identified as presumptive *Listeria spp.* must be confirmed by biochemical and serological testing[11].

After 48 hours incubation, typical *Listeria spp.* form colonies that are approximately 2mm in diameter, grey-green in colour with a black sunken centre and a black halo against a cherry-red medium background.

Occasional *Enterococcus* or *Staphylococcus* strains develop on PALCAM Medium to form grey colonies with a brown-green halo or yellow colonies with a yellow halo.

The medium is not totally selective for *L. monocytogenes*. Any typical *Listeria* colonies must be confirmed by biochemical and serological methods[12]. However, in a study comparing PALCAM and Oxford Agars, PALCAM Medium was seen to be consistently more effective than Oxford Medium in suppressing other micro-organisms in a variety of meat products[11].

Colony appearance of Listeria spp. *on PALCAM Agar.*

Storage conditions and Shelf life
Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the selective supplement in the dark at 2-8°C and use before the expiry date on the label.

Store the prepared medium at 2-8°C and use as soon as possible.

Quality Control
Positive control:
Listeria monocytogenes ATCC® 19112

Negative control:
Escherichia coli ATCC® 25922
Staphylococcus aureus ATCC® 25923
Streptococcus faecalis ATCC® 29212

Precautions
For in vitro diagnostic use

Acriflavine hydrochloride is activated by light which may cause it to become inhibitory to *Listeria spp*.

References

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10 Cassiday, P.K. and Brackett, R.E. (1989) *J. Food Prot.* **52,** 207-214.
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Identification Tests

Detailed procedures for identification of *Listeria species* are beyond the scope of this publication. However, the following brief summary of tests may be helpful.

Simple tests to identify *Listeria*
Colonies that appear to be *Listeria spp.* on plating media can be confirmed using three simple tests.

1 ***Motility***

Heavily inoculate Brain Heart Infusion (CM225) or Nutrient Broth No. 2 (CM67) and incubate at room temperature.

Examine microscopically at 4-6 hours. Tumbling, rotating motility is characteristic of *Listeria spp.* If motility is not seen, re-examine at 18 hours before discarding as negative.

Note: *Listeria spp.* do not form flagellae above 30-33°C and motility may not occur if cultures are incubated above 30°C.

2 ***Catalase***

Emulsify a colony in a drop of hydrogen peroxide on a glass slide. Immediate bubbling indicates a positive catalase test.

Listeria spp. are catalase positive.

Note: False positive catalase reactions may occur if a colony is taken from a medium containing blood.

3 ***Microscopy***

Examine a Gram-stain of growth from a suspected colony.

Listeria cells have a distinctive appearance and disposition. They are short Gram-positive rods which occur as straight pairs, pairs arranged in V formation and pairs adjacent to each other.

Most non-*Listeria spp.*can be eliminated by these three screening tests.

Confirmation of *L. monocytogenes*

L. monocytogenes must be differentiated from other *Listeria spp.* by the CAMP test and biochemical reactions. The tests to be done and the results shown by *L. monocytogenes* are given in Tables 8 and 9.

Gram stain of L. monocytogenes.

