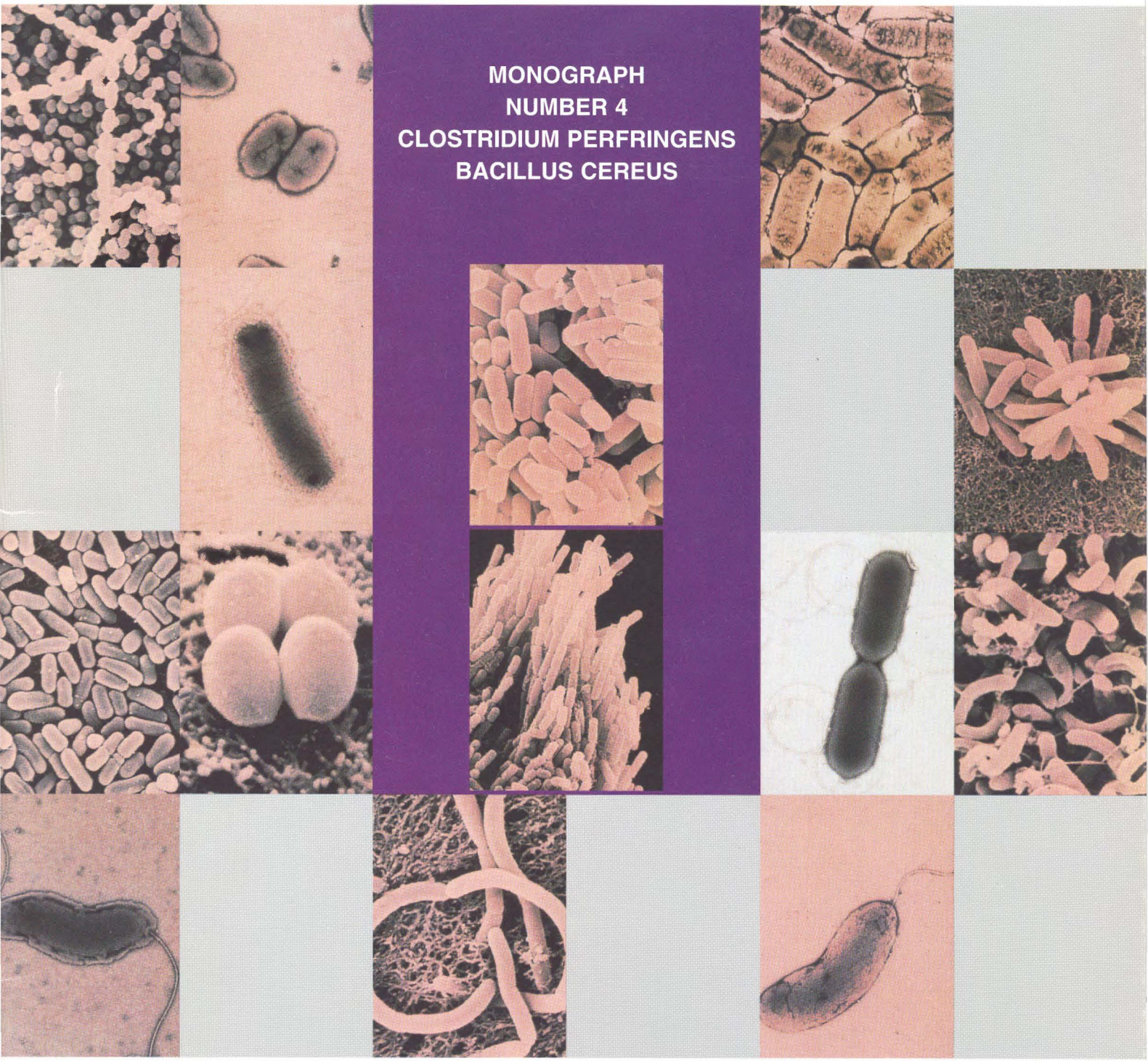
Food-borne Pathogens



OXOID

SETTING STANDARDS

FOOD-BORNE PATHOGENS MONOGRAPH NUMBER 4 CLOSTRIDIUM PERFRINGENS BACILLUS CEREUS

D. E. POST

Technical Support Manager

Price £5.00

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Introduction

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Clostridium perfingens

Clostridium perfringens (previously known as *Clostridium welchii*) was first described in detail in 1892 by Welch and Nuttall.[1] It was recognised as a cause of food-borne illness as early as 1895[2] and the link was established firmly through epidemiological studies by McClung in 1945.[3] The identification of an enterotoxin was reported in 1969 by Duncan and Strong.[4] In addition to enteritis, *Clostridium perfringens* is responsible for necrotising tissue infections in humans and animals and severe enterotoxaemia in some animals.

Clostridium perfringens is a Gram-positive, anaerobic, sporulating bacillus, unusual amongst the clostridia in being non-motile. It appears to be a natural inhabitant of the human gut but, because it possesses a number of necrotising and lethal enzymes and toxins, it has considerable pathogenic potential in both man and animals. *Cl. perfringens* strains are sub-divided into 5 types, A, B, C, D and E, (Table 1) dependent on the presence or absence of 4 major toxins, lethal for mice, and a variety of minor toxins. Enterotoxin responsible for food poisoning in humans is produced by type A strains. Rarely, type C strains may cause a much more severe necrotic enteritis (Pig-Bel) which may be fatal.

Food poisoning arises as a result of ingesting large numbers of viable organisms which, typically, have multiplied in meat dishes prepared in large quantities and which have received insufficient heating. Because of their bulk, cooling will have been slow, thus allowing maximum opportunity for rapid multiplication. *Cl. perfringens* enteritis is not a classical intoxication because toxin is not preformed in the food, although this has been observed on rare occasions. Instead, it is necessary for viable organisms to be ingested in numbers great enough to survive passage through the stomach. Enterotoxin is subsequently produced when sporulation occurs in the gut. Symptoms of intoxication are abdominal pain and diarrhoea 8 to 24 hours after eating the contaminated food. Vomiting and fever does not occur. Duration of illness is short, usually only 12 to 24 hours. On rare occasions fatalities may occur, particularly amongst the elderly, as a result of dehydration.

Cl. perfringens has the ability to multiply over a temperature range of 15 to 50°C and at the optimal temperature of about 45°C, cell numbers will double in about 12 minutes. Because many of the organisms that accompany *Cl. perfringens* in food are inhibited at temperatures above 40°C the competition for nutrients is much reduced and *Cl. perfringens* is able to multiply without hindrance.

Prevention of *Cl. perfringens* food poisoning is achieved by proper use of refrigeration during storage and rapid cooling of cooked foods containing meat, poultry or fish.

More recently a different form of *Cl. perfringens* enterotoxin-associated diarrhoea has been recognised.[5] Cases occur sporadically and the evidence indicates that the condition is an infection, and not food poisoning, although ingestion of the strain responsible with food cannot be excluded. The condition is similar to *Cl. difficile* antibiotic-associated colitis and diarrhoea and was recognised when a cytotoxin other than that produced by *Cl. difficile* was detected and confirmed as *Cl. perfringens* enterotoxin in tissue culture neutralisation tests. The disease appears to be due to small bowel colonisation by *C. perfringens* and in character is significantly different from *C. perfringens* food poisoning. Further work has shown that cases can arise in the absence of antibiotic treatment.[6]

Bacillus cereus

Bacillus cereus, like *Cl. perfringens*, is a sporulating Gram-positive bacillus and differs in growing aerobically. Its ability to cause food poisoning has been recognised since the early 1900's when the role of "Bacillus peptonificans" in an outbreak of gastroenteritis was described.[7] The recognition that there are two distinct disease syndromes caused by separate toxins that cause vomiting or diarrhoea is much more recent.

The emetic toxin causes nausea and vomiting 1 to 5 hours after eating the contaminated food, frequently rice. The diarrhoeal toxin is slower to act, with an incubation period of 8 to 16 hours. It is commonly associated with reheated, spiced, meat-casserole dishes. Spices may be heavily contaminated with heat-resistant Bacillus spores which germinate during cooking.

Cereals other than rice have been implicated and other vehicles are pasta, milk puddings and pasteurised cream.

In addition to its capacity for producing food poisoning, *Bacillus cereus* has been shown to infect various body sites including wounds[8] and eyes.[9]

Bacillus species are ubiquitous in the environment and consequently are frequently present in food. Prevention of food poisoning is dependent on controlling spore germination and growth of the organisms. This is most easily done by avoiding storage of rice after cooking. Rapid cooling and refrigeration may not always be effective because psychrophilic strains exist, capable of producing toxin at refrigeration temperatures.[10]

Although *Bacillus cereus* is the species most commonly associated with food poisoning, *B. licheniformis* and *B. subtilis* have also been implicated.[11] *B. subtilis* food poisoning is generally emetic and has a very rapid onset. It has been associated with wheat and pastry dishes such as sausage rolls and meat pies.

A possible role for *B. thuringiensis* in gastroenteritis has been suggested following an investigation into an outbreak in a chronic care institution.[12]

This publication is concerned with *Clostridium perfringens* and *Bacillus cereus* and is intended as a guide to culture media and methodology available for their detection.

References

1 Welch, W.H. and Nuttall, G.H.F. (1892) *Johns Hopkins Hosp. Bull.* **3,** 81.
2 Klein E. (1895) *Zbl. Bakt.* (Abt. 1, Orig.) **18,** 137. Cited in Hobbs, B.C. (1965) *J. Appl. Bact.* **28,** 74–82.
3 McClung, L. (1945) *J. Bact.* **50,** 229–231.
4 Duncan, C.L. and Strong, D.H. (1969) *J. Bact.* **100,** 86–94.
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6 Mpamugo, O., Donovan, T. and Brett, M.M. (1995) *J. Med. Microbiol.* **43,** 442–445.
7 Lübenau, C. (1906) *Zbl. Bakt., I.* **40,** 433–437.
8 Akesson, A., Hedström, S.A. and Ripa, T. (1991) *Scand. J. Inf. Dis.* **23,** 71–77.
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12 Jackson, S.G., Goodbrand, R.B., Ahmed, R. and Kasatiya, S. (1995) *Lett. Appl. Microbiol.* **21,** 103–105.

Table 1 – The major toxins produced by the five types of *Cl. perfringens*.

Major Toxins

TYPE	ALPHA	BETA	EPSILON	IOTA
A	+	–	–	–
B	+	+	+	–
C	+	+	–	–
D	+	–	+	–
E	+	–	–	+

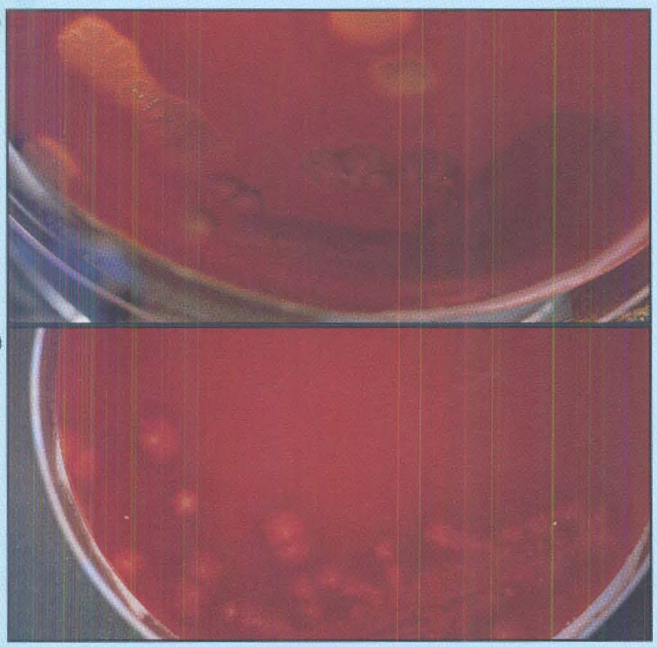
Note:

1 It is generally necessary only to detect the 4 major toxins to differentiate types A to E. The presence of a number of additional minor toxins may be determined if necessary.[1]
2 Food poisoning strains of Type A possess an enterotoxin distinct from any of the toxins named above.

Reference

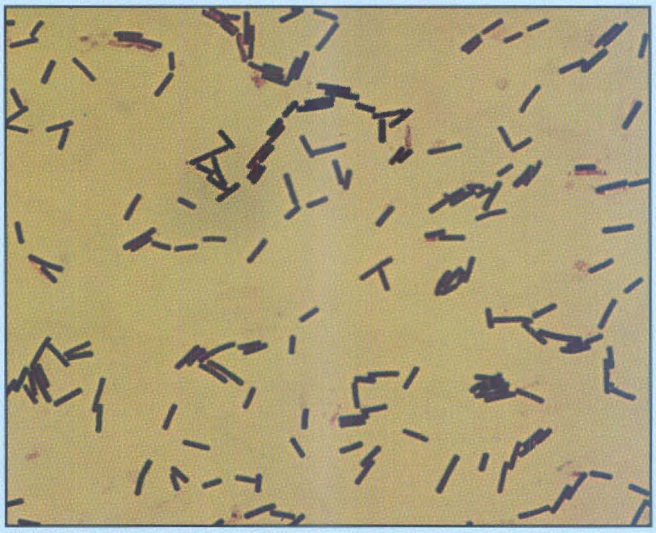
1 Brooks, M.E., Sterne, M. and Warrack, G.H. (1957) *J. Pathol. Bacteriol.* **74,** 185.

(a)
(b)

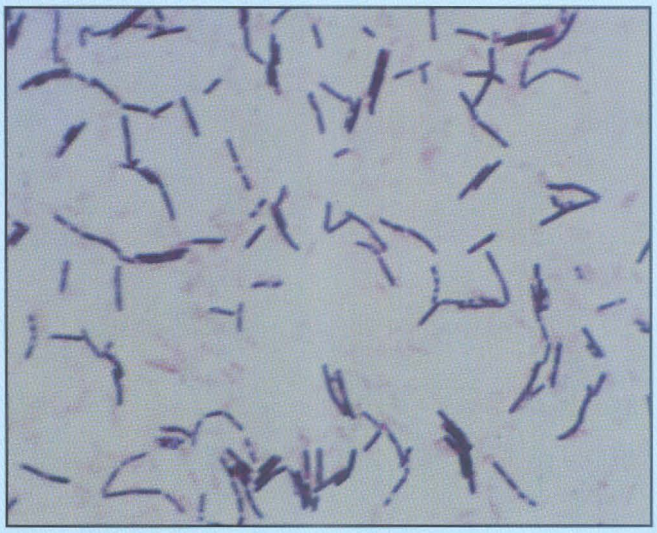


Typical appearance of *Clostridium perfringens* on blood agar. (a) haemolytic strain; (b) non-haemolytic strain.

Typical appearance of *Bacillus cereus* on blood agar.



Clostridium perfringens. Gram stain.



Bacillus cereus. Gram stain.

The Ocurrence of *Clostridium perfringens* and *Bacillus cereus* in Foods

Clostridium perfringens

Cl. perfringens is commonly present in the gut of man and animals and in soil. Surveys have shown that 80–100% of healthy humans harbour type A. Types B, C, D and E are generally associated with animals, probably as obligate parasites, but type A is a normal component of the microflora of both soil and intestinal tracts of a variety of animals.

The carcasses of food animals and poultry are readily contaminated in abattoirs during processing and the natural presence of *Cl. perfringens* in soil results in contamination of vegetables.

Low-grade faecal contamination leads to the frequent occurrence of *Cl. perfringens* in milk. The heat-resistant spores will survive pasteurisation but food poisoning by milk products is rare.

Consumption of contaminated fish may result in food poisoning. Salmon seems to present a greater hazard than other fish. The organism appears to be normally present on the body surface and in the alimentary canal of a number of fish species, but it is also probable that access to the fish occurs from outside during processing or final preparation and serving.

Outbreaks of *Cl. perfringens* food poisoning are caused by enterotoxin-producing strains of type A and are typically associated with institutional catering. Problems may arise as a result of advance preparation of large quantities of foods such as stews, casseroles, pies and gravies which provide a rich source of nutrients, favourable pH and aw values and an anaerobic milieu resulting from driving off air during cooking. However, as *Cl. perfringens* is not a strict anaerobe it will thrive even if very low Eh values are not achieved. Cells may survive the cooking process if heating is not rapid and uniform, and multiply rapidly when suitable temperatures are reached on cooling. The effect will be exacerbated if inefficient refrigeration allows only slow temperature reduction. Reheating from cold must be very thorough in order to kill vegetative cells present.

Cured meat products are rarely incriminated in food poisoning.

Pig-Bel

Enteritis necroticans is a form of serious, sometimes fatal, food poisoning caused by beta toxin produced by *Cl. perfringens* type C. It is generally associated with the eating of pork. *Cl. perfringens* type C poisoning is particularly associated with communal feasting in the Highlands of Papua New Guinea where it is known as Pig-Bel.

The disease predominately affects young adults and is the most common cause of death in hospitalised children after the first year of age.

Pig-Bel is very similar to "Darmbrand" which occurred in Germany soon after the end of World War 2 in conditions of poor hygiene and nutrition. Cases of Pig-Bel continue to arise but Darmbrand has not recurred.

A detailed review of Pig-Bel has been written by Walker.[1]

Bacillus cereus

Bacillus species, including *B. cereus*, are widespread in the environment, and can be found in soil, dust, water and the air. Consequently there is considerable opportunity for them to be present in, or on, foods. The natural habitat of most species is soil. Because of their frequent occurrence, care must be taken to ensure that the correct significance is given to isolation of *Bacillus* species; the spores are very hardy and isolation does not necessarily imply that a species has a major ecological role in the habitat in which it is found. Small numbers of *Bacillus cereus* present in foods may be inconsequential in some circumstances.

B. cereus and other species may be present on fresh meat, probably as a result of contamination from soil. Spiced meat products may show an even higher incidence, cells in naturally-contaminated seasonings contributing to the population. The organism may be detected on a high percentage of poultry carcasses.

Highly seasoned dishes, such as goulash, prepared from meat and vegetables are associated with *B. cereus* food poisoning, probably because of the presence of large numbers of spores in the seasoning spices and the favourable conditions created for germination and growth during cooking.

B. cereus has for many years been recognised as a spoilage organism of fresh milk and may be isolated from pasteurised and even ultra-heat-treated milk. It may be found in cream and has also been found in yoghurt. Rarely, it may survive the manufacture of cheese.

B. cereus is commonly present in dried milk and may be found in foods manufactured using dried milk, eg. some kinds of confectionery.

Cereals may contain *B. cereus* in large numbers. Boiled rice stored at room temperature before consumption is particularly associated with emetic food poisoning. *B. cereus* may also be found in wheat and foods made from it, eg. pasta, and corn starch used as a thickening agent in sauces and confectionery.

Other dried foods in which *B. cereus* may be found are egg, leguminous and other vegetables and fruits. It may survive the manufacture of dried soups and sauce mixes. The actual numbers of *B. cereus* spores present is very variable and they are often of no consequence unless cooking procedures are favourable to their multiplication.

Isolation of *B. cereus* from food under suspicion of having caused food poisoning is, by itself, not sufficient to prove the association because of the frequency with which the organism occurs naturally in a wide range of foods. It is necessary to show that the strains isolated from the patient and the food are the same.

A biotyping scheme to do this, based on the ability of isolates to produce acid from various carbohydrates, has been shown valuable in determining the source of strains and may be useful in epidemiological investigations.[2]

B. subtilis and *B. licheniformis*, both of which have been associated with food poisoning, are common in wheat flour and are recognised spoilage organisms. *B. licheniformis* has been implicated in food poisoning following consumption of bread which has started to spoil.

References

1 Walker, P.D., Pig-Bel. In: *Clostridia in Gastrointestinal Disease*. Borriello, S.P. (Ed). CRC Press Inc. Florida.

2 Jha, N.K. and Narayan, K.G. (1995) *J. Food Sci. Technol.* **32,** 231–232.

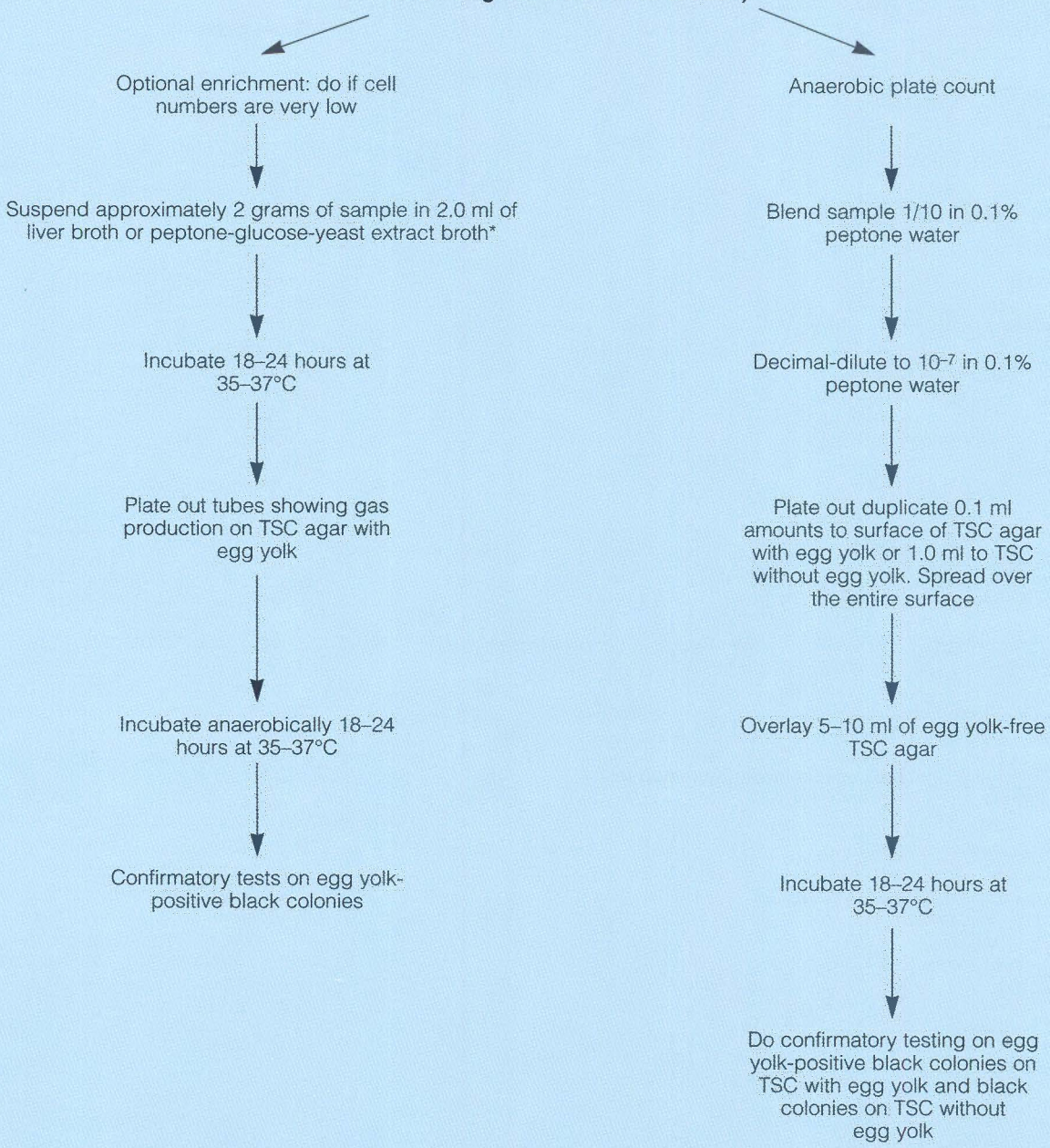
Table 2 – Some regulatory bodies that specify detection procedures for *Clostridium perfringens* and the culture media to be used. The codes for Oxoid dehydrated culture media available from Unipath are in parentheses.

The appropriate documents should be consulted for the composition of the other media specified in the Standards.

Regulatory Body	Detection Media	Other Media
AOAC/FDA Bacteriological Analytical Manual (BAM) (1992)	Tryptose-sulphite-cycloserine agar (TSC) (CM587 + SR88)	Thioglycollate medium (CM173) Modified cooked meat medium or chopped liver broth Iron-milk medium Lactose-gelatin medium Buffered motility-nitrate medium Sporulation broth Spray's fermentation medium AE sporulation medium Duncan-Strong sporulation medium
Agriculture Canada (1988) Methods Manual	Tryptose-sulphite-cycloserine agar (TSC) (CM587 + SR88)	Modified cooked meat medium Fluid thioglycollate medium (CM173) Nitrate-motility medium Lactose-gelatin medium
German Institute of Normalisation (DIN 10165) adopted by the Official Collection of Investigative Procedures L06-00-20 (1984)	Tryptose-sulphite-cycloserine agar (TSC) (CM587 + SR88)	Thioglycollate medium (CM173) Nitrate-motility medium Lactose-gelatin medium
British Standards Institution (BSI) BS5763: Part 9: 1986 (1992) and ISO 7937 BS4285: Part 3: Section 3–13 (1990) *BSI Draft Document 95/502681 (BS 5763: Part 9 revision (BS EN ISO 7937)	Tryptose-sulphite-cycloserine agar (SC agar) (CM587 + SR88) Tryptose-sulphite-cycloserine agar (SC agar) (CM587 + SR88)	Fluid thioglycollate medium (CM173) Nitrate-motility medium Lactose-gelatin medium Lactose-sulphite medium (LS)
Standards Australia Committee on Food Microbiology AS 1766.2.8. (1991)	(a) Tryptose-sulphite-cycloserine (TSC) agar with egg yolk (CM587 + SR88 + SR47) (b) TSC agar without egg yolk (CM587 + SR88)	(a) Neomycin-cooked meat medium (b) Lactose-egg yolk agar (LEY) (c) Lactose-gelatin medium (LG) (d) Nitrate-motility medium (NM)
Normalisation Française (AFNOR) V08-056 (1994)	Tryptose-sulphite-cycloserine agar (TSC) (CM587 + SR88)	Fluid thioglycollate medium (CM173) Lactose-sulphite medium (LS)
Spanish Ministeria de Sanidad y Consumo Instituto de Salidad, Carlos III. Technical Manual	(a) Tryptose-sulphite-cycloserine agar (TSC) (b) TSC agar with egg yolk (CM587 + SR88 + SR47) (c) Oleandomycin-polymyxin-sulphadiazine Perfringens agar (OPSP) (CM543 + SR76 + SR77) (d) Tryptone-sulphite-neomycin agar (TSN)	(a) Lactose fermentation agar (b) Tryptone-yeast extract agar (c) Lactose-milk-egg yolk agar (d) Reinforced clostridial medium (RCM) (CM149) with added neomycin sulphate (100 µg/ml) (e) Cooked meat-neomycin medium (f) Neomycin-blood agar (CM55 + 7% horse blood with added neomycin sulphate (100 µg/ml))

*This revision of BS 5763: Part 9 is quoted for information only to draw attention to a proposed change in the method for identifying *Clostridium perfringens*. It is a draft and must not be regarded or used as a British Standard.

Procedure for isolation and quantitation of *Clostridium perfringens* (based on APHA. Compendium of Methods for Microbiological Examination of Food)



***Peptone-glucose-yeast extract broth**

	grams/litre
Tryptone (L43)	50.0
Peptone (L34)	5.0
Yeast extract (L21)	20.0
Glucose	4.0
Disodium phosphate	5.0
Sodium thioglycollate	1.0
Water	1000 ml

pH 7.0 ± 0.2

When the testing specification requires the use of a different Standard Method the Standard should be consulted for the technique, which may differ in detail from the above.

Liquid Media for *Clostridium Perfringens*

Enrichment

Cl. perfringens is very widely distributed in the environment and consequently occurs in raw foods and food ingredients. Additionally, because of its ability to produce resistant spores, *Cl. perfringens* may survive exposure to some food processing procedures and persist in low numbers. Enrichment is therefore essential to ensure detection in samples which contain few cells and, because the organism may be greatly outnumbered by other microbes, it is very often necessary to employ selectivity.

Numerous media have been recommended for enrichment including some which incorporate sulphite and iron to give the familiar blackening reaction as a presumptive identification feature. However, blackening is not limited to *Cl. perfringens* and, also, failure to blacken is not necessarily proof of the absence of *Cl. perfringens*. Some workers have therefore disregarded blackening in their development of enrichment procedures.

The comparative heat resistance of food poisoning strains has been recognised and heating of food samples in non-selective media such as Robertson's cooked meat, liver broth or reinforced clostridial medium is commonly employed when the number of cells is likely to be small.[1] This process of pasteurisation destroys accompanying heat-sensitive organisms enabling *Cl. perfringens* to grow as a pure, or considerably less mixed, culture. However, spores of other clostridia present will also survive and germinate. Attempts to make the process elective for *Cl. perfringens* have met with little success.

Antibiotics have been used in some applications to confer selectivity. A tryptone-glucose-yeast extract enrichment medium containing cycloserine at a concentration of 400 µg/ml, (TPYG), was used to detect *Cl. perfringens* on the surface of red meat carcasses.[2]

Debevre[3] employed fluid thioglycollate medium without dextrose, containing 400 µg/ml of cycloserine, in a method for detecting small numbers of vegetative cells or spores of *Cl. perfringens* in unspecified foods. Incubation conditions were 46°C for 18 hours and the medium was then inoculated on plates of iron sulphite agar. The plates were also incubated at 46°C for 18 hours to obtain typical black colonies for confirmatory testing.

Poumeyrol and Billon[4] recommend the use of Perfringens Enrichment Medium (PEM). The medium is based on casein hydrolysate and, like Debevre's formula, omits dextrose to minimise acid production and the consequent fall in pH to values that would inhibit *Cl. perfringens*. Cycloserine is included for selectivity. The medium is intended for recovery of vegetative or sporulated cells which have been damaged by heat or ionic preservatives.

The formulae of TPYG, PEM and Debevre's fluid thioglycollate medium are given on pages 8 and 9.

An enrichment method that does not require culture media may be employed in the examination of red meats packed under vacuum.[5] Adequate water and nutrients are provided by the meat itself and the packaging provides conditions for anaerobic growth. The packaged meat is incubated at 43 to 45°C and *Cl. perfringens* present produces large quantities of gas, causing the packs to swell. Moisture is taken from the swollen packs and plated on a selective medium to obtain colonies for further testing.

Detection of Cl. perfringens *by Most Probable Number (MPN)*

Liquid media may also be employed for enumeration of *Cl. perfringens* by the Most Probable Number (MPN) method as an alternative to plate counts on agar.[6]

Despite the inherent disadvantages of inaccuracy associated with the MPN method, several compensating advantages have been claimed for it.[7] It is useful where the number of viable cells is very low and as a consequence, colony counts fail or lose their accuracy. Higher recoveries of clostridia have been obtained from liquid rather than solid culture media and incubation in specialised anaerobic apparatus is not necessary.

A variety of different media including Cooked Meat and Reinforced Clostridium media have been recommended for the MPN technique to detect *Cl. perfringens* amongst other sulphite-reducing clostridia. A more recent formulation is lactose-sulphite broth (LS medium)[8] formulated to select *Cl. perfringens* by using its specific biochemical and cultural characteristics. The medium is also important in confirming presumed cultures of *Cl. perfringens*. A somewhat older medium is Rapid Perfringens Medium (RPM).[9] This consists of a mixture of litmus milk and fluid thioglycollate broth fortified with a number of additions. Selectivity is achieved by the incorporation of polymyxin B and neomycin.

Stormy fermentation (the "stormy clot" reaction) is used as presumptive evidence that *Cl. perfringens* is present.

Green and Litsky[10] improved on the enumeration results they had been obtaining with SPS agar when they devised a new liquid medium (TYD-C) for use in a "mimic" MPN method. This uses a very selective confirmatory medium to identify which tubes of the primary MPN investigation contain sulphite-reducing clostridia. *Cl. perfringens* grows faster than other non-clostridial organisms in TYD-C medium at 45°C with the production of gas.

At the same time that tubes of TYD-C medium were inoculated from the primary MPN tubes, plates of SPS agar were also inoculated to obtain isolated colonies for further identification.

The formulae of LS, RPM and TYD-C media are given on page 8. Oxoid products that can be used when making them are named.

References

1 *Practical Food Microbiology* (1995) Section 6.5. Roberts, D., Hooper, W. and Greenwood, M. (Eds). Public Health Laboratory Service, London.

2 Smart, J.L., Roberts, T.A., Stringer, M.F. and Shah, N. (1979) *J. Appl. Bact.* **46,** 377–383.

3 Debevre, J.M. (1979) *Eur. J. Appl. Microbiol. Biotechnol.* **6,** 409–414.

4 Poumeyrol, M. and Billon, J. (1995) Chapter 21. In: *Microbiological Control for Foods and Agricultural Products.* Bourgeois, C.M. and Levan, J.Y. (Eds). V.C.H. Publishers Ltd.

5 Mead, G.C., Adams, B.W., Roberts, T.A. and Smart, J.L. "Isolation and Enumeration of *Clostridium perfringens*". In: *Isolation and Identification Methods for Food Poisoning Organisms* (1982). Pages 99–110. Corry, J.E.L., Roberts, D. and Skinner, F.A. (Eds). Society for Applied Bacteriology, Technical Series Number 17. Academic Press.

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9 Erickson, J. and Deibel, R.H. (1978) *Appl. Environ. Microbiol.* **37,** 567–571.
10 Green, J.H. and Litsky, W. (1966) *J. Food Sci.* **31,** 610–614.

Perfringens Enrichment Medium (PEM)

	grams/litre
Tryptone (L42)	15
Yeast extract (L21)	5
Sodium chloride	2.5
Sodium thioglycollate	0.5
L-cysteine	0.5
Resazurin	0.001 g
Agar	0.75
Water	1000 ml

pH 7.1 ± 0.1

Cycloserine	400 mg

Reference

Poumeyrol, M. and Billon, J. (1995) Chapter 21. In: *Microbiological Control for Foods and Agricultural Products.* Bourgeois, C.M. and Levan, J.Y. (Eds). V.C.H. Publishers Ltd.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Tryptone-Yeast Extract-Dextrose Clostridium Medium (TYD-C)

	grams/litre
Tryptone (L42)	40.0
Yeast extract (L21)	10.0
Dextrose (L70)	5.0
Soluble starch	1.0
Sodium thioglycollate	2.0
Thiamine hydrochloride	1.0 mg
Agar (L11)	0.15
BBL Clostrisel broth	3.0
Water	1000 ml

pH 7.2 ± 0.2

Reference

Green, J.H. and Litsky, W. (1966) *J. Food Sci.* **31,** 610–614.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Lactose Sulphite (LS) Broth

	grams/litre
Tryptone (L42)	5
Yeast extract (L21)	2.5
Sodium chloride (L5)	2.5
Lactose (L70)	10
L-cysteine hydrochloride	0.3
Water	1000 ml

pH 7.1 ± 0.1

Add the following before use, to freshly boiled medium:

(a) Sodium metabisulphite (anhydrous) 1.2% solution.

(b) Ferric ammonium citrate 1%.

Sterilise both solutions by filtration immediately before use. Add 0.5 ml for each solution to 8 ml in tubes or 5 ml of each solution to 80 ml in flasks.

Reference

Beerens, H., Romond, C.L., Lepage, C. and Criquelion, J. (1982) *Isolation and Identification Methods for Food Poisoning Organisms.* Corry, J.E.L., Roberts, D. and Skinner, F.A. (Eds). SAB Technical Series number 17. Academic Press.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Rapid Perfringens Medium (RPM)

Solution A

Litmus milk (CM45, prepared as directed)	1000 ml
Neomycin sulphate	150 mg
Polymyxin B sulphate	25 mg

Solution B

Thioglycollate medium USP (CM173)	1000 ml
Gelatin (L8)	120 grams
Peptone (L34)	10
Dextrose (L71)	10
Dipotassium hydrogen phosphate	10
Yeast extract (L21)	6
Sodium chloride (L5)	3
Ferrous sulphate	1

Boil gently to dissolve the gelatin. Dispense 5 ml volumes into tubes and autoclave (conditions not stated).

Aseptically add 5 ml of solution A to each 5 ml tube of solution B. Cap tightly and store at 2–8°C.

Reference

Erickson, J.E. and Deibel, R.H. (1978) *Appl. Environ. Microbiol.* **36,** 567–571.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Tryptone-Peptone-Yeast Extract-Glucose Medium (TPYG)

	grams/litre
Tryptone (L42)	2
Peptone (L34)	5
Yeast extract (L21)	2
Glucose	4
Water	1000 ml
Cycloserine	800 mg/litre

These amounts are for double-strength medium.

Reference

Smart, J.L., Roberts, T.A., Stringer, M.F. and Shah, N. (1979) *J. Appl. Bact.* **46,** 383.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Fluid Thioglycollate Medium Without Dextrose

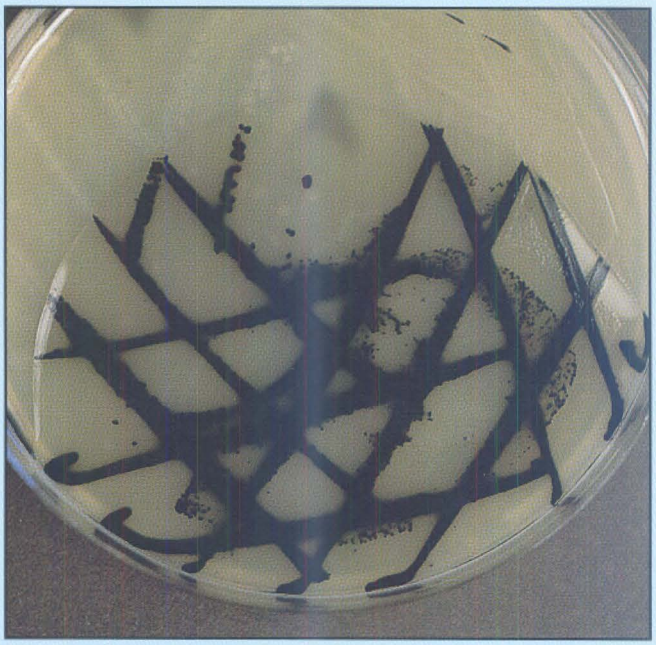
	grams/litre
Tryptone (L42)	15.0
Yeast extract (L21)	5.0
Sodium chloride (L5)	2.5
L-cysteine	0.5
Sodium thioglycollate	0.5
Agar (L11)	0.75
Resazurin	1.0 mg
Water	1000 ml

pH 7.1 ± 0.2

Reference

Debevre, J.M. (1979) *Euro. J. Appl. Microbiol. Biotechnol.* **6,** 409–414.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.



Effect of an agar overlay on sulphite reduction by *Clostridium perfringens.*

Colonies arising from surface-plated inocula on sulphite-containing media may not blacken or may blacken poorly unless covered by an overlay of agar.

This illustration of growth on SFP Agar which has been partially covered clearly shows the transition from black to white colonies at the boundary of the overlay.

The Evolution of Agar Media for Detection of *Clostridium perfringens* in Food

Culture media that have been developed to detect *Clostridium perfringens* in foods have almost all used the ability of the organism to reduce sulphite and produce lecithinase (phospholipase c) so that the appearance of black colonies surrounded by opacity can be used for presumptive identification. These characteristics are shared by other foodborne organisms and the necessity to use selective processes to separate clostridia from other sulphite reducers quickly became obvious if the two reactions were to be of real use. When it became apparent that *Clostridium perfringens* causes food poisoning, specialised media that would favour this species over other sulphite-reducing clostridia were necessary.

This review examines the process of gradual improvements in media that has led to the formulations currently used in National and International Standard Methods.

Neuberg and Nord[1] demonstrated that *B. welchii*, (as the organism was then known) is able to reduce sodium sulphite but would appear not to have applied their discovery to any practical purpose. Wilson and Blair[2] used sulphite reduction in their work aimed at detecting *Salmonella species* in water and subsequently showed that some anaerobic spore-forming bacilli produced black colonies on a nutrient-glucose agar containing sodium sulphite and ferric chloride.[3] The medium was used in petri dishes and the inoculum of water was overlaid with a further layer of medium. Ratios of vegetative cells to spores were determined by testing unheated and heated water samples, but, in practice no vegetative cells were found. The same technique was applied to milk samples but the inconsistent results prevented the establishment of satisfactory bacterial standards. Further work by the same authors[4] confirmed the usefulness of sulphite reduction in detection of *Clostridium perfringens* because the colonies had a characteristic appearance. Colonies with the appearance of *Clostridium perfringens* were picked off and inoculated into milk to demonstrate the disruptive "coagulated-stormy clot" fermentation which developed on incubation.