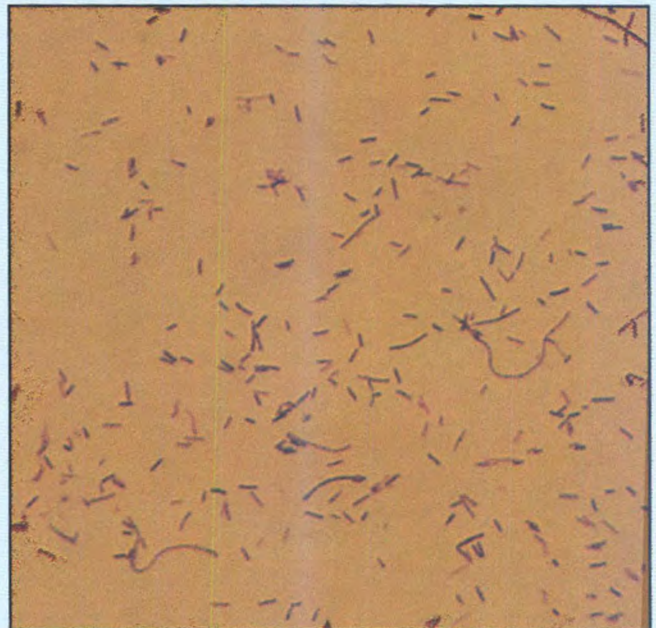


TABLE 8

Test	*L. monocytogenes*
Beta haemolysis (Horse Blood)	+
Camp Test	
Staphylococcus aureus	+
Rhodococcus equi	–
Catalase	+
Oxidase	–
Nitrate reduction	–
Methyl red (MR)	+
Voges-Proskauer (VP)	+
H_2S	–
Urea	–
Acid from:	
Glucose	+
Xylose	–
Rhamnose	+
Mannitol	–
Aesculin	+

β *haemolysis of horse blood in McBride Agar.*

Strains identified as *L. monocytogenes* should be tested for their serological group.

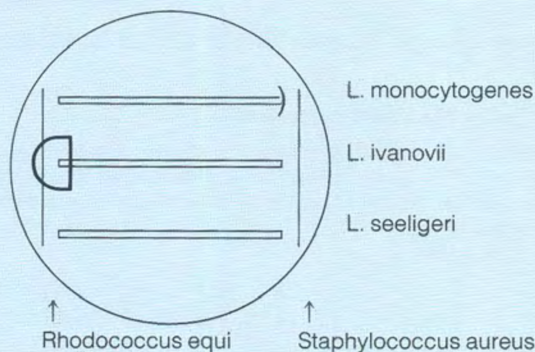
The CAMP Test (Christie-Atkins-Munch-Peterson)
Prepare sheep blood agar plates by pouring a thin layer of 5% v/v blood agar made with washed sheep cells, SR51, on to the surface of nutrient agar base plates. Allow to set and dry before use.

Streak cultures of *Staphylococcus aureus* NCTC 1803 and *Rhodococcus equi* NCTC 1621 across the sheep blood agar plate. Then streak the test strains at right angles to the *Staph. aureus* and *Rhod. equi.* leaving a minimum of 1-2mm between cultures. Incubate at 37°C overnight.

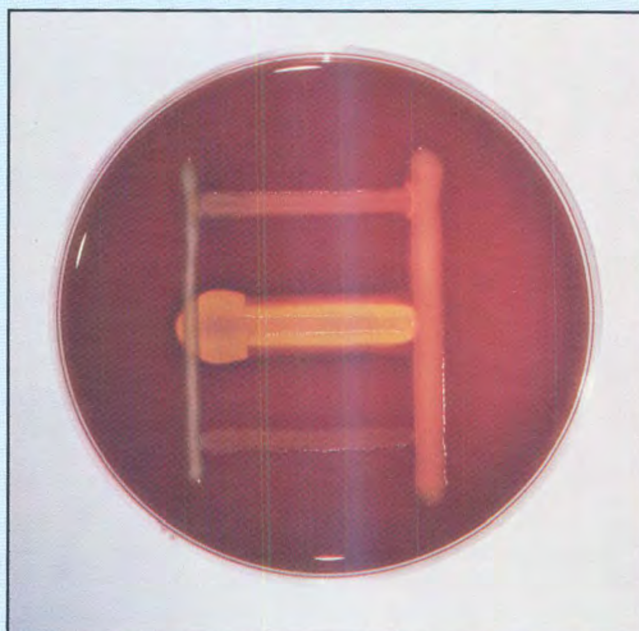
Results are recorded as positive when an enhanced zone of haemolysis between the two cultures occurs. Use of known control *Listeria spp.* on a separate sheep blood agar plate is recommended.

TABLE 9 – CAMP reactions for the haemolytic species of *Listeria*.

	Staphyloccus aureus	***Rhodococcus equi***
L. monocytogenes	+	–
L. seeligeri	+	–
L. ivanovii	–	+



Species differentiation by the CAMP test.



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