The detection of lecithinase activity towards lecithovitellin present in serum and egg yolk contained in culture media, as an aid to recognition of *Clostridium perfringens*, arose from Nagler's work with toxins.[5] Nagler observed that an opalescence developed as a result of growth in a liquid medium containing serum. Subsequently, other workers showed that egg yolk gives a stronger reaction than serum. Hayward,[6] in a study of the use of the Nagler reaction for rapid identification of *Clostridium perfringens*, noted that cultures on agar containing human serum produced a well-defined opacity extending from the edge of the colony. Later, Nagler[7] further developed the reaction named after him using solid medium and McClung and Toabe[8] used the egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and other gas gangrene-producing clostridia, including *Clostridium perfringens*. Their work confirmed the observations of others that the egg yolk reaction is not specific to *Clostridium perfringens* as was originally thought. Even so, the authors concluded that the egg yolk reaction in agar plates was of considerable value in presumptive identification.

At this time the stimulus for formulating media for *Clostridium perfringens* arose primarily because of the importance of the organism as a cause of gas gangrene. Recognition of the role of *Clostridium perfringens* in food poisoning brought with it the need for media and methodology more suited to detection in faeces and foods. Mossel and colleagues[9] modified Wilson and Blair's sulphite-iron medium by omitting glucose after observing that this sugar stimulated "blowing" and acidification of the medium and was found not to be essential for the growth of clostridia. It was also found necessary to reduce the sulphite content following the realisation that some clostridia were inhibited by the original amount. The means, then current, of selecting clostridia by pasteurising samples to destroy non-sporulating organisms was unsatisfactory because a high proportion of the clostridial cells occurred in the vegetative state and consequently did not survive the heating process. Attempts to make the medium selective using sodium azide were only partially successful because, although many interfering organisms, including proteolytic clostridia, were inhibited, the strains of *Clostridium perfringens* which were particularly associated with food poisoning were also affected. An attempt to replace azide by sorbic acid failed completely. Even so, the medium was satisfactorily used in Miller-Prickett tubes.

Mossel[10] further advanced the development of a satisfactory selective medium when, three years later, he described the addition of polymyxin B to the basal medium used previously. Miller-Prickett tubes were still used to hold the medium and for further studies isolates were heated at 80°C and subcultured on poured plates of sulphite-polymyxin agar. Polymyxin B had been chosen after investigation of a number of antibiotics and lithium chloride.

Following the demonstration that polymyxin B could be used to make a successful selective medium, alternative antibiotics were investigated by other workers in a search for improved sensitivity and selectivity. It was also desirable that any new medium could be used with conventional plate-counting procedures because of the inconvenience associated with the use of Miller-Prickett tubes.

Angelotti and his co-workers[11] sought to improve Mossel's polymyxin-sulphite-iron agar to make it suitable for quantitative recovery from foods and to restrict the formation of black colonies to *Clostridium species*. To achieve the latter it was necessary to eliminate the growth of Proteus and Salmonella. A variety of non-sulphite-reducing organisms including staphylococci and enterococci also grew on Mossel's medium and, clearly, improvements in performance depended on inhibiting these. Sulphadiazine was already known to suppress growth of *Proteus spp.*, coliforms and *Pseudomonas spp.* on media used for counting salmonella. It was reasoned that, because many of the sulphite-reducing organisms found normally in foods are members of the enterobacteriaceae, the addition of sulphadiazine to sulphite-polymyxin agar to make SPS Agar appeared appropriate. This was shown to be so and although other species of clostridia apart from *Clostridium perfringens* were capable of growing on the medium, the formation of black colonies was restricted to members of the genus. In order to differentiate *Clostridium perfringens* from other species of Clostridium the authors devised a number of tests to demonstrate motility, nitrate reduction and spore production.

Marshall, Steenbergen and McClung[12] noted that Lowbury and Lilly[13] had found neomycin to be very effective in the selective isolation of *Clostridium perfringens* from burns cases and used it in the formulation of a new medium, TSN Agar, which was a modification of SPS Agar. As part of the overall selective activity, the medium was incubated at 46°C. Their particular concern was to inhibit *Clostridium bifermentans* which was able to grow on SPS Agar making additional testing necessary to differentiate it from *Clostridium perfringens*.

Spencer[14] found that some food poisoning strains were relatively susceptible to neomycin and warned against using the concentrations present in some existing media.

Further doubts about the reliability of SPS Agar arose when Shahidi and Ferguson[15] observed that commercially-manufactured SPS media varied considerably in performance. *Clostridium perfringens* was sometimes inhibited and often the organisms failed to produce black colonies, apparently as a result of unstable components in the medium. Additionally it was difficult to demonstrate consistent nitrate reduction and sporulation of *Clostridium perfringens*, both key confirmatory tests used as part of the procedure with SPS Agar. Shahidi and Ferguson disregarded TSN Agar because of the lack of independent evidence of its effectiveness in the study of foodborne outbreaks and developed their own selective medium, SFP Agar. This contained stable ingredients and provided rapid quantitative and qualitative analysis of *Clostridium perfringens* in foods. At the same time they developed Lactose-Motility (LM) Agar as a single confirmatory test. SFP Agar used polymyxin and kanamycin for selectivity and incorporated egg yolk for presumptive identification. The method used an overlayer of the same medium, but without the egg yolk, following observation that without the addition of the overlayer the development of black colonies was unreliable. Incubation was at 35°C. The use of an overlay would probably have been unnecessary if they had used pour-plates instead of spread-plates but, in practice, it was found that after 24 hours incubation the egg yolk reaction was more intense on spread plates. Other egg yolk-positive and hydrogen sulphide-producing clostridia that grew on the medium could readily be differentiated by the use of LM Agar.

A comparison of SPS, TSN and SFP agars[16] showed all three to have limitations in their selectivity and sensitivity. Later that year the same authors described Tryptose-Sulphite-Cycloserine Agar[17] which consisted of SFP agar basal medium with added egg yolk. Because of the observation by Füzi and Csukás[18] that blood agar containing cycloserine is very selective for *Clostridium perfringens*, the polymyxin and kanamycin used in SFP Agar were replaced in TSC by cycloserine. TSC Agar allowed virtually complete recovery of *Clostridium perfringens* strains but inhibited nearly all the facultative anaerobes tested. Subsequently it was recognised that the use of egg yolk in TSC Agar was accompanied by serious disadvantages[19] and TSC Agar is now generally used without egg yolk. Hauschild et al reported protection of *Cl. celatum* from the inhibitory activity of cycloserine by the lysozyme present in egg yolk.[20]

The superiority of SFP and TSC agars (containing egg yolk) over earlier media was recognised by Handford.[21] However, the ability of other species of clostridia apart from *Clostridium perfringens*, particularly *Clostridium bifermentans*, which also is egg yolk-positive, to spread and completely blacken the medium made colony counting difficult. Handford developed OPSP Agar containing polymyxin, oleandomycin, (an antibiotic not previously used in media for *Clostridium perfringens*) and the sulphonamide sulphadiazine. Although the medium successfully inhibited *Clostridium bifermentans* it was subsequently found to be more inhibitory for *Clostridium perfringens* than TSC Agar used without egg yolk. More recently, Hood and co-workers[22] have formulated Blood-*Clostridium Perfringens* agar (BCP) with the particular intention of devising a medium which resuscitates damaged cells. The medium contains pyruvate to neutralise toxic oxygen derivatives. It also contains inositol to enable *Clostridium perfringens* to be identified presumptively. Inositol is used in place of lactose because the authors, like Handford, found that lactose had an adverse effect on the development of the egg yolk reaction. Inositol has no such effect and its fermentation by *Clostridium spp.* is as similarly restricted as that of lactose. Unusually, selective activity of the medium is optional and, where necessary to assist in enumeration of *Clostridium perfringens* in the presence of a large amount of accompanying flora, neomycin or cycloserine may be added. The medium is incubated initially at 37°C to assist repair of injured organisms and the temperature then raised to 43–45°C which contributes to selectivity, and enhances the egg yolk reaction and inositol fermentation.

The medium may also be used for detection and enumeration of *Bacillus cereus*, but despite its apparent advantages would appear not yet to have been widely accepted.

The formulae of the more important media not in the Oxoid product range are given on page 13. OPSP, SFP and TSC agars are described on pages 14–16. All three media are available from Unipath.

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Table 3 – Antibiotics contained in culture media for selective isolation of *Clostridium perfringens* and presumptive identification systems employed

Amounts in µg/ml except where stated as International Units							
Medium	**Polymyxin B sulphate**	**Sulphadiazine**	**Neomycin**	**Cycloserine**	**Oleandomycin**	**Kanamycin**	**Identification System**
Sulphite-Iron Polymyxin Agar	10						Sulphite reduction
Sulphite-Polymyxin Sulphadiazine (SPS) Agar	10	120					Sulphite reduction
Tryptone-Sulphite Neomycin (TSN) Agar	20		50				Sulphite reduction
Shahidi-Ferguson Perfringens (SFP) Agar	30 i.u.					12	Lecithinase Sulphite reduction
Tryptose-Sulphite-Cycloserine (TSC) Agar				400			Lecithinase (optional) Sulphite reduction
Oleandomycin-Polymyxin-Sulphadiazine-Perfringens (OPSP) Agar	10 i.u.	100			0.5		Lecithinase Sulphite reduction
Blood-Free Pyruvate-Perfringens (BCP) Agar			50 (optional) OR	400 (optional)			Lecithinase Inositol fermentation

Blood-Free-Pyruvate Clostridium Perfringens (BCP) Agar

	grams/litre
Blood Agar Base No. 2 (Oxoid CM271)	40
Inositol	10
Mannitol	10
Sodium pyruvate	1
1% bromocresol purple in ethyl alcohol	4 ml
Egg yolk emulsion (50% in 0.9% sodium chloride)	100 ml
Neomycin	50 mg
Or Cycloserine	400 mg
Water	1000 ml

Reference

Hood, A.M., Tuck, A. and Dane, C.R. (1990) *J. Appl. Bact.* **69,** 359–372.

Sulphite-Iron-Polymyxin Agar

	grams/litre
Tryptone (L43)	15
Yeast extract (L21)	10
Ferric citrate	0.5
Sodium sulphite (Na_2SO_3–$7H_2O$)	0.5
Agar (L11)	15
Polymyxin B sulphate	10 mg
Water	1000 ml

Reference

Mossel, D.A.A. (1959) *J. Sci. Food Agric.* 10. December 1959, 662–669.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Sulphite-Polymyxin-Sulphadiazine (SPS) Agar

	grams/litre
Tryptone (L43)	15
Yeast extract (L21)	10
Ferric citrate	0.5
Sodium sulphite (Na_2SO_3–$7H_2O$)	0.5
Polymyxin B sulphate	10 mg
Sodium sulphadiazine	120 mg
Agar (L11)	15
Water	1000 ml

Reference

Angelotti, R., Hall, H.E., Foter, M.J. and Lewis, K.H. (1962) *Appl. Microbiol.* **10,** 193–199.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Tryptone-Sulphite-Neomycin (TSN) Agar

	grams/litre
Tryptone (L43)	15
Yeast extract (L21)	10
Ferric citrate	0.5
Sodium sulphite	0.4
Polymyxin B sulphate	20 mg
Neomycin sulphate	50 mg
Agar (L11)	15
Water	1000 ml

Reference

Marshall, R.S., Steenbergen, J.F. and McClung, L.S. (1965) *Appl. Microbiol.* **13,** 559–563.

The code numbers of Oxoid products that can be used in making the medium are given in parentheses.

Perfringens Agars (TSC and SFP)

Perfringens Agar (TSC and SFP)
Code: CM587

A basal medium for use with selective agents to make either TSC agar or SFP agar for the presumptive identification and enumeration of Clostridium perfringens.

Formula

	grams/litre
Tryptose	15.0
Soya peptone	5.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
Sodium metabisulphite	1.0
Ferric ammonium citrate	1.0
Agar	14.0

Final pH 7.6 ± 0.2

Perfringens (SFP) Selective Supplement
Code: SR93

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

Kanamycin sulphate	6 mg
Polymyxin B	15000 IU

Perfringens (TSC) Selective Supplement B
Code: SR88

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

D-cycloserine	200 mg

Directions

To Prepare the Agar Base
Suspend 23 g in 500 ml of distilled water and heat gently until the agar is completely dissolved. Sterilise by autoclaving at 121°C for 10 minutes. Allow the medium to cool to 50°C.

To Prepare Tryptose Sulphite Cycloserine Agar (TSC Agar)
To 500 ml of agar base cooled to 50°C add the rehydrated contents of 1 vial of TSC supplement, SR88 and 25 ml of egg yolk emulsion, SR47. Mix well and pour into sterile petri dishes.

To Prepare Egg Yolk Free TSC Agar
To 500 ml of agar base cooled to 50°C add the rehydrated contents of 1 vial of TSC supplement SR88. Mix well and pour into sterile petri dishes.

To Prepare Shahidi-Ferguson Perfringens Agar (SFP Agar)
To 500 ml of agar base cooled to 50°C add the rehydrated contents of 1 vial of SFP supplement, SR93 and 25 ml of egg yolk emulsion, SR47, mix well and pour into sterile petri dishes.

To Prepare Agar for an Overlay
For TSC or SFP Agar used as an overlay, the egg yolk emulsion, SR47, is omitted. Its inclusion does not improve the lecithinase reaction and diminishes the visibility of the colonies.

Description
Perfringens Agar Base (TSC and SFP) CM587 is a nutrient medium to which is added egg yolk emulsion SR47 and the appropriate antibiotic supplement to prepare either Shahidi-Ferguson Perfringens (SFP) Agar using SR93 to Tryptose Sulphite Cycloserine (TSC) Agar using SR88.

An egg yolk free TSC agar has been described[4,5] which has the advantage that smaller colonies are formed. This can simplify the counting of plates with high numbers of colonies. Higher counts have been demonstrated by using it with a pour plate technique. The differences were thought to be due to exposure of the *Cl. perfringens* cells to high oxygen tension in the surface plating procedure.[4]

Shahidi-Ferguson Perfringens Agar is based on the formulation developed by Shahidi and Ferguson.[1] The medium utilises kanamycin sulphate (12 mg/litre) and polymyxin B sulphate (30,000 U/litre) as the selective agents to give a high degree of selectivity and specificity for *Cl. perfringens*.

Tryptose Sulphite Cycloserine Agar was developed using the same basal medium as SFP Agar[2] but with 400 mg/litre of D-cycloserine as the selective agent.

Sodium metabisulphite and ferric ammonium citrate are used as an indicator of sulphite reduction by *Cl. perfringens* which produces black colonies in both media.

Trials[3] have indicated that polymyxin B and kanamycin sulphate used in SFP Agar allow a greater recovery of both vegetative cells and spores of *Cl. perfringens* than either polymyxin B and sulphadiazine used in Sulphite Polymyxin Sulphadiazine Agar, or neomycin, used in Tryptone Sulphite Neomycin Agar. However, a greater number of non-specific colonies were found on SFP Agar.

In another study[2], *Serratia marcescens* and *Streptococcus lactis* were the only faculative anaerobes to grow on TSC Agar, whereas SFP Agar also allowed the growth of *Enterococcus*, *Proteus* and *Enterobacter* strains, but allowed a slightly higher rate of recovery of *Cl. perfringens* than TSC Agar.

Both SFP Agar and TSC Agar permitted growth of other sulphite-reducing *Clostridium* species tested, with the exception of *Cl. sordellii* which was completely inhibited and *Cl. bifermentans* which was partially inhibited on TSC Agar. Both strains grew on SFP Agar.

Some strains of *Cl. perfringens* may produce an opaque zone around the colony due to lecithinase activity, but this is not considered to be universal for all *Cl. perfringens* strains after overnight incubation[4] and both black lecithinase-positive and black lecithinase-negative colonies should be considered as presumptive *Cl. perfringens* on TSC or SFP Agars and confirmatory tests carried out. Egg yolk positive facultative anaerobes may grow on SFP agar to produce completely opaque plates thus masking the egg yolk reaction of *Cl. perfringens*.

Technique

1 Make up the medium according to the directions and prepare plates containing approximately 20 ml of a basal layer of TSC or SFP Agar containing egg yolk.

2 Prepare 0.1 ml aliquots of a suitable series of dilutions of the homogenised test sample and spread over the surface of the basal layer using a sterile swab.

3 Overlay with an additional 10 ml of egg yolk free TSC or SFP Agar.

4 Incubate the plates at 35°C for 18-24 hours with an anaerobic Gas Generating Kit, BR38, in a gas-jar. Alternatively use Anaerogen AN025A or AN035A. Anaerogen does not require the addition of water or a catalyst.

Alternatively, pour-plates using approximately 25 ml per plate of TSC or SFP Agar containing egg yolk may be prepared using 1 ml aliquots of a suitable series of dilutions of the homogenised test sample. Mix the plates well before the agar gels. With this technique, lecithinase activity of *Cl. perfringens* colonies is more difficult to see.

Cl. perfringens colonies may be seen as large, black (2–4 mm diameter) colonies within the depth of the agar.

Egg yolk free TSC agar is used with the techniques described above. *Cl. perfringens* colonies are black but in the absence of egg yolk no lecithinase activity can be detected.

Tests for confirmation are described in a study initiated by the International Commission on Microbiological Specifications for Foods[6] involving nitrate reduction, lactose fermentation, gelatin liquefaction and the absence of motility. All black colonies growing on TSC or SFP agars should be tested.

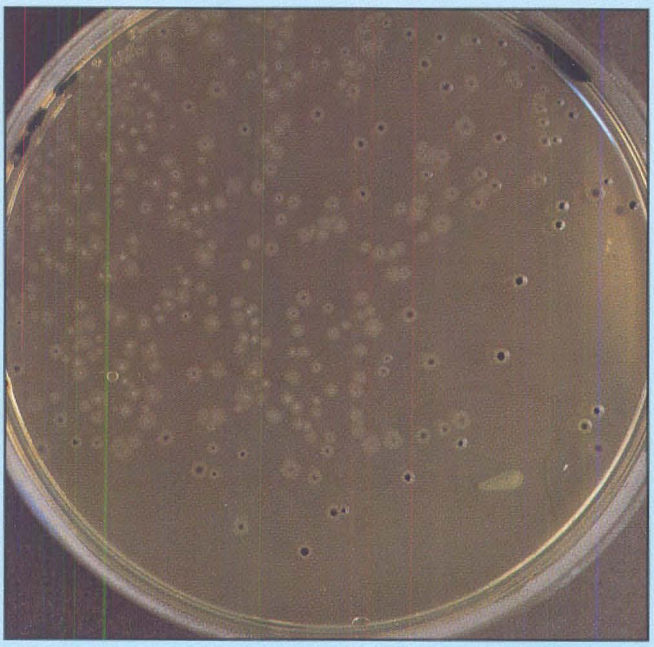
Storage Conditions and Shelf Life
Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2-8°C.

Quality Control
Positive control:
Clostridium perfringens ATCC® 13124

Negative control:
Clostridium sordellii ATCC® 9714

Precautions
Black colonies appearing on these two media may be organisms other than *Cl. perfringens*.



Appearance of *Cl. perfringens* on Perfringens Agar (TSC) without egg yolk.

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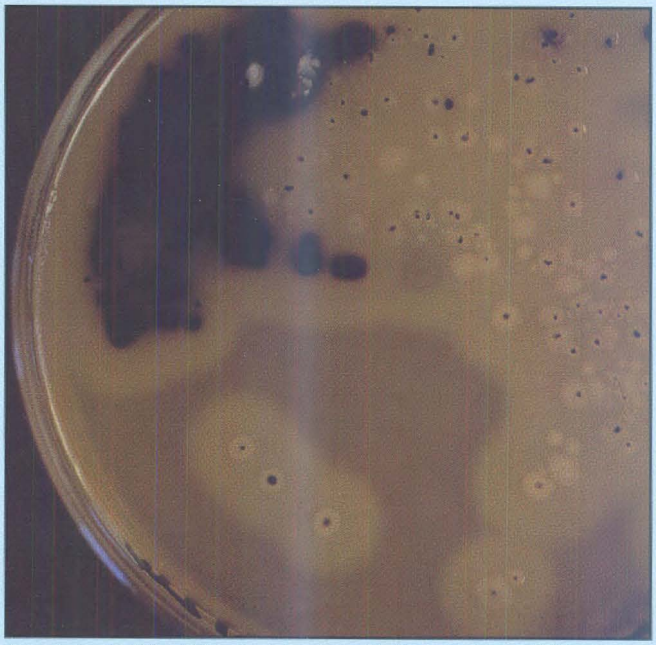
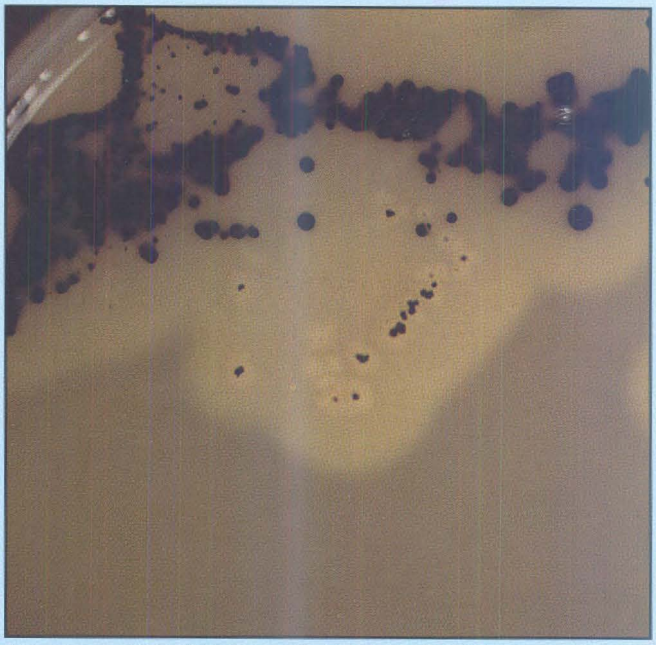


Appearance of *Clostridium perfringens* on Perfringens Agar (TSC) containing egg yolk.



Appearance of *Cl. perfringens* on Perfringens Agar (SFP).

Perfringens Agar (OPSP)

Perfringens Agar (OPSP)
Code: CM543

For the enumeration of *Cl. perfringens* in foods.

Formula

	grams/litre
Tryptone	15.0
Yeast extract	5.0
Soya peptone	5.0
Liver extract	7.0
Ferric ammonium citrate	1.0
Sodium metabisulphite	1.0
Tris buffer	1.5
Agar	10.0
pH 7.3 ± 0.2	

Perfringens (OPSP) Selective Supplement A
Code: SR76

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

Sodium sulphadiazine	50 mg

Perfringens (OPSP) Selective Supplement B
Code: SR77

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

Oleandomycin phosphate	0.25 mg
Polymyxin B	5000 IU

Directions
Suspend 22.8 g in 500 ml of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Allow to cool to 50°C and aseptically add the contents of one vial each of Perfringens Agar (OPSP) supplements A and B SR76 and SR77 which have been rehydrated by the addition of 2 ml of sterile distilled water. Mix well and pour into sterile dishes.

Description
Oxoid Perfringens Agar (OPSP) CM543, is based on the formulation developed by Handford.[1]

The medium utilises sulphadiazine (100 µg/ml), oleandomycin phosphate (0.5 µg/ml) and polymyxin B sulphate (10 U/ml), presented as freeze-dried supplements SR76 and SR77 to give a high degree of selectivity and specificity for *Clostridium perfringens*.

Sodium metabisulphite and ammonium ferric citrate are used as an indicator of sulphite reduction by *Cl. perfringens* which produces black colonies on this medium when using a pour plate technique.

Tests for confirmation of *Cl. perfringens* are described in a study initiated by the International Commission on Microbiological Specifications for Foods (I.C.M.S.F.).[2]

Sulphite-reducing bacteria other than *Cl. perfringens* such as salmonellae, *Proteus* species and *Citrobacter freundii*, as well as staphylococci and *Bacillus* spp, are inhibited on OPSP Agar.

Perfringens Agar (OPSP) has the advantage of inhibiting growth of *Cl. bifermentans* and *Cl. butyricum*. These sulphite reducing organisms grow readily on Shahidi-Ferguson Perfringens Agar (SFP)[3] and Tryptone-Sulphite-Neomycin Agar (TSN)[4] as black colonies with a tendency to spread and obscure the whole surface of the medium.

Occasional strains of enterococci will grow on Perfringens Agar (OPSP) as white colonies, easily distinguished from the large black colonies of *Cl. perfringens*.

Cl. perfringens enumeration media which include egg yolk in order to detect lecithinase activity have not proved satisfactory partly because *Cl. perfringens* colonies may frequently fail to produce haloes and thus appear falsely to be negative, and partly because counting is rendered impractical as the organism often grows in the form of large spreading colonies which completely blacken the medium.[5]

Technique
Make up the medium according to the directions. Prepare pour plates, containing approximately 25 ml per plate, using 1 ml aliquots of a suitable series of dilutions of the homogenised test sample. Mix well before setting.

Incubate the plates at 35°C for 18–24 hours with a H_2/CO_2 Gas Generating Kit pack BR38 in a conventional gas-jar. Alternatively use Anaerogen AN025A or AN035A. Anaerogen does not require the addition of water or a catalyst.

Cl. perfringens may be seen as large black colonies (2–4 mm diameter) within the depth of the agar.

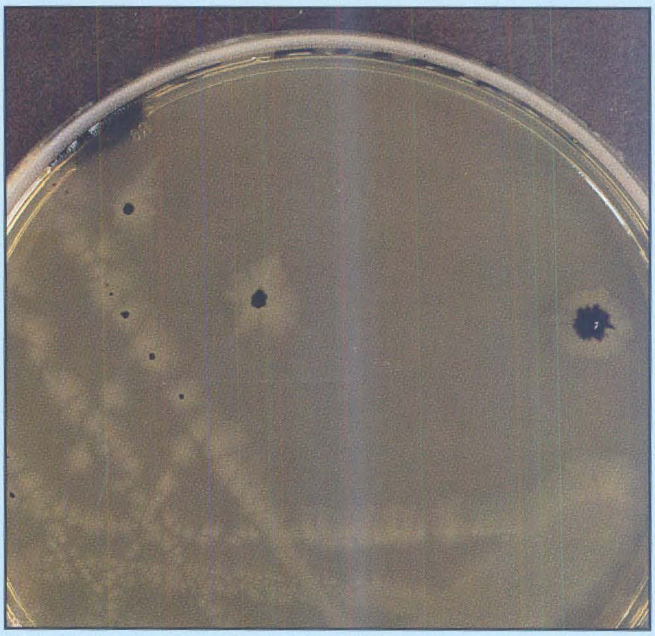
Occasional strains of *Enterococcus faecalis* which may grow on Perfringens Agar (OPSP) as small colourless colonies are easily distinguished from *Cl. perfringens*.

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:
Clostridium perfringens ATCC® 13124

Negative control:
Clostridium bifermentans ATCC® 638



Appearance of *Cl. perfringens* on Perfringens Agar (OPSP).

Precautions

The production of black colonies on this medium is a presumptive identification of *Cl. perfringens*. Further identification tests must be carried out.

References

1 Handford, P.M. (1974) *J. Appl. Bact.* **37,** 559–570.

2 Hauschild, A.H.W., Gilbert, R.J., Harmon, S.M., O'Keefe, M.F. and Vahlefeld, R. (1977) ICMSF Methods Studies VIII, *Can. J. Microbiol.* **23,** 884–892.

3 Shahidi, S.A. and Ferguson, A.R. (1971) *Appl. Microbiol.* **21,** 500–506.

4 Marshall, R.S., Steenbergen, J.F. and McClung, L.S. (1965) *Appl. Microbiol.* **13,** 559–562.

5 Hauschild, A.H.W. and Hilsheimer, R. (1974) *Appl. Microbiol.* **27,** 78–82.

Identification of *Clostridium perfringens*

A detailed description of identification methods for *Clostridium perfringens* lies outside the scope of this Monograph. However, the following information may be useful in helping to distinguish *Clostridium perfringens* from other sulphite-reducing clostridia with which it is commonly associated.

Morphological and cultural characteristics of *Cl. perfringens* greatly assist in the initial recognition of the organism on selective differential media. Production of black colonies surrounded by haloes of lecithinase activity in a medium containing egg yolk indicates organisms that should be examined further.

Microscopically, *Cl. perfringens* appears as Gram-positive, straight, square or blunt-ended rods that occur singly or in pairs. Generally spores are absent.

Initial confirmation that an isolate is an anaerobe can be shown by demonstrating susceptibility to metronidazole using Oxoid diagnostic discs code DD8 which are impregnated with 15 mcg of metronidazole. The susceptibility test plates must be incubated under anaerobic conditions at a temperature of 30°C for up to 2 days. Inhibition zones measuring 4 mm or greater around the discs confirm the organism as anaerobic. However, some *Bacillus licheniformis* strains may show zones of about 2.5 mm and doubtful colonies should be checked by subculturing to a non-selective nutrient medium e.g. Tryptone Soya Agar and incubated under anaerobic conditions. Anaerobic organisms will not grow in aerobic culture. Alternatively, growth of a suspected anaerobe can be subcultured on two plates of medium, incubating one anaerobically and the other in air.

A number of sulphite-reducing *Clostridium spp.* share with *Clostridium perfringens* the ability to produce lecithinase and show a positive egg yolk reaction. The Nagler test, which uses *Clostridium perfringens* type A antitoxin to neutralise lecithinase activity is useful, but positive results are not confined entirely to *Clostridium perfringens*. A greater level of differentiation is obtained by combining the Nagler test with a test for lactose fermentation as in the medium of Willis and Hobbs.[1] The possibility that a strain of *Clostridium perfringens* may not produce lecithinase must always be considered.

The reversed CAMP test is an alternative to the Nagler test and is generally more satisfactory because it is specific for *Clostridium perfringens*. Details of the test and the appearance of a positive result are shown on page 19. Although the test is very reliable it is not applicable to non-haemolytic strains.

In a comparative study of Standard Methods,[2] the reversed CAMP test correctly identified 94.2% of all investigated colonies as *Clostridium perfringens*. The combination of tests for motility, lactose fermentation, gelatin liquefaction and nitrate reduction correctly identified 89.2% of colonies and LS medium only 43.1%.

Detection of acid phosphatase[3] was as efficient as the reversed CAMP test. The authors conclude that a combination of the reversed CAMP test and detection of acid phosphatase would be the most suitable combination of tests for the identification of *Cl. perfringens*.

The production of the characteristic "stormy clot" in litmus milk culture is strongly indicative of *Clostridium perfringens*. Lactose fermentation produces acid which causes a coagulum to form from the milk in the medium and gas production disrupts the coagulum.

Minimum confirmatory testing requires the inoculation of two media, one of which combines tests for lactose fermentation and gelatin liquefaction and the other, motility and reduction of nitrate to nitrite.

LS medium which detects both sulphite reduction and lactose fermentation is proposed in standard methodology as an alternative to lactose/gelatin and motility/nitrate media. The tests have greater specificity for *Clostridium perfringens* when conducted at 46°C.

Clostridium perfringens may also be identified immunologically using immunofluorescence microscopy.

The tests and results which serve to differentiate *Clostridium perfringens* from other sulphite-reducing clostridia often found in association with it are shown in table 5.

A membrane filtration immunostaining technique for detection and direct enumeration of enterotoxigenic *Cl. perfringens* has been developed for examination of pre-cooked beef. The procedure combines growth of microcolonies on membranes with an indirect alkaline phosphatase-conjugated antibody system that permits microcolonies of enterotoxigenic strains to be detected by their colour.[4]

References

1 Willis, A.T. and Hobbs, G. (1959) *J. Pathol. and Bacteriol.* **77,** 511.

2 Schalch, Von Barbara., Eisgruber, H., Pia Geppert and Stolle, A. (1996) *Archiv. für Lebensmittel Hygiene.* **47,** 1–32.

3 Ueno, K., Fujii, H., Marui, T., Takahshi, J., Sugitani, T., Ushijima, T. and Suzuki, S. (1970) *Japan J. Microbiol.* **14,** 171–173.

4 Baez, L.A. and Juneja, V.K. (1995) *J. Rapid Methods and Automation in Microbiology* **3,** 165–175.

Litmus milk medium showing "stormy clot" fermentation (left).

Lactose-gelatin medium indicating lactose fermentation (left).

Nitrate-motility medium
(a) Positive tests for nitrate reduction shown by the red reaction at the surface of the medium (centre and right).
(b) Non-motile organism shown by the sharp line of the stab inoculum.

The Nagler Test

The Nagler test demonstrates inhibition of lecithinase by antitoxin prepared against *Cl. perfringens* alpha toxin.

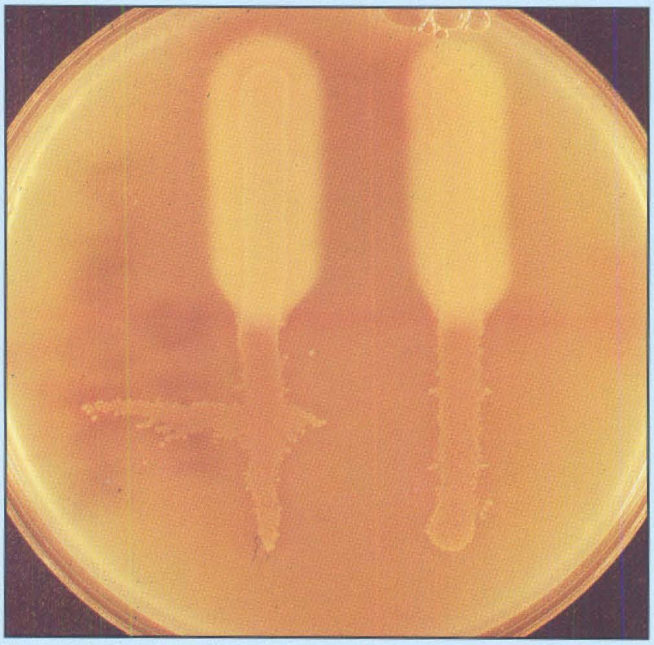
The test is not specific for *Cl. perfringens*. *C. bifermentans*, *C. sordellii* and *C. barati* will also give a positive reaction.

Technique
Before inoculating an egg yolk agar plate, swab one half of the plate with *Cl. perfringens* type A antitoxin and allow it to dry.

Streak the inoculum across both halves of the plate, starting on the half without antitoxin. Incubate 24–48 hours anaerobically.

Inhibition of lecithinase production on the half of the plate treated with antitoxin indicates a positive Nagler test.

Egg yolk agar
500 ml of nutrient agar containing 25 ml of Egg Yolk Emulsion code SR47.



Nagler test. The egg yolk turbidity caused by lecithinase produced by *Cl. perfringens* is absent from the half of the plate on which *Cl. perfringens* type A antitoxin has been spread to neutralise the toxin.

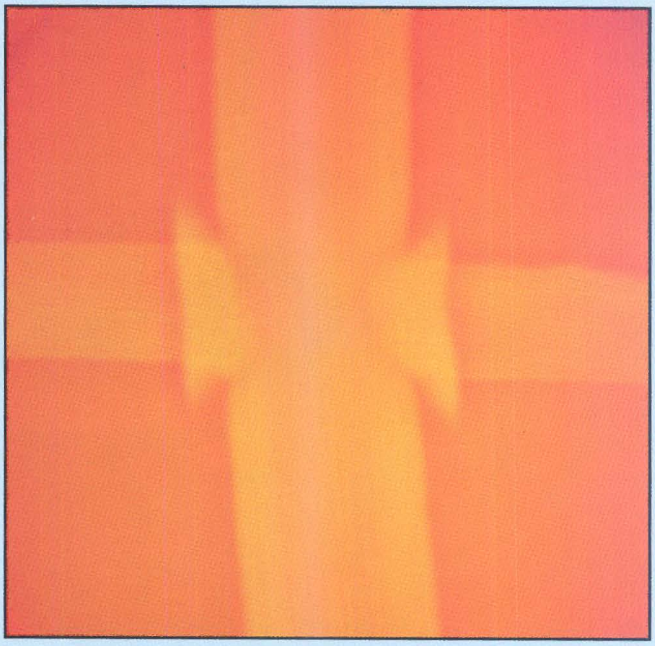
Photograph supplied by Mr M. W. Wren, University College Hospital, London.

THE REVERSED CAMP TEST

Technique
Inoculate a sheep blood agar plate made of Columbia Blood Agar Base code CM331 containing 5% sheep blood in Alsever's solution SR53, by streaking a pure culture of *Streptococcus agalactiae* in a single line across the plate. Streak the suspected *Clostridium perfringens* culture perpendicular to, but not touching, the line of *Strep. agalactiae*. (More than one test can be inoculated on each plate.)

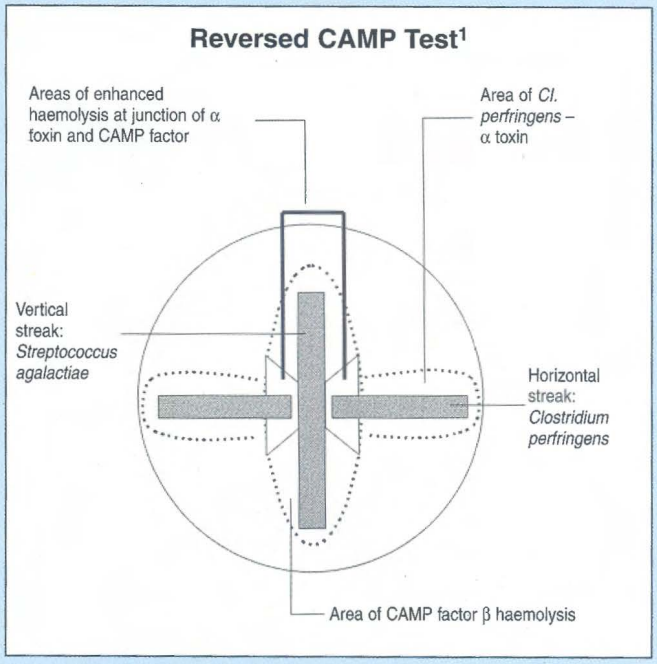
Incubate at 37°C for 24 hours under anaerobic conditions.

A positive result is indicated by arrow-shaped areas of synergistic enhanced haemolysis at the junction of the *Strep. agalactiae* and *Cl. perfringens* cultures.



A positive reversed-CAMP test showing typical arrow-headed areas of enhanced haemolysis.

An explanation of the appearance of the test plate is given in the diagram below. Photograph supplied by Mr M. W. Wren, University College Hospital, London. Diagram adapted from Vandepitte et al., Basic Laboratory Procedures in Clinical Bacteriology, WHO Geneva.



Reference
Hansen, M.V. and Elliot, L.P. (1980) *J. Clin. Microbiol.* **12,** 617–619.

Table 4 – Confirming characteristics of presumptive-positive *Cl. perfringens*

Motility	Negative
Nitrate reduction	Positive
Gelatin liquefaction	Positive within 44 hours
Lactose fermentation	Positive
Raffinose fermentation	Positive
Salicin fermentation	Negative

Table 5 – Differentiation of *Cl. perfringens* from closely similar species

	Lecithinase	Nagler test: inhibition by antitoxin	Motility	Gelatin liquefaction	Nitrate reduced to Nitrite	Fermentation of:			Indole	Urease
						Lactose	Raffinose	Salicin		
Cl. perfringens	+	+	–	+	+	+	+	–	–	–
Cl. barati (Cl. para-perfringens)	+	+	–	–	+	+	–	+	–	–
Cl. bifermentans	+	+	+	+	–	–	–	–	–	–
Cl. sordellii	+	+	+	+	–	–	–	–	+	±
Cl. absonum	+	–	– or weak+	+ >44 hours	+	+	–	+	*	*
Cl. celatum	–	–	–	–	+	+	–	+	*	*
Cl. perenne	±	–	–	–	+	+	–	±	*	*
Cl. sardiniensis	+	–	+ weak	+ >44 hours	+	+	–	+	*	*

*No data available

Table 6 – Some regulatory bodies that specify detection procedures for *Bacillus cereus* and the culture media to be used. The codes for Oxoid dehydrated culture media available from Unipath are in parentheses

Regulatory Body	Detection Media	Other Media
Spanish Ministeria de Sanidad y Consumo Instituto de Salidad Carlos III. Technical Manual	Mannitol-egg yolk agar (MYA) Mannitol-egg yolk polymyxin agar (MYP)	Anaerobic agar without glucose and indicator Glucose broth without phosphate Glucose agar Nutrient agar
German Institute of Normalisation (DIN 10198) adopted by the Official Collection of Investigative Procedures 00.00-25 (1992)	Polymyxin-pyruvate-egg yolk-mannitol bromothymol blue agar (PEMBA) (CM617 + SR99) Mannitol-egg yolk polymyxin agar (MYP)	Glucose medium Voges-Proskauer medium Nitrate medium
Standards Australia AS 1766.2.6-1991 Food Microbiology Method 2.6	Polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) (CM617 + SR99) Tryptone-soy-polymyxin broth (MPN technique)	
AOAC/FDA BAM (1992)	Mannitol-egg yolk-polymyxin agar (MYP) Tryptone-soy-polymyxin	Phenol red-glucose broth Nitrate broth Modified VP medium Tyrosine agar Lysozyme broth
Agriculture Canada (1988) Methods Manual	Mannitol-egg yolk-polymyxin agar (MYP)	Tryptone soya agar (CM131) Sheep blood agar Modified VP medium Nitrate broth
BS 5763: Part II (1988) ISO 7932 (1987) BS 4285: Part 3: Section 3.12 1989 (1994)	Mannitol-egg yolk-polymyxin agar (MYP)	Glucose agar Voges-Proskauer (VP) medium Nitrate medium
Normalisation Française (AFNOR) XPV 08-058 (1995)	Mannitol-egg yolk-polymyxin agar (MYP)	Blood agar (CM271 + defibrinated horse blood or sheep blood) Motility agar Voges-Proskauer medium Nitrate broth

A Typical Procedure for Detection of *Bacillus cereus*

(a) **Vegetative cells**

Make an initial 1/10 dilution of the sample in an appropriate diluent (e.g. phosphate buffer saline, Maximum Recovery Diluent, peptone water)

↓

Decimal dilute to 10^{-6}

↓

Inoculate duplicate plates of *Bacillus cereus* Selective Medium (PEMBA) with 0.1 ml of 10^{-3} to 10^{-6} dilutions

↓

Spread the inoculum over the entire surface

↓

Incubate aerobically for 24–28 hours at 35–37°C

↓

Examine for peacock-blue colonies with blue egg yolk precipitate zone

↓

Confirm with the rapid staining procedure (see page 30)

↓

If necessary verify with biochemical tests

Note: Where test specifications require the use of a Standard Method the Standard should be consulted for the technique which may differ in detail from the above.

(b) **Spores**

Sometimes it may be necessary only to count spores. If so, the sample must first be treated with heat or alcohol to destroy vegetative cells.

(1) *Heat treatment*
Heat the initial 1/10 suspension of sample for 15 minutes at 70°C. Proceed as for detection of vegetative cells.

(2) *Alcohol treatment*
Dilute the initial suspension 1:1 in 95% ethyl alcohol. Leave for 30 minutes at room temperature. Proceed as for detection of vegetative cells, adjusting the dilutions to account for the 1:1 dilution of the sample suspension in alcohol.

The Use of Liquid Media in the Detection of *Bacillus cereus*

Enrichment

Because *Bacillus* species are ubiquitous and small numbers present in foods are not generally of significance, enrichment culture is not normally undertaken. However, there are circumstances in process control or sterility testing where low levels must be detected and enrichment is necessary. Robertson's Cooked Meat Medium may be used where overgrowth by accompanying organisms is not likely to be a problem.[1] Addition of polymyxin B at a concentration of 100 international units (iu) per millilitre to cooked-meat medium or other nutrient broth is likely to be successful where selective enrichment appears advisable.

Enumeration by Most Probable Number

A most probable number (MPN technique)[2,3] is available as an alternative to the direct plate count for examining foods that are expected to contain less than 10^3 *B. cereus* cells in a gram of food. Tubes of Tryptone Soya Broth containing polymyxin B sulphate (TSP) are inoculated with dilutions of the food homogenised in phosphate buffer. Presumed positive cultures showing the dense growth characteristic of *B. cereus* are plated on MYP agar for confirmation. PEMBA Agar may also be used.

References

1 Kramer, J.M. and Gilbert, R.J. (1989) In: *Foodborne Bacterial Pathogens*, page 38, Doyle, M.P. (Ed) Marcel Dekker Inc. New York.

2 Lancette, G.A. and Harmon, S.M. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 581–586.

3 Harmon, S.M., Goepfert, J.M. and Bennett, R.W. (1992) *Bacillus cereus*. In: *Compendium of Methods for the Microbiological Examination of Foods*. 3rd Edition. Vanderzart, C. and Splittstoesser, D.F. (Eds) APHA. Washington D.C.

Tryptone Soya Polymyxin Broth (TSP)

	grams/litre
Tryptone	17.0
Mycological peptone	5.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Dextrose	2.5
Polymyxin B sulphate	89,000 units
Water	1000 ml

pH 7.3 ± 0.1

Dispense 15 ml amounts into glass tubes. Sterilise by autoclaving at 121°C for 15 minutes.

Polymyxin B Supplement

Before use, add to each tube 0.1 ml of a solution of 500,000 units of Polymyxin B sulphate in 37.5 ml of water.

Reference

Lancette, G.A. and Harmon, S.M. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 581–586.

Broth culture of *Bacillus cereus* showing typical heavy growth which may form a pellicle at the medium surface or may sink to the bottom.

The Evolution of Agar Media for Detection of *Bacillus cereus* in Foods

Nearly all plating media for detection and enumeration of *B. cereus* possess major common features in their design. These are:

1 Demonstration of phospholipase-c activity.

2 Failure to produce acid from mannitol.

The two key characteristics together serve to distinguish the species from almost all others in the genus.

Phospholipase-c production is readily observed from the formation of zones of opacity around the bacterial colonies. This is due to the effect of the enzyme on lecithovitellin present in egg yolk contained in the medium.

Inability of *B. cereus* to produce acid from mannitol is seen by failure of the pH indicator in the medium to change colour.

Most media formulae incorporate polymyxin B to confer selectivity.

Prior to the formulation of mannitol-egg yolk-polymyxin (MYP) medium by Mossel and his co-workers in 1967[1] no specific medium for the enumeration of *B. cereus* in foods had been described. The examination of food ingredients for *B. cereus* had usually been carried out by subjecting dilutions to heat before plating out which was invariably successful in destroying non-sporulated cells and allowing unrestricted growth from spores which had survived the treatment. However, the spore population in foods may be very low and it was apparent that a selective differential medium was desirable for enumeration of both spores and vegetative *B. cereus* cells in the presence of much higher numbers of other bacteria.

In earlier developments, McClung and Toabe[2] described a non-selective medium to demonstrate the Nagler reaction for presumptive identification of *Clostridium perfringens*. As this test is dependent on demonstrating neutralisation of phospholipase-c activity by specific *Cl. perfringens* antiserum the same medium (but not neutralising serum) could be used to detect *B. cereus* colonies by the opacity surrounding them.

Donovan[3], working on *B. cereus*, the cause of "bitty cream" in milk, described a selective medium capable of detecting as few as 1 or 2 cells, as spores or vegetative cells, in 1 ml of raw milk in a direct plating technique. This medium had been devised to overcome the problem of heat destruction of vegetative *B. cereus* cells in the existing technique for investigating "bitty cream". Egg yolk was incorporated in a peptone-meat extract basal medium to enable colonies of *B. cereus* cells to be seen because of their production of opaque zones in the medium.

Accompanying flora was inhibited by the inclusion of lithium chloride and polymyxin. Donovan preferred this medium to others she tried because it was found that the ease of differentiating the egg yolk reaction in the presence of milk could be increased by the inclusion in the formula of sodium citrate which serves to increase the transparency of the yolk and milk in the agar gel.

Following inoculation of the medium a thin overlayer of water agar was added to prevent spreading of resistant organisms presumed to be *Proteus spp.*

Mossel, Koopman and Jongerious[1] followed Donovan in rejecting heat pre-treatment of food samples when considering the need for a *B. cereus* enumeration medium. They had found that on occasions, as few as one spore-bearing cell or spore was present in 10^4 viable cells of *B. cereus*. In their early work on formulating a medium they investigated sodium chloride, ethyl alcohol and polymyxin B for inhibitory activity. They do not explain why they did not follow Donovan's idea of using lithium chloride. Polymyxin B was found to be superior. Egg yolk was included for detection of phospholipase-c. *B. cereus* characteristically dissimilates arabinose, xylose and mannitol and although any of the three could be used, mannitol was chosen because of its greater stability. Phenol red was chosen as the pH indicator. Colonies on this medium are large and pink, indicating that acid has not been produced from mannitol, and generally surrounded by opacity.

Kim and Goepfert[4] initially employed MYP agar in a survey of dry food products for presence of *B. cereus*. They found that mannitol utilisation was not significantly helpful in differentiating *B. cereus* from other species and also did not find the confirmatory tests suggested by Mossel et al[1] to be of much value. Their work on devising an improved confirmatory procedure indicated that a serological test based on detecting spore antigens was better. This necessitated formulation of a medium (KG medium) which gave faster spore formation than MYP to enable early confirmation of *B. cereus*. Phenol red is present in KG medium solely to accentuate the appearance of the opaque zones surrounding the colonies of *B. cereus*. KG Agar and MYP Agar were reported to possess similar capability of detecting low numbers of *B. cereus*.

Kramer and his co-workers[5] successfully used Columbia blood agar with added polymyxin for selective isolation of *B. cereus* from the mixed bacterial populations normally encountered in faeces and vomitus when investigating suspected food poisoning. Polymyxin-blood agar was preferred because it can be freshly prepared; blood agar is almost always immediately available due to its wide range of uses. As a consequence there are unlikely to be "shelf life" problems which can occur with more specialised media which are only occasionally used. Also the characteristic colony appearance of *B. cereus* on blood agar is retained as a familiar identification feature and colony types are readily differentiated for further testing.

Kramer et al also recommend another plating medium, BC medium, containing egg yolk and mannitol. Isolates showing the typical colony appearance of *B. cereus* on blood agar–positive egg yolk reaction, failure to ferment mannitol to produce acid from glucose in a medium containing an ammonium salt as a source of inorganic nitrogen are presumed to be *B. cereus*.

Holbrook and Anderson[6] reviewed the performance of McClung, KG and MYP media and concluded that further improvement was necessary in provision of media for *B. cereus* in food microbiology. They gave as reasons that MYP agar may show poor differentiation of mannitol-fermenting colonies, the egg yolk reaction in KG agar is often weak and differentiation of *B. cereus* colonies from those of other organisms on both KG and McClung agar may be poor. Arising from these observations they formulated polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) to meet the requirements for a medium that is sufficiently selective to detect low numbers of *B. cereus* spores and vegetative cells in the presence of large numbers of other organisms and possessing obvious diagnostic features that could be rapidly and easily confirmed. Sporulation was improved by minimising the peptone content.

Pyruvate reduced the tendency for *B. cereus* to form rhizoid colonies, improved the egg yolk reaction and encouraged sporulation within 24 hours. Cycloheximide was added to polymyxin to improve selective activity by inhibiting mould growth. *B. cereus* grows on PEMBA medium as characteristic peacock-blue colonies. *B. thuringiensis*, amongst other *Bacillus spp.* able to grow on the medium, forms colonies with the same appearance. *Bacillus cereus* can easily be differentiated microscopically from other non-mannitol dissimilating species. This may be achieved microscopically by examining cells stained by a special technique to demonstrate lipid granules and spores in the cytoplasm. Lipid granules are present only in *B. cereus* and the combination of cell lipid and the peacock-blue colony is diagnostic.

Szabo, Todd and Rayman[7] observed that lipid stain-confirmed isolates were often atypical with respect to colony colour after 24 hours incubation and occasionally with respect to egg yolk reaction. They improved performance by substituting bromocresol purple for bromothymol blue and found the modification provided consistently reliable colony colour and typical egg yolk reaction. The improvement enabled routine incubation time to be reduced from 24–48 hours to 18–22 hours.

Hood, Tuck and Dane[8] returned to McClung's principle of one medium for both *Cl. perfringens* and *B. cereus* in their development of a blood-free egg yolk medium which they designated BCP. This was designed primarily to improve the recovery of stressed *Cl. perfringens* spores and contains pyruvate, inositol, mannitol and bromocresol purple indicator in Blood Agar Base Number 2.

The inclusion of mannitol also allows the medium to be used for the presumptive identification of *B. cereus*, showing performance similar to BC medium.[5]

Vasconcellos and Rabinovitch[9] recently published a formula for a medium that does not contain antibiotics, claiming that it is more selective, simple to make and less costly than the *B. cereus* media already described. The new medium inhibits *B. megaterium* and enables presumptive quantification of *B. cereus* in foods in less than 24 hours. It contains egg yolk and selectivity is achieved by using a combination of Tris buffer, maintaining pH at approximately 8.2, and resazurin. *B. cereus* and *B. thuringiensis* reduce the resazurin producing characteristic cream-coloured colonies, the centres of which show more intense colouration. The zone of egg yolk reaction is pale pink in contrast to the darker pink of the rest of the medium.

Descriptions of MYP and PEMBA media are given on pages 26/27 and formulae for the other media named in this review on page 29. Oxoid products which may be used in making the media are listed alongside the formulae.

References

1 Mossel, D.A.A., Koopman, M.J. and Jongerius, E. (1967) *Appl. Microbiol.* **15,** 650–653.
2 McClung, L.S. and Toabe, R. (1947) *J. Bact.* **53,** 139–147.
3 Donovan, K.O. (1958) *J. Appl. Bact.* **21,** 100–103.
4 Kim, H.U. and Goepfert, J.M. (1971) *Appl. Microbiol.* **22,** 581–587.
5 Kramer, J.M., Turnbull, P.C.B., Munshi, G. and Gilbert, R.J. (1982) In: *Isolation and Identification Methods for Food Poisoning Organisms.* Corry, J.E.L., Roberts, D. and Skinner, F.A. (Eds). SAB Technical Series 17, Academic Press.
6 Holbrook, R. and Anderson, J.M. (1980) *Can. J. Microbiol.* **26,** 753–759.
7 Szabo, R.A., Todd, E.C.D. and Rayman, M.K. (1984) *J. Food Prot.* **47,** 856–860.
8 Hood, A.M., Tuck, A. and Dane, C.R. (1990) *J. Appl. Bact.* **69,** 359–372.
9 Vasconcellos, F.J M. and Rabinovitch, L. (1995) *J. Food Prot.* **58,** 235–238.

Table 7 – Selective agents and identification systems used in media for *Bacilus cereus*

Medium	Selective Agents	Identification System	Reference*
PEMBA	Polymyxin B 10 µg/ml Cycloheximide 40 µg/ml (optional)	Acid from mannitol Lecithinase Rapid staining procedure	Holbrook & Anderson (1980)
MYP	Polymyxin B 10 µg/ml	Acid from mannitol lecithinase	Mossel, Koopman and Jongerius (1967)
K.G.	Polymyxin B 10 µg/ml	Lecithinase	Kim and Goepfert (1971)
PEMPA	Polymyxin B 10 µg/ml	Acid from mannitol Lecithinase	Szabo, Todd and Rayman (1984)
BCP		Acid from mannitol Lecithinase	Hood, Tuck and Dane (1990)
VRM	TRIS buffer, pH 8.2 Resazurin	Lecithinase Resazurin reduction	Vasconcellos and Rabinovitch (1995)

* See above for complete references.

Mannitol-Egg Yolk-Phenol Red Agar (MYP)

MYP Agar, described by Mossel and his co-workers[1] was the first specific selective and differential medium developed for the enumeration of *Bacillus cereus* in foods. Examination for *B. cereus* had generally been carried out by subjecting dilutions of samples to preliminary heat treatment to destroy non-sporulating accompanying flora. This is invariably a successful procedure when testing foods suspected of causing food poisoning because the number of viable cells is usually so high that isolation is not a problem. However, when examining foods or food ingredients in which, if *B. cereus* is present at all, there are few cells, preliminary heat treatment is not a valid procedure because the proportion of spores present may be very low; Mossel et al confirmed Nikodemusz' earlier observation[2] that in certain foods there may be as few as one spore or spore-bearing cell in 10^4 total viable cells of *B. cereus*. A selective differential medium is therefore essential when searching for *B. cereus* amongst large populations of other bacteria.

Mossel et al initially experimented with sodium chloride and ethyl alcohol as selective agents but found neither to be satisfactory. Following Donovan's earlier work[3] showing that polymyxin B is effective for selection of *B. cereus*, this antibiotic was investigated and finally used at a concentration of 10 µg/ml. Coliforms are suppressed by *Proteus spp.* and Gram-positive cocci may not be inhibited.

Differential properties were given to the medium by exploiting the failure of *B. cereus* to produce acid from mannitol and the ability of most strains to produce lecithinase.

B. cereus grows on MYP Medium as rough, dry colonies with a violet-red background. The colonies are surrounded by a halo of dense white precipitate. Mossel et al used a number of biochemical tests to confirm the identity of isolates including acid production, under both aerobic and anaerobic conditions, from glucose, D-mannitol and xylose, nitrate reduction and production of acetyl-methyl-carbonol. Strains were also tested for their ability to grow in the presence of 0.25% chloral hydrate and 10% sodium chloride.

The medium has been shown to be very effective for detecting *B. cereus*, even for ratios as challenging as one cell of *B. cereus* to 10^6 cells of other organisms.

MYP Agar is specified in a number of Standard Methods for detection of *B. cereus* in foods.

References

1 Mossel, D.A.A., Koopman, M.J. and Jongerius, E. (1967) *Appl. Microbiol.* **15,** 650–653.
2 Nikodemusz, I. (1958) *Z. Hyg. Infektionskrankl.* **145,** 335–338.
3 Donovan, K.O. (1958) *J. Appl. Bact.* **21,** 100–103.

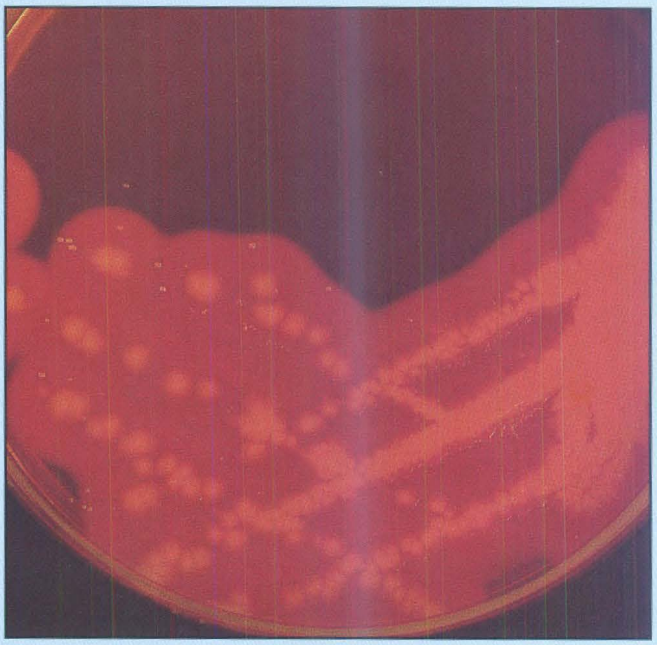
Mannitol-Egg Yolk-Phenol Red Agar (MYP)

A selective differential medium for the detection and enumeration of *B. cereus* in foods.

	grams/litre
Meat extract (Lab-Lemco, L29)	1.0
Peptone (L37)	10.0
D-Mannitol	10.0
Sodium chloride (L5)	10.0
Phenol red	0.025
Agar (L11)	15.0
Supplement: Polymyxin B	10 mg/litre

pH 7.2 ± 0.2

Oxoid products that may be used in making this medium are given in parentheses.



Appearance of *B. cereus* on MYP agar.

Bacillus cereus Selective Agar

Polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA)

Base Medium
Code: CM617

Formula

	grams/litre
Peptone	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium sulphate	0.1
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromothymol blue	0.12
Sodium pyruvate	10.0
Agar	14.0

pH 7.2 ± 0.2

***Bacillus cereus* Selective Supplement**
Code: SR99

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

Polymyxin B	50,000 IU

Directions
Suspend 20.5 g in 475 ml of distilled water and 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 Oxoid Bacillus cereus Selective Supplement SR99 reconstituted with 2 ml of sterile distilled water, then add 25 ml of sterile Egg Yolk Emulsion SR47. Mix well and pour into sterile petri dishes.

Description
Bacillus cereus Selective Agar CM617, is based on the highly specific diagnostic and selective PEMBA medium, developed by Holbrook and Anderson[1] for the isolation and enumeration of *Bacillus cereus* in foods. It meets the requirements of a medium that is sufficiently selective to be able to detect small numbers of *B. cereus* cells and spores in the presence of large numbers of other food contaminants. The medium is also sufficiently diagnostic that colonies of *B. cereus* are readily identified and confirmed by microscopic examination.

The role of *B. cereus* in food poisoning, particularly from the consumption of contaminated rice, is now well documented.[2,3,4] The organism has also been implicated in eye infections[5,6] and a wide range of other conditions including abscess formation, meningitis, septicaemia and wound infection. *B. cereus* is recognised as a significant pathogen in post-operative and post-traumatic wounds of orthopaedic patients.[7]

Amongst veterinarians, *B. cereus* is a known cause of disease, especially mastitis, in ewes and heifers.[8]

In the formulation of Bacillus cereus Selective Agar a peptone level of 0.1% and the addition of sodium pyruvate improve egg yolk precipitation and enhance sporulation. Bromothymol blue is added as a pH indicator to detect mannitol utilisation. The medium is made selective by addition of Bacillus cereus Selective Supplement SR99, which gives a final concentration of 100 IU of polymyxin B per ml of medium. Polymyxin B as a selective agent for the isolation of *B. cereus* has been previously suggested by Donovan[9] and found to be satisfactory by Mossel.[10] It is recommended that, where large numbers of moulds are expected in the inoculum, filter-sterilised cycloheximide is added to the medium at a final concentration of 40 µg/ml.

The primary diagnostic features of the medium are the colonial appearance, precipitation of hydrolysed lecithin and the failure of *B. cereus* to utilise mannitol.

The typical colonies of *B. cereus* are crenated, about 5 mm in diameter and have a distinctive turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same colour.

These features distinguish *B. cereus* from other *Bacillus* species except *B. thuringiensis*. Other egg yolk-reacting organisms which can grow on the medium, including *Staphylococcus aureus*, *Serratia marcescens* and *Proteus vulgaris* are distinguished from *B. cereus* by colony form and colour. These organisms also produce an egg yolk-clearing reaction in contrast to egg yolk precipitate produced by *B. cereus*.

Microscope examination for presence of lipid globules in the vegetative cells is recommended as a rapid and confirmatory test for *B. cereus* and replaces the need for biochemical testing. Holbrook and Anderson[1] have confirmed that only *B. cereus* of the *Bacillus* species are capable of possessing lipid globules in their vegetative cells when grown on the selective medium. One further advantage of this test is that strains of *B. cereus* that react only weakly or not at all with egg yolk can be detected and confirmed.

Technique
1 Homogenise 10 g of the food sample for 30 seconds in 90 ml of 0.1% Peptone Water CM9 using a Stomacher. Dried foods should first be rehydrated by soaking 20 g in 90 ml of Tryptone salt solution (Tryptone L42 0.3% and sodium chloride 0.8%, pH 7.3) for 50 minutes at room temperature. Add a further 90 ml of 0.1% peptone water to give a final dilution of 10^{-1}. Homogenise for 30 seconds using the Stomacher.

2 Further dilutions of the homogenate should be made in 0.1% peptone water.

3 Inoculate 0.1 ml amounts of the 10^{-1} and higher dilutions on to the surface of the medium.

4 Incubate the plates at 35°C for 24 hours.

5 Examine for typical colonies of *B. cereus*.

6 Leave the plates for a further 24 hours at room temperature in order to detect all the *Bacillus cereus* colonies.

7 Confirm the presumptive identification of *B. cereus* by the Rapid Confirmatory Staining Procedure.

8 Report the results as the number of *B. cereus* colonies per gram weight of the food sample.

The medium may also be used for detecting *B. cereus* in milk. When necessary, decimal dilutions of the samples should be made in 0.1% peptone water. Undiluted and diluted samples are inoculated directly on to plates of agar and incubated. An incubation temperature of 30°C for 18 hours is recommended as optimal for promoting the growth of *B. cereus* relative to that of other organisms.[9]

For examining clinical specimens plates may be inoculated in the usual way.

Rapid Confirmatory Staining Procedure
This staining method was developed by Holbrook and Anderson[1] combining the spore stain of Ashby[11] and the intracellular lipid stain of Burdon.[12]

Procedure

1 Prepare films from the centre of a 1 day old colony or from the edge of a 2 day colony.

2 Air-dry the film and fix with minimal heating.

3 Flood the slide with aqueous 5% w/v malachite green and heat with a flaming alcohol swab until steam rises. Do not boil.

4 Leave for 2 minutes without re-heating.

5 Wash the slide with running water and blot dry.

6 Flood the slide with 0.3% w/v Sudan black in 70% ethyl alcohol. Leave for 15 minutes.

7 Wash the slide with running Citroclear* from a wash bottle for 5 seconds. For reasons of safety, Citroclear replaces xylene in the original technique.

8 Blot dry using filter paper.

9 Flood the slide with aqueous 0.5% w/v safranin for 20 seconds.

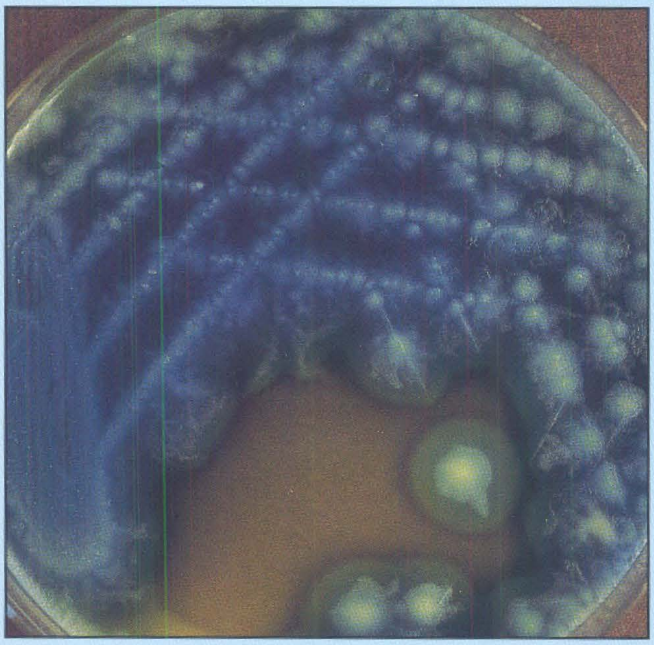
10 Wash under running water.

11 Blot dry and examine under the microscope using the oil immersion lens. A blue filter may be used to accentuate the appearance of the lipid granules but this will give a blue colour cast to the red of the cytoplasm.

Characteristic appearance of *B. cereus* vegetative cells
The cells are 4–5 micron long and 1.0–1.5 micron wide with square ends and rounded corners.

Spores stain pale to mid green, are central or paracentral in position and do not swell the sporangium.

Vegetative cytoplasm stains red. Lipid granules within the cytoplasm are black.



Appearance of *B. cereus* on Bacillus cereus Selective Agar (PEMBA).

The appearance, together with the typical colony form, confirms the identification of *B. cereus*.

Note

1 The amount of stained lipid present is strain-variable.

2 If a strain has been repeatedly subcultured on PEMBA medium it may be necessary to increase staining time with malachite green to up to 10 minutes, heating twice (P. Butler, personal communication).

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

The prepared medium may be stored at 2–8°C.

Quality Control
Positive Control:
Bacillus cereus ATCC® 10876
Bacillus subtilis ATCC® 6633 (Should be readily differentiated by colony form and colour).

Negative Control:
Bacillus coagulans ATCC® 7050

Precautions
On this medium *B. cereus* is indistinguishable from *B. thuringiensis*.

Identify *B. cereus* by colony form, colour, egg yolk hydrolysis and confirm with cell and spore morphology.[13]

Occasional strains of *B. cereus* show weak or negative egg yolk reactions.

References

1 Holbrook, R. and Anderson, J.M. (1980) *Can. J. Microbiol.* **26** (7), 753–759.
2 *Brit. Med. J.*, 15 January 1972. 189.
3 *Brit. Med. J.*, 22 September 1973. 647.
4 Mortimer, P.R. and McCann, G. 25 May, 1974, *Lancet*, 1043–1045.
5 Davenport, R. and Smith, C. (1952) *Brit. J. Ophthal.* **36,** 39.
6 Bouza, E., Grant, S., Jordan, C., Yook, R. and Sulit, H. (1979) *Arch. Ophthalmol.* **97,** 498–499.
7 Akesson, A., Hedström, S.A. and Ripa, T. (1991) *Scan. J. Inf. Dis.* **23,** 71–77.
8 Wohlgemuth, K., Kirkbridge, C.A., Bicknell, E.J. and Ellis, R.P. (1972) *J. Amer. Vet. Med. Ass.* **161,** 1691–1695.
9 Donovan, K.O. (1958) *J. Appl. Bacteriol.* **21** (1), 100–103.
10 Mossel, D.A.A., Koopman, M.J. and Jongerius, E. (1967) *J. Appl. Microbiol.* **15** (3), 650–653.
11 Ashby, G.K. (1938) *Science* **87,** 433–435.
12 Burdon, K.L. (1946) *J. Bacteriol.* **52,** 665–678.
13 Deák, T. and Timár, E. (1988) *Int. J. Food Microbiology* **6,** 115–125.

*Citroclear is available from:
H. D. Supplies
44 Rabans Close
Rabans Lane Industrial Estate
Aylesbury
Buckinghamshire
HP19 3RS

K.G. Agar

	grams/litre
Peptone (L34)	1.0
Yeast extract (L21)	0.5
Phenol red	0.025
Agar (L11)	18.0

pH 6.8 ± 0.2

Add to 900 ml of medium:

Egg yolk emulsion (SR47)	100 ml
Polymyxin B sulphate	10 µg/ml

Reference

Kim, H.U. and Goepfert, J.M. (1971) *Appl. Microbiol.* **22,** 581–587.

Oxoid products that may be used in making this medium are given in parentheses.

PEMPA Medium

	grams/litre
Tryptone (L42)	1.0
D-mannitol	10.0
Magnesium sulphate: $7H_2O$	0.1
Sodium chloride	2.0
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromocresol purple	0.06
Agar (L11)	18.0

pH 6.9 ± 0.2

Add to 90 ml volumes of medium:

Sodium pyruvate 20% w/v	5 ml
Egg yolk emulsion	5 ml

Polymyxin B 8260 USP units/mg to a final concentration of 10 µg/ml.

Reference

Szabo, R.A., Todd, E.C.D. and Rayman, M.K. (1984) *J. Food Prot.* **47,** 856–860.

Oxoid products that may be used in making this medium are given in parentheses.

VRM Medium

	grams/litre
Tryptone (L42)	10.0
TRIS buffer	1.21
Sodium chloride	5.0
Magnesium sulphate: $7H_2O$	0.2
Resazurin	0.01
Agar (L11)	14.0
Fresh egg yolk	50 ml

pH 8.2 ± 0.1

Reference

Vasconcellos, F.J.M. and Rabinovitch, L. (1995) *J. Food Prot.* **58,** 235–238.

Oxoid products that may be used in making this medium are given in parentheses.

BCP Agar

This medium is also used for isolation and enumeration of *Cl. perfringens*. The formula is given on page 13.

Reference

Hood, A.M., Tuck, A. and Dane, C.R. (1990) *J. Appl. Bact.* **69,** 359–372.

Identification of *Bacillus cereus*

1 Modified rapid staining procedure for growth taken from PEMBA medium (Holbrook and Anderson).

(a) Prepare films from the centre of a 1 day old colony or from the edge of a 2 day colony.

(b) Air-dry the film and fix with minimal heating.

(c) Flood the slide with aqueous 5% w/v malachite green and heat with a flaming alcohol swab until steam rises. Do not boil.

(d) Leave for 2 minutes without re-heating.

(e) Wash the slide with running water and blot dry.

(f) Flood the slide with 0.3% w/v Sudan black in 70% ethyl alcohol. Leave for 15 minutes.

(g) Wash the slide with running Citroclear* from a wash bottle for 5 seconds. For reasons of safety, Citroclear replaces xylene in the original technique.

(h) Blot dry using filter paper.

(i) Flood the slide with aqueous 0.5% w/v safranin for 20 seconds.

(j) Wash under running water.

(k) Blot dry and examine under the microscope using the oil immersion lens. A blue filter may be used to accentuate the appearance of the lipid granules but this will give a blue colour cast to the red of the cytoplasm.

Characteristic appearance of *B. cereus* vegetative cells

The cells are 4–5 micron long and 1.0–1.5 micron wide with square ends and rounded corners.

Spores stain pale to mid green, are central or paracentral in position and do not swell the sporangium.

Vegetative cytoplasm stains red. Lipid granules within the cytoplasm are black.

Microscope preparations may contain many green-staining spores free of cytoplasm

Note:

1 The amount of lipid present is strain-variable.

2 If a strain has been repeatedly subcultured on PEMBA medium it may be necessary to increase staining time with malachite green to up to 10 minutes, heating twice (P. Butler, personal communication).

2 Appearance of the spores

Species within the genus Bacillus can be differentiated into 3 morphological groups according to the appearance and position of the spores within the cell.

B. cereus is contained within morphological group 1 with the following defining characteristics:

(a) Sporangia are not swollen, or only very slightly swollen, by the endospores.

(b) The spores are ellipsoidal or cylindrical with central or terminal positioning in the sporangium.

(c) The sporangium will stain Gram-positive or Gram-variable.

3 Biochemical tests

Bacillus spp. may generally be differentiated by a series of tests of biochemical activity carried out in conjunction with detection of lecithinase and tolerance to elevated temperatures.

Suggested tests and the reactions of species associated with food poisoning are given in table 8.

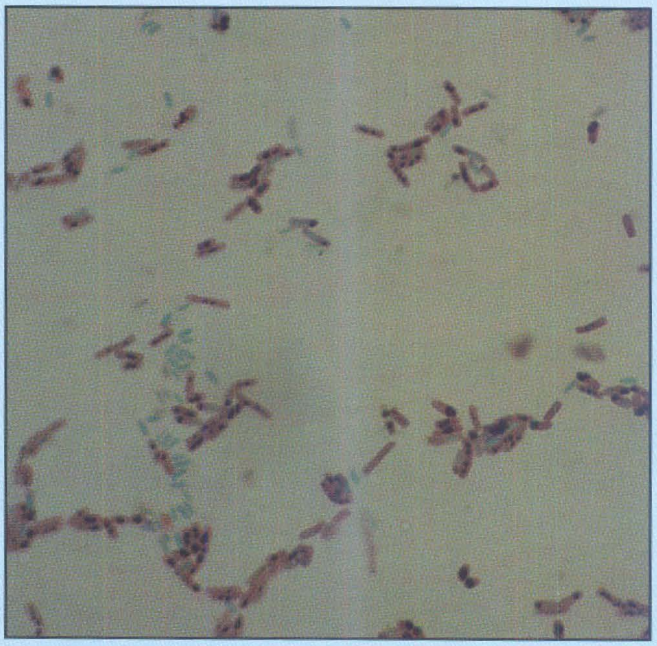
Specialist texts should be consulted for detailed methodology.[1]

*Citroclear is available from:

H. D. Supplies
44 Rabans Close
Rabans Lane Industrial Estate
Aylesbury
Buckinghamshire
HP19 3RS

Reference

1 Parry, J.M., Turnbull, P.C.B. and Gibson, J.R. (1983) *A Colour Atlas of Bacillus species*, Wolfe Medical Publications, London.



Appearance of *B. cereus* stained by the rapid confirmatory staining procedure from growth on PEMBA medium.

Foodborne Illness Caused by other *Bacillus* Species

There is accumulating evidence to implicate species of Bacillus other than *B. cereus* in food poisoning. A number of reports have pointed to *B. licheniformis* and *B. subtilis* in particular and there are suspicions about the possible significance in food poisoning of *B. thuringiensis*, an insect pathogen that is closely similar to *B. cereus*.

A species of Bacillus identified as *B. subtilis*, but which may have been *B. licheniformis*, was recovered in large numbers in almost pure culture from the remains of turkey and the faeces of patients who developed gastroenteritis in an American hospital. There have been numerous other reports of isolation of large numbers of *B. licheniformis* in the absence of known pathogens in outbreaks of food poisoning. The foods involved, incubation time and major symptoms suggest a similarity to the food poisoning caused by *Clostridium perfringens*.

Other reported episodes of food poisoning strongly indicate *B. subtilis* as the cause. In contrast to *B. licheniformis*-related cases, most of the *B. subtilis*-related cases were notable for unusually short incubation periods before onset of nausea, vomiting and stomach cramps. Additionally, diarrhoea occurred in approximately 50% of those affected. The combined reports name a wide variety of food vehicles and frequently *B. subtilis* was present in high numbers.

B. licheniformis and *B. subtilis* are widely distributed in the environment and are particularly abundant in soil and on vegetation. Both species can be isolated easily using routine non-selective culture media. Both grow well on PEMBA medium appearing very similar to each other but distinctively different from *B. cereus*. Species of Bacillus are a concern in food processing and canning because of their association with food spoilage. The increasing evidence of an occasional role in food poisoning may add a further dimension to the importance of their detection in foods and raw materials.

Evidence suggesting a possible role for *B. licheniformis* and *B. subtilis* in food poisoning has been published by Kramer and Gilbert[1] in a review of *B. cereus* as a foodborne pathogen.

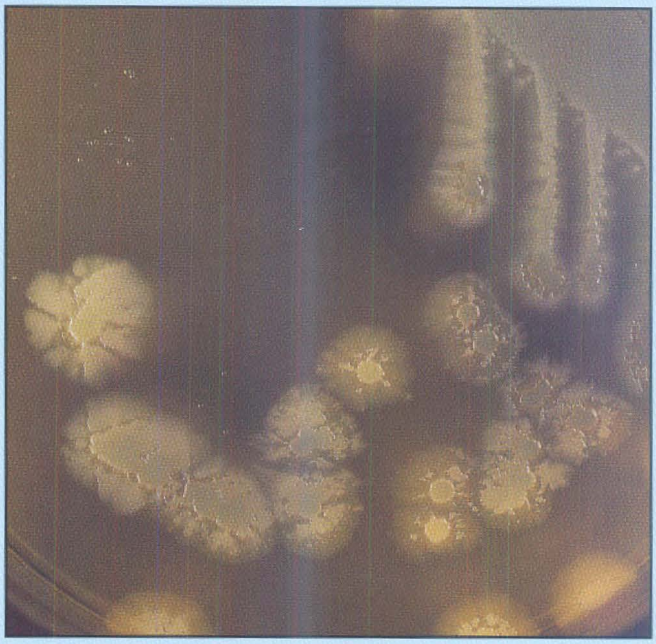
A preliminary report[2] of toxin formation by *B. cereus* and other *Bacillus spp.*, using a modified cell cytotoxicity assay for toxin detection, has shown that some strains of *B. mycoides*, *B. thuringiensis*, *B. circulans* and *B. lentus*, as well as *B. licheniformis*, are able to produce toxin. No information is available concerning the significance in food poisoning of this finding.

References

1 Kramer, J.M. and Gilbert, R.J. (1989) Chapter 2 in *Foodborne Bacterial Pathogens*. Doyle, M.P. (Ed). Marcel Dekker Inc. New York and Basel.

2 Beattie, S.H. and Williams, A.G. Abstracts of the Summer Conference of the Society for Applied Bacteriology, Bradford, 1996. Supplement to *Journal of Applied Bacteriology* (1996) **81** (2), XXIV.

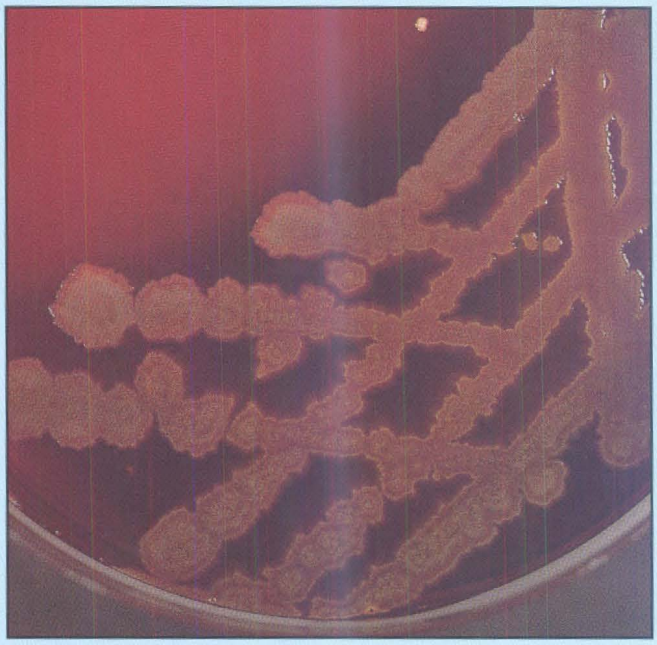
Appearance of *B. licheniformis* on PEMBA medium.



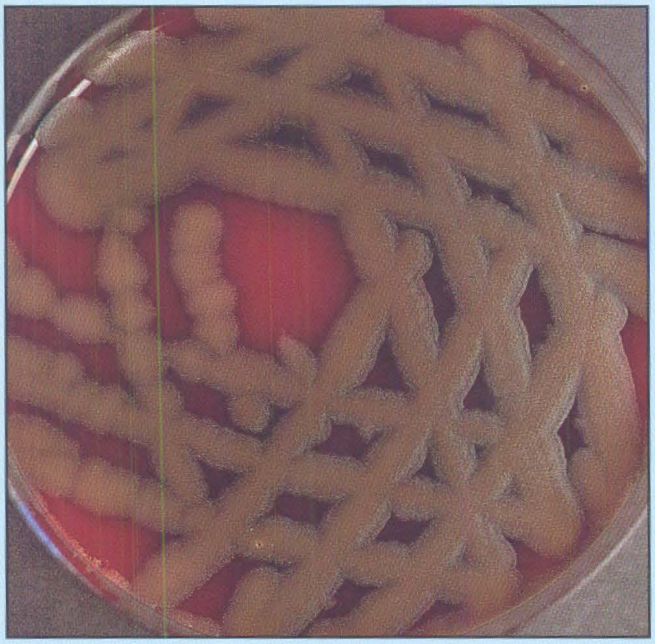
Appearance of *B. subtilis* on PEMBA medium.

Table 8 – Differentiation of Food Poisoning Species of Bacillus

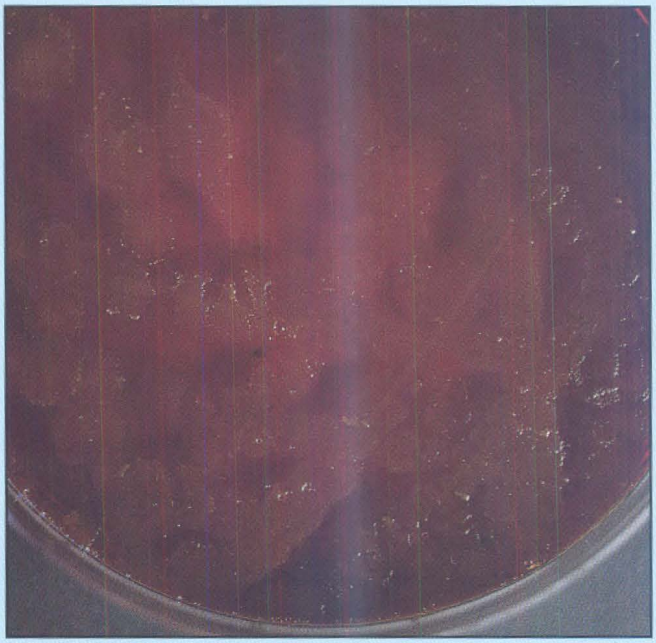
	Bacillus cereus	***B. licheniformis***	***B. subtilis***
Lipid granules seen by rapid staining procedure	+	–	–
Spore morphology group	1	1	1
Lecithinase production	+	–	–
Anaerobic growth	+	+	–
Growth at 50°C	–	+	+
Growth at 55°C	–	±	+
Acid from:			
Mannitol	–	+	+
Glucose	+	+	+
Arabinose	–	+	+
Xylose	–	+	+
Nitrate reduction	+	+	+
Hydrolysis of:			
Starch	+	+	+
Gelatin	+	+	+
Tyrosine	+	–	–
pH of VP Broth	4.3–5.6	5.0–6.5	5.0–8.0



Appearance of *B. licheniformis* on blood agar.



Appearance of *B. cereus* on blood agar.



Appearance of *B. subtilis* on blood agar.

Food Poisoning Toxins of *Clostridium perfringens* and *Bacillus cereus* and their Detection

Food poisoning by *Cl. perfringens* and *B. cereus* occurs as a result of intoxication by metabolic products formed by the organisms in the gut or in foods before consumption.

Cl. perfringens enterotoxin is generally produced in the intestine by cells that have survived passage through the stomach. Toxin production in food has been reported but this is a rare event. The toxin is a protein with a molecular weight of 35,000. Its action is to damage epithelial cells on the villi and inhibit absorption of glucose. There is an efflux of sodium chloride and water into the gut lumen causing diarrhoea.

Cl. perfringens occurs commonly in the human intestine and the presence of organisms in faeces, even in high numbers, cannot be considered proof that *Cl. perfringens* is the causative organism in a particular case. Determination of faecal enterotoxin is necessary to confirm the role of *Cl. perfringens*. Detection of high numbers of cells in food and enterotoxin in the faeces of subjects who have eaten the food and become ill with the typical symptoms, is very convincing evidence of cause and effect.

A number of biological methods are available for detection of *Cl. perfringens*. The rabbit ligated ileal-loop test is very effective and has been widely used. Other *in vivo* tests have been applied but, in general, tests involving live animals are unattractive. Latterly, tests which employ tissue culture cells have been found to be a useful alternative.

Immunology-based assays are increasingly applied. Commonly used earlier techniques included gel diffusion and counterimmunoelectrophoresis. More recently ELISA and Reverse Passive Latex Agglutination have found favour as relatively simple and sensitive techniques. A detailed review of testing methods has been published by Stringer.[1]

B. cereus causes two distinct types of food poisoning, one emetic the other diarrhoeal. The two syndromes are the result of different toxins; it is rare for both forms of poisoning to occur together.

The emetic toxin is a low molecular weight protein, apparently heat stable and probably associated with spore formulation. The mode of action appears similar to that of staphylococcal enterotoxin.

B. cereus diarrhoeal toxin is a protein with a molecular weight of approximately 50,000 and is thought to be similar in action to cholera toxin.

The emetic toxin has a much shorter incubation period than the diarrhoeal toxin, and recovery from both intoxications is usual within 24 hours. Differential diagnosis of the two types of *B. cereus* food poisoning can be difficult unless bacteriological examination of faeces is also carried out. The diarrhoeal symptoms closely resemble *Cl. perfringens* poisoning and the emetic syndrome can be confused with staphylococcal poisoning.

The role of *B. cereus* in food poisoning and procedures for toxin detection are described by Kramer et al.[2]

Reversed Passive Latex Agglutination (RPLA) kits for detection of *Cl. perfringens* enterotoxin and *B. cereus* diarrhoeal toxin are described in the following pages.

References

1 Stringer, M.F. in *Clostridia in Gastrointestinal Disease*, Chapter 6, Borriello, 117–143, S.P. (Ed). CRC Press Inc, Florida.

2 Kramer, J.M., Turnbull, P.C.B., Munshi, G. and Gilbert, R.J. (1982) In *Isolation and Identification Methods for Food Poisoning Organisms*, 261–286, Corry, J.E.Ll., Roberts, D. and Skinner, F.A. (Eds). SAB Technical Series 17, Academic Press.

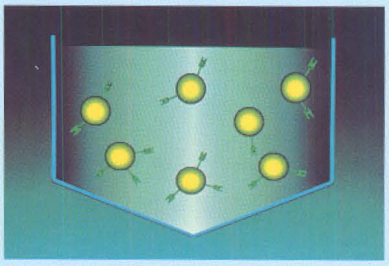
The Principle of Reversed Passive Latex Agglutination (RPLA)

Standard agglutination tests involve the reaction between a soluble antibody and a particulate antigen e.g. bacterial cells.

In reversed agglutination assays, antibody is attached to inert carrier particles e.g. latex. The union of antibody to soluble antigen will link the latex particles in a lattice structure to form visible agglutination.

The latex particles are described as passive since they do not have an active role in the antigen/antibody reaction.

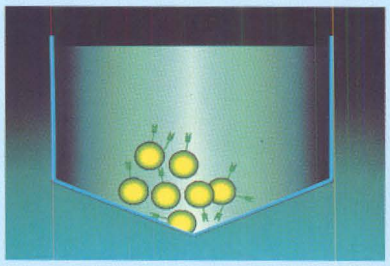
Diagrammatic Representation

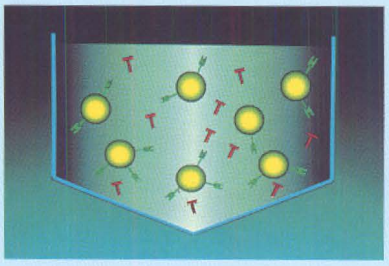


Latex particles (in suspension) sensitised with antibodies to the toxin.

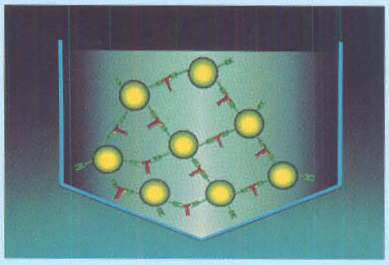
No antigen in sample.

Latex particles settle in base of well (tight button).

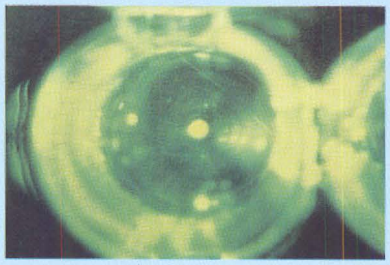




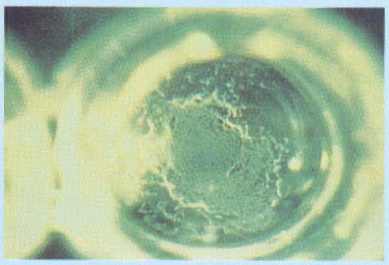
Add soluble antigen (toxin in solution – normally culture filtrate).



Antibodies bind to antigen causing latex particles to cross-link. A lattice is formed.



Negative RPLA test.
View from above.



Positive RPLA test.
View from above.

Clostridium perfringens RPLA Toxin Detection Kit PET-RPLA

Code: TD930

Application

This kit is used for the detection of *Clostridium perfringens* enterotoxin in faecal samples or culture filtrates by reversed passive latex agglutination.

It detects *Clostridium perfringens* type A enterotoxin which causes food poisoning.

Direct detection from faeces is accomplished within 24 hours.

The test is simple to use, easy to interpret and provides a clearly visible endpoint.

Sensitivity is 2 ng/ml in culture filtrates.

INTRODUCTION

Perfringens poisoning usually arises as a result of the temperature abuse of prepared foods. Spores may survive cooking, germinate and multiply to dangerous levels before the food is eaten.

Ingestion of vegetative cells of *Clostridium perfringens* type A can lead to perfringens poisoning, which is characterised by diarrhoea and abdominal cramps. Illness typically begins about 6–24 hours after ingestion of foods containing at least 10^8 *Clostridium perfringens* type A cells per gram. Ingested cells that survive passage through the stomach multiply and then produce spores in the small intestine. Illness is caused by the production of enterotoxin which is associated with this spore-forming process. It is therefore important to detect the enterotoxin, either in faecal specimens obtained from the patient or in the culture fluid of bacterial isolates to confirm the diagnosis of food poisoning.

Reversed passive latex agglutination has been reported to be a reliable method for the detection of *Clostridium perfringens* enterotoxin.[1]

The direct detection of *Clostridium perfringens* enterotoxin in faeces is the preferred approach due to the much larger amount of toxin formed *in vivo*. The culture method is less reliable because toxin may not be produced *in vitro*.

Test Principle

The test is performed in V-well microtitre plates.

Latex particles are sensitised with purified antiserum taken from rabbits immunised with purified *Clostridium perfringens* type A enterotoxin. In the presence of this enterotoxin, the latex particles will agglutinate forming a clearly visible lattice structure.

If *Cl. perfringens* enterotoxin is absent, or present at a concentration below the assay detection level, a lattice structure will not form and the latex particles settle in a tight button in the base of the well.

Positive and negative RPLA tests are illustrated on page 34.

Procedure

Toxin extraction from faeces

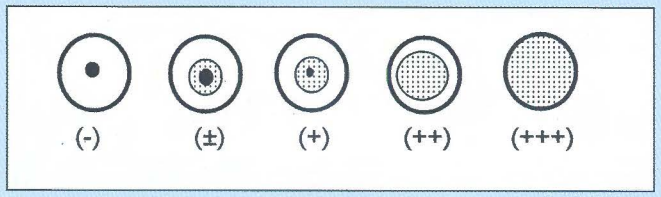
Extraction from faeces is accomplished by mixing faeces with a similar volume of phosphate-buffered saline (Oxoid BR14A) and homogenising. The test sample may then be extracted by means of centrifugation or filtration.

Production of enterotoxin in culture fluid

Isolates should be cultured in Cooked Meat Medium (Oxoid CM81) at 37°C for 18–20 hours, and then vegetative cells inactivated by heating at 75°C for 20 minutes. The spores remaining should then be subcultured at 37°C for 24 hours on a medium designed for the promotion of enterotoxin production.[2,3] After incubation, the test sample may be extracted by means of centrifugation or filtration.

Assay Method

1 Shake reagents thoroughly.
2 Position V-well microtitre plates so that each row consist of 8 wells. (Each sample requires 2 such rows.)
3 Dispense 25 µl diluent in each well of the 2 rows, except for the 1st well in each row.
4 Add 25 µl test sample to the 1st and 2nd well of each row.
5 Pick up 25 µl from the 2nd well and perform doubling dilutions along each row, ending at the 7th well. The last well should contain diluent only.
6 Add 25 µl sensitised latex (TD931) to each well in row 1.
7 Add 25 µl control latex (TD932) to each well in row 2.
8 Agitate the plate by hand or a micromixer to mix the contents of each well.
9 Cover the plates and leave undisturbed for 20–24 hours.
10 Examine each well for agglutination against a black background. Dispose of all items in hypochlorite solution (>1.3% w/w).



Interpretation of Results

Agglutination should be assessed by comparison with the above illustration. For more detailed procedure and interpretation information, refer to the pack insert.

Negative

Results classified as (-) and (±) are considered to be negative.

Positive

Results classified as (+), (++) and (+++) are considered to be positive.

References

1 Harmon, S. and Kautter, D. (1986) *J. Food Prot.* **49,** 523.
2 Duncan, C. and Strong, D. (1968) *App. Microbiol.* **1,** 82.
3 Harmon, S. and Kautter, D. (1986) *J. Food Prot.* **49,** 706.

Bacillus cereus RPLA Toxin Detection Kit BCET-RPLA TD950

Application

This kit is used for the detection of protein constituents of *Bacillus cereus* diarrhoeal enterotoxin in foods and culture filtrates by reversed passive latex agglutination.

- Direct detection from food samples is accomplished within 24 hours. Also suitable for testing culture filtrates.
- The test is simple to use, easy to interpret and provides clearly visible endpoint reaction.
- Sensitivity of 2 ng/ml in culture filtrates.

INTRODUCTION

In both its spore and vegetative forms, the organism *Bacillus cereus* is common in the environment, and can easily contaminate food.

If contaminated foods are not cooled sufficiently after cooking and there is delay between the preparation and consumption of food, then surviving heat-resistant spores can germinate, enabling the organism to multiply and produce toxins. Under these circumstances, *B. cereus* can cause food poisoning.

Rice, pasta, meat, poultry, vegetable dishes, various soups, puddings and sauces have been implicated in *B. cereus* food poisoning.[1,2,3]

Two distinct types of illness can be caused by this organism: An acute-onset "emetic-syndrome" type which is mainly associated with cooked rice, and the longer-onset "diarrhoeal-syndrome" type in which a wide range of foods has been implicated. Separate toxins are responsible for the characteristic symptoms of the two forms of illness: the emetic toxin and the diarrhoeal enterotoxin.[4]

The BCET-RPLA test kit was developed for the purpose of detecting the diarrhoeal enterotoxin by means of reversed passive latex agglutination. With increasing knowledge of the complexity of *B. cereus* enterotoxin there is currently uncertainty about the exact component that is being detected.

The RPLA kit may be used to detect the presence of enterotoxigenic *B. cereus* in a variety of foods and to give a semi-quantitative result.

The test may also be used to demonstrate the ability of *B. cereus* to produce enterotoxin when grown in culture.

Test Principle

The test is performed in V-well microtitre plates.

Latex particles are sensitised with purified antiserum taken from rabbits immunised with purified *B. cereus* diarrhoeal enterotoxin. In the presence of this enterotoxin, the latex particles will agglutinate forming a clearly visible latex structure.

If *B. cereus* enterotoxin is absent, or present at a concentration below the assay detection level, a lattice structure is not formed and the latex particles settle in a tight button in the base of the well.

Positive and negative RPLA tests are illustrated on page 34.

Summary of Procedure

Toxin extraction from food matrices

Extraction is accomplished by blending 10 g of sample with 10 ml of 0.9% sodium chloride solution, and centrifuging the blended sample at 4°C for 30 minutes. The resultant supernatant is then filtered, and the filtrate is used for assay of toxin content.

This procedure may be modified according to the nature of the food. To obtain a representative sample of a batch of food products, a series of 10 g portions may be collected from different locations within the batch.

Production of enterotoxins in culture fluids

B. cereus may be recovered from food or faecal samples and identified using standard methods. The use of *Bacillus cereus* Selective Agar (Oxoid CM617 and SR99) will aid the isolation and presumptive identification of *B. cereus* prior to toxin detection.

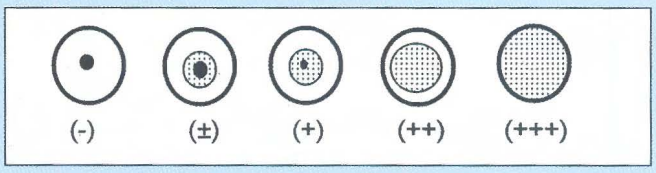
Isolates should be inoculated into Brain-Heart Infusion Broth (CM225) and incubated at 32°C for 6–18 hours. After growth has occurred, the test sample may be extracted by means of centrifugation or filtration.

For full protocol details please see pack insert.

Assay Method

1 Shake reagents thoroughly.
2 Position V-well microtitre plates so that each row consists of 8 wells. (Each sample requires 2 such rows.)
3 Dispense 25 µl diluent in each well of the 2 rows, except for the 1st well in each row.
4 Add 25 µl test sample to the 1st and 2nd well of both rows.
5 Pick up 25 µl from the 2nd well and perform doubling dilutions along each row, ending at the 7th well. The last well should contain diluent only.
6 Add 25 µl sensitised latex (TD951) to each well in row 1.
7 Add 25 µl control latex (TD952) to each well in row 2.
8 Agitate the plate by hand or a micromixer to mix the contents of each well.
9 Cover the plate and leave undisturbed for 20–24 hours.
10 Examine each well for agglutination against a black background. Dispose of all items in hypochlorite solution (>1.3% w/w).

Full details of the test protocol are printed in the product insert.



Interpretation of Test Results

Agglutination should be assessed by comparison with the above illustration. For more detailed procedure and interpretation information, refer to pack insert.

Negative

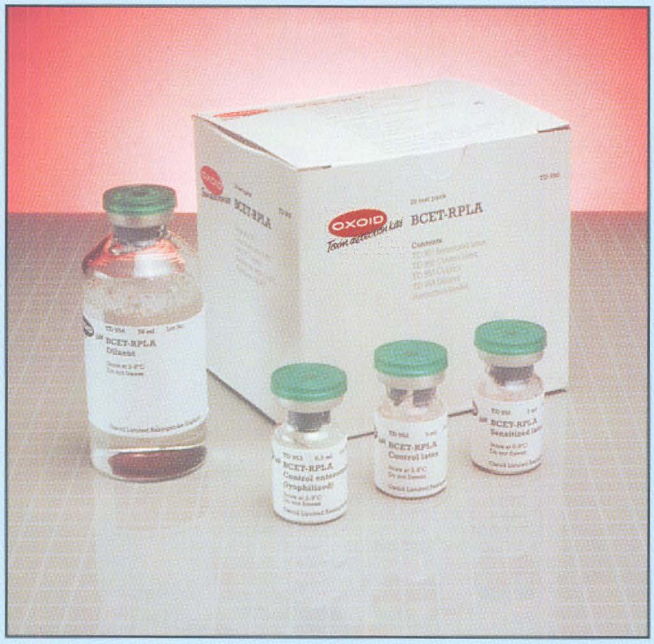
Results classified as (-) and (±) are considered to be negative.

Positive

Results classified as (+), (++) and (+++) are considered to be positive.

References

1 Kramer, J. and Gilbert, R. (1988) In: *Foodborne Bacterial Pathogens* (ed. M. P. Doyle) pp. 21–70. Marcel Dekker Inc. New York.
2 Hauge, S. (1955) *J. Appl. Bacteriol.* **18,** 591–595.
3 Mortimer, P. and McCann, G. (1974) *Lancet* **1,** 1043–1045.
4 Turnbull, P. (1986) In: *Pharmacology of Bacterial Toxins* (ed. F. Dorner and J. Drews) 367–448. Pergamon Press, Oxford.
5 Holbrook, R. and Anderson, J. (1980) *Can. J. Microbiol.* **26,** 753–759.



An Oxoid RPLA Kit.

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Modern Food Microbiology, 4th Edition 1992. Jay, J.M. Van Nostrand Reinhold, New York.

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Foodborne Pathogens. An Illustrated Text (1991) Varnam, A.H. and Evans, M.G. Wolfe Publishing Ltd., London.

Appendix: Oxoid Products for Anaerobic Incubation

Introduction

Recognition that some microorganisms require oxygen-free conditions in order to thrive came early in the history of microbiology. Leewenhoek demonstrated that some of his "animalcules" could exist and grow in gases other than oxygen. Pasteur discovered anaerobiosis and introduced the descriptive terms aerobes and anaerobes for, respectively, organisms that require oxygen and those that do not tolerate it.

Much ingenuity and effort has been put into developing and improving means of achieving anaerobiosis and new ways of doing so continue to evolve, driven by the needs for efficacy, convenience, safety and economy. The first procedures evolved by Pasteur involved boiling of solutions to remove oxygen but the limitations very quickly became apparent. Pasteur, with Roux, then used vacuum to eliminate air as far as possible and with it oxygen. When the technical difficulties of working with vacuum became obvious they abandoned this approach and then adopted the principle of displacing air using carbon dioxide or hydrogen.

McIntosh and Fildes, in their anaerobic jar, introduced the concept of removing active oxygen by using catalytic activity to combine it with hydrogen to form molecules of water. Anaerobic jars have been so successful since their introduction that improvements continue to be made and the operating principle has been extended to large cabinets that serve as anaerobic work stations.

Many other principles have been applied to anaerobic culture, including physical restriction of oxygen access to cultures by overlaying inoculated petri dishes with a second agar layer, and filling media into tubes to create deep columns that restrict oxygen ingress to the top of the column. A variety of chemical reducing agents including glucose, pyrogallol and sulphur-containing compounds, and biological reducing agents, e.g. animal tissues and respiring microorganisms have been, and still are, employed. The examples just given constitute a very incomplete list of ways and means which have been used for anaerobic culture. The subject is broadly reviewed by Anderson and Fung,[1] who also provide a very comprehensive list of references for access to much more detailed information than space allows in the review.

Unipath has played an active part in recent development of anaerobic technique with an anaerobic jar that possesses a number of features which earned the jar a British Design Council award shortly after it was first marketed. The Oxoid Anaerobic Jar, its supporting products and the new AnaeroJar™ which does not employ the principles of hydrogen generation or catalysis are described on the following pages.

Reference

1 Anderson, K.L. and Fung, D.W C. (1983) *J. Food Prot.* **46,** 811–822.

The Oxoid Anaerobic Jar HP11

A 3.4 litre capacity Anaerobic Jar of advanced design that gives great flexibility in use by coping equally well with Gas Generating Envelopes or gas cylinders.

Both jar and lid are of robust construction and used with the Low Temperature Catalyst BR42 ensure unprecedented protection to operator and equipment.

The Oxoid Anaerobic Jar has a number of novel design features and for greater convenience in use it is supplied with a corrosion resistant plate carrier that greatly reduces the time and effort needed to load the jar. A test tube carrier is available as an optional extra minimising the risk of spillage of broth cultures.

Anaerobiosis is achieved rapidly, safely and efficiently using the Gas Generating Kit BR38 or hydrogen obtained from cylinders.

The Oxoid Anaerobic Jar may also be used for the isolation of microaerophilic and CO_2 dependent organisms by using the Campylobacter Gas Generating Kit BR56 or the CO_2 Gas Generating Kit BR39.

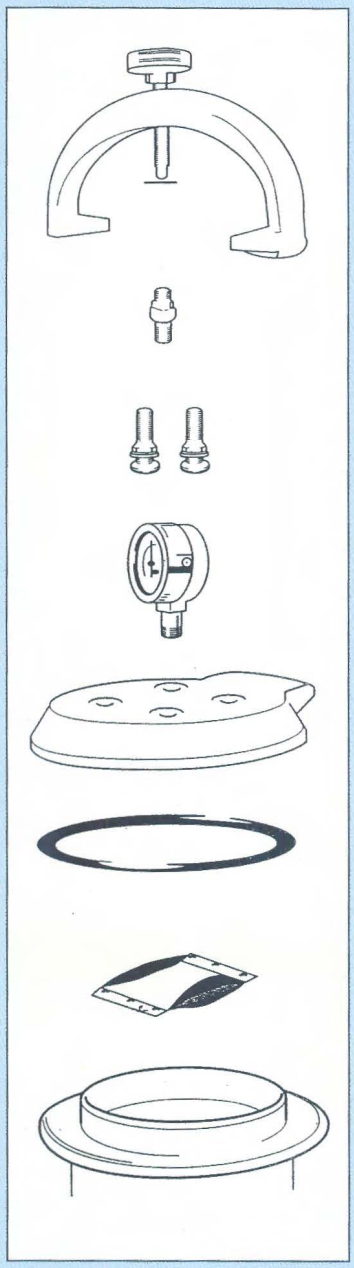
Catalytic activity may be checked both by the pressure gauge for an immediate indication of efficiency and by the Anaerobic Indicator BR55 which provides supporting evidence of the pressure changes. These checks ensure that the absence of growth does not reflect poor anaerobic incubations.

The Oxoid Anaerobic Jar HP11 is part of the complete Oxoid Anaerobic System consisting of:

- 3.4 Litre Anaerobic Jar of advanced design.
- Gas Generating Kit which is superior in design to any other on the market.
- A new, safe, low temperature catalyst.
- An Anaerobic Indicator that is reliable and reacts faster than other equivalent products.

The Oxoid Anaerobic Jar code HP11.

Design Features of the Oxoid Anaerobic Jar HP11



Strong metal clamp

Pressure release valve

Schrader® valves

Easy-to-read pressure gauge

Corrosion-resistant coloured rigid metal lid

O-ring to give a gas-tight seal

Effective and safe low temperature catalyst

Heavy duty transparent jar

Oxoid Gas Generating Kit code BR38

The Oxoid Gas Generating Kit code BR38 is a laminated foil envelope within which is a container holding tablets of sodium borohydride, sodium bicarbonate and tartaric acid. The tablets are activated by the addition of water causing hydrogen and carbon dioxide to be produced.

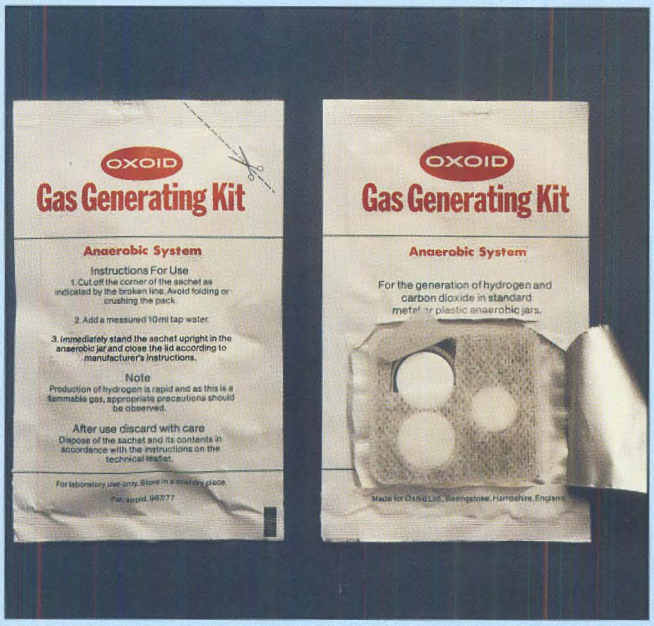
Gas production takes place smoothly and reproducibly because the porous membrane of the inner container regulates the passage of water inwards and gas outwards.

Each individual Gas Generating Kit, when activated with water, evolves sufficient hydrogen for the catalytic removal of oxygen present in the air and leaves the final internal pressure approximating to that of the atmosphere.

Carbon dioxide is also evolved to give a final concentration of 10% v/v in the anaerobic atmosphere.

Gas generation is completed within 30 minutes and, because the resultant solution is acid, it does not reabsorb the carbon dioxide so necessary for the growth of fastidious anaerobes.

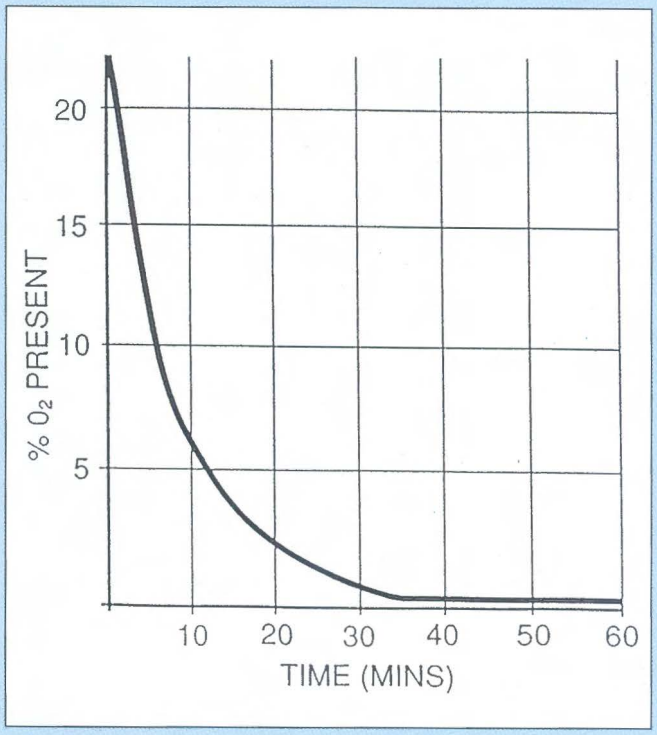
Catalytic activity may be checked both by the pressure gauge for an immediate indication of efficiency and by the Anaerobic Indicator, code BR55, which provides supporting evidence of the pressure changes. These checks ensure that the absence of growth does not reflect poor anaerobic incubations.



The Oxoid Anaerobic Gas Generating Kit code BR38.

The illustration shows a Gas Generating Kit envelope cut to demonstrate the inner sachet holding tablets of sodium borohydride, sodium bicarbonate and tartaric acid. The system is activated by adding water to the envelope. The porous membrane covering the inner sachet regulates water flow inwards and gas flow outwards to give controlled and reproducible evolution of hydrogen and carbon dioxide.

Figure 1 – Rate of oxygen catalysis when using the Gas Generating Kit code BR38. It shows the rapidity with which the O_2 level is lowered in the Oxoid Anaerobic Jar.



The Oxoid Anaerobic Catalyst code BR42

The Oxoid low temperature Catalyst code BR42 is of patented design[1] and is a safer and more efficient version of the cold catalytic devices which are used universally to create low-oxygen atmospheres within anaerobic gas jars.

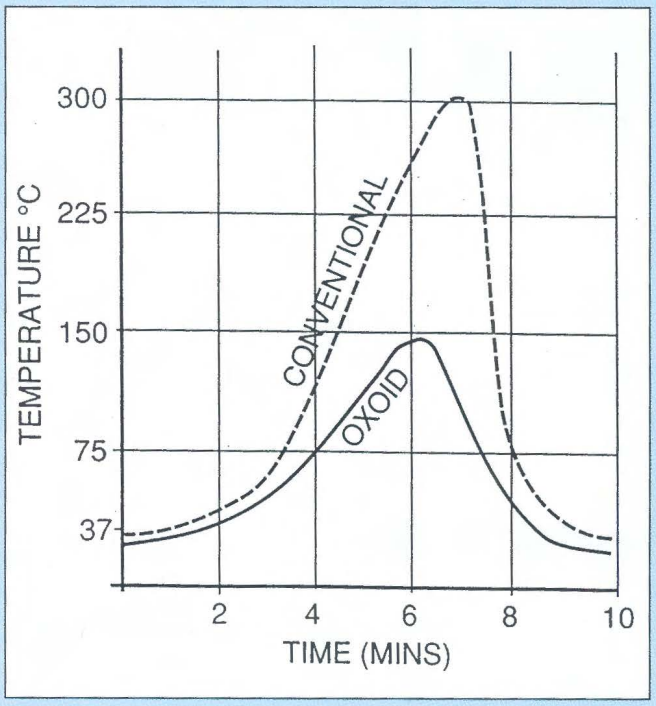
Each Oxoid Catalyst contains 4 grams of palladium-coated pellets and is suitable for use in anaerobic jars up to 3.5 litres effective volume. This follows the recommendation of the United Kingdom, Department of Health and Social Security[2] that there should be not less than 1 gram of catalyst for each litre volume of anaerobic jar. The extra-large charge of activated palladium in the Oxoid catalyst is wrapped in a special foil and enclosed in a large surface area capsule, woven from very fine stainless steel wire.

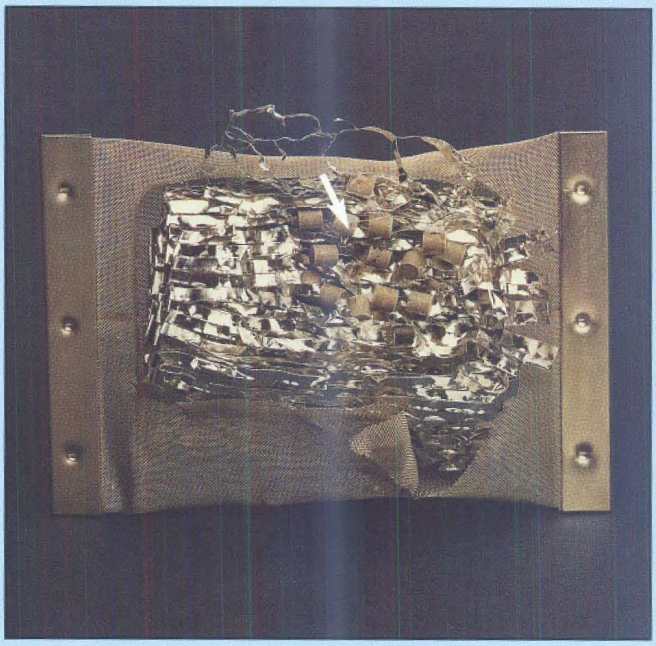
The graph shows the difference in operating temperature between a conventional catalyst (unprotected pellets enclosed in wire gauze) and the Oxoid catalyst. The description 'safe' applied to the Oxoid catalyst refers to the reduction in risk of an explosion in the Oxoid anaerobic jar. Explosions in anaerobic systems although rare, can occur when hydrogen and oxygen are present in critical proportions. Such explosions are triggered by extremely hot catalyst envelope temperature or by glowing particles which have become detached from the main body of catalyst. The Oxoid catalyst design protects users from both of these possibilities.

References

1 Patent application 54354/7 developed by Don Whitley Scientific Limited.
2 United Kingdom Department of Health and Social Security, February 1979.

Figure 2 – Difference in operating temperature between a conventional catalyst (unprotected pellets enclosed in wire gauze) and the Oxoid Catalyst code BR42.





The Oxoid Low Temperature Catalyst code BR42.

The illustration shows an Oxoid catalyst cut to demonstrate its construction.

Palladium catalyst pellets (arrowed) are held separated from each other in a wrapping of shredded aluminium foil which immobilises the pellets and absorbs heat generated during catalysis. The catalyst package is contained within a securely sealed fine wire mesh sachet to prevent escape of small particles of active catalyst into the jar atmosphere.

The Oxoid Anaerobic Indicator code BR55

The Oxoid Anaerobic Indicator code BR55 consists of a cotton strip impregnated with a redox indicator solution enclosed in a laminated foil envelope. This formulation and the purity of the cotton strip gives a reproducible redox colour change in a shorter time than similar products that are available. Use of the Oxoid Anaerobic Indicator will support the evidence of pressure changes which occur with active catalysts and ensure that absence of growth does not reflect poor anaerobic incubation.

AnaeroJar™ AG025

AnaeroJar has been specially designed for use with AnaeroGen and CampyGen.

The 2.5 litre Jar will hold up to 12 disposable plastic petri dishes at a time, and forms an integral part of the complete Oxoid Atmosphere Generation System.

Convenient and safe

The jar's practicality complements the innovative technology which makes AnaeroGen and CampyGen sachets so easy to use.

The polycarbonate base is secured to the lid by means of four spring loaded clips that make it simple to open and close. The clips themselves allow venting to occur in the unlikely event of a pressure increase, thus maintaining optimum conditions within the jar.

A handle has been designed as an integral part of the lid and folds into the lid circumference when not in use. Consequently it does not add to the overall dimensions of the jar. The handle can quickly be adjusted into place to maximise safety when carrying the jar.

Vacuum relief feature

An easy to operate vacuum relief screw permits the ingress of air to the jar, thus overcoming any difficulty in opening the lid because of negative pressure.

Space-saving design

The AnaeroJar is lightweight yet robust. Its uncomplicated design makes it easy to maintain and load, minimising the possibility of malfunction.

It saves space in the incubator: there are no external clamps or protruding valves, and the carrying handle does not occupy additional space.

The Oxoid AnaeroJar™ shown with AnaeroGen™ and the Anaerobic Indicator code BR55.

Anaerogen™

CODE: AN25 FOR 2.5 LITRE JARS

CODE: AN35 FOR 3.5 LITRE JARS

Description

An AnaeroGen sachet placed in a sealed jar rapidly absorbs atmospheric oxygen and simultaneously generates carbon dioxide. This novel method differs from those commonly used in that the reaction proceeds without evolution of hydrogen and therefore does not require a catalyst. Furthermore, addition of water is not needed to activate the reaction. The active component is ascorbic acid.

When used as directed, an AnaeroGen sachet will reduce the oxygen level to below 1% within 30 minutes. Carbon dioxide content will increase to 9%–13%.

Precautions

This product is for *in vitro*-use only.

As soon as the AnaeroGen paper sachet is exposed to air the reaction will start. It is therefore essential that the paper sachets are placed in the jar without delay and the jar sealed within 1 minute.

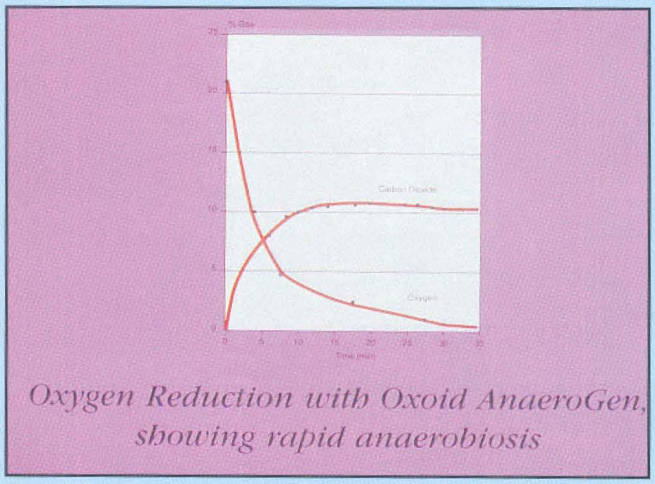
The ascorbic acid and oxygen react exothermically. The sachet will become warm to the touch but the temperature will not exceed 65°C.

Directions

AN35 is designed for use in 3.5 litre jars. It is therefore suitable for the Oxoid Anaerobic Jar code HP11 and for other jars of similar capacity.

AN25 is designed for use in 2.5 litre jars such as the Oxoid AnaeroJar™ code AG25 and other jars of similar capacity.

1 Place the inoculated plates in the anaerobic jar. Disposable plastic petri dishes should be of the vented variety to aid gas transfer between the interior and exterior of the plates.



Oxygen Reduction with Oxoid AnaeroGen, showing rapid anaerobiosis

Rate of oxygen reduction by Anaerogen™. It shows the rapidity with which anaerobic conditions are created in the Anaerojar™.

2 Tear open an appropriate AnaeroGen foil sachet at the tear-nicked indicated and remove the paper sachet from within.

3 Immediately position the paper sachet in the clip on the plate carrier in the jar. The sachet will become warm to the touch on exposure to air.

4 Close the jar lid immediately.
N.B. The time taken between opening the foil sachet and sealing the jar should not exceed 1 minute. Extended exposure will result in loss of reactivity and consequently satisfactory anaerobic conditions may not be achieved.

5 After incubation, examine the plates for the presence of anaerobes. If continued incubation is needed, a fresh AnaeroGen sachet must be used. Repeat steps 2–5.

6 After incubation, the exhausted AnaeroGen sachet should be discarded with appropriate laboratory waste.

Control Testing

It is recommended that an Oxoid Anaerobic Indicator (BR55) is put in the jar as a visual check that anaerobic conditions have been achieved and maintained.

Users should check their anaerobic system periodically for its ability to provide adequate conditions for growth of appropriate bacteria. The following strains are recommended:

Clostridium novyii	ATCC® 9690 – growth
Micrococcus luteus	ATCC® 9341–no growth

Disposal

On removal from the jar after incubation, the AnaeroGen paper sachet will retain a small amount of reactivity and will become warm. The sachets should therefore be allowed to cool to room temperature prior to disposal.

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