Part II

Particular techniques

6

Culture methods

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6.1 Introduction

Culture methods for detecting pathogens in food have existed for more than a century. To this day, culture methods still form the foundation of nearly all methods for detecting pathogens in food. In fact, in the majority of situations, culture methods still form the detection method in its entirety. In a recent survey of 312 food testing laboratories within Europe more than 99 per cent of respondents said they were using conventional culture methods for the isolation of most food pathogens (Chris Baylis, personal communication). In the same survey, respondents listed universal acceptance, cost effectiveness, reliability and ease of use as the main reasons for choosing culture methods.

In this chapter, the key components of culture medium design will be reviewed. The principles of culture method structure, both quantitative and qualitative, will be discussed and examples given. Recent, continuing and future innovations in culture methods will be presented.

6.2 Culture medium design

6.2.1 Basic culture medium components

Culture methods, according to their definition, are methods that involve the growing or keeping alive of a collection of microorganisms in or on a nutrient medium. In the most basic sense nutrient media comprise up to six key components disolved in water:

- amino-nitrogen compounds
- energy sources

- buffer salts
- mineral salts and metals
- growth promoting factors
- gelling agents.

Amino-nitrogen compounds (peptones, other protein hydrolysates, infusions or extracts)
Relatively few microorganisms can utilise heated and denatured protein as a source of amino-nitrogen as this would depend on the excretion of extracellular proteolytic enzymes. Therefore, pre-digested or acid-hydrolysed protein, or aqueous infusions/extracts of protein rich materials are used. Peptones are water-soluble mixtures of polypeptides, oligopeptides and single amino acids, together with the other water-soluble compounds present in the original proteinaceous substrate. The peptides formed are of varying chain lengths and amino acid sequences, which depend on the protein substrate and the process of hydrolysis. Proteolytic enzymes hydrolyse proteins by splitting specific peptide bonds, thus producing characteristic peptones. It follows, therefore, that a wide variety of peptones can be made by allowing different proteolytic enzymes to act on different proteins. Commercially, only three enzymes are generally used: papain, pancreatin and pepsin. The variety of protein sources, however, is large including meat (fresh, frozen, dried), casein (precipitated from milk with lactic or hydrochloric acids), gelatin, fish (fresh, dried), keratin (horn, feather), soya and many types of vegetable derived material.

Energy sources (e.g. glucose)
The most common components used for this purpose are glucose or similar easily utilised carbohydrates intended to provide a readily accessible source of energy. Media that do not contain an identifiable carbohydrate source provide peptones or similar compounds, which are utilised by the organisms as sources of carbon and energy.

Buffer salts (e.g. soluble phosphate salts, acetates and citrates)
Buffering agents are commonly added to media supplemented with energy sources, to maintain pH stability if fermentation is likely to take place. They are also used to prevent adverse shifts in pH when sample materials such as food are added to the medium.

Mineral salts and metals (phosphates, sulphates, calcium, magnesium, iron, manganese and trace metals)
Essential metals are normally present as contaminants in other medium components, at adequate levels to improve growth. Specific supplements are often not made, unless to overcome the chelating effects of competing compounds in the medium. A common reason for the use of these additives is to enhance and identify a particular biochemical reaction. In these circumstances they act as indicator substances, e.g. iron and sulphites (Wilson, 1923).

Growth promoting factors (blood, serum, vitamins, NADH, etc.)
There is a diverse assortment of complex, often heat-labile substances, essential for the growth of fastidious or particularly demanding microorganisms, which are not present in unsupplemented media. These essential factors are not always solely acting in a nutritional role, e.g. blood in culture media for the isolation of *Campylobacter* also acts as a protective agent against reactive oxygen species and influences dissolved oxygen levels (Bolton *et al*., 1984).

Gelling agents (agar, gelatin, alginate, gums, etc.)
Agar is the predominant gelling agent used to provide a solid matrix for the visualisation of growing microorganism colonies. It is rarely a pure ingredient and it contributes metals and minerals as well as exerting a significant effect on water availability. Gelling agents can also be used as selective components by controlling the movements of motile microorganisms (De Smedt *et al*., 1986).

6.2.2 Selective culture medium components

In food microbiology, when detecting specific pathogens in a sample containing many different species, it is often essential to incorporate one or many selective features into a culture medium in addition to the basic components listed above. Selective agents preferentially or selectively allow for the growth of the target group from a sample. Healthy bacteria of the target group should be resistant to the selective compounds at the concentrations used. The more common selective agents of choice can be divided into four groups:

- inorganic salts
- dyes
- surface-active agents
- antibiotics.

Inorganic salts
Examples include sodium azide active against Gram negative bacteria (Mossel *et al*., 1957), bismuth sulphite active against coliforms (Wilson and Blair, 1926), lithium chloride active against Gram negatives and enterococci (van Netten *et al*., 1989), potassium tellurite active against Gram negatives (Baird-Parker, 1962), tetrathionate active against Gram positives and coliforms (Knox *et al*., 1943), and sodium selenite active against Gram positives and coliforms (Leifson, 1936).

Dyes
Acridines and triphenylmethane dyes have been known, since the beginning of the last century, to possess outstanding powers of bacteriostasis. Examples include acriflavine (van Netten *et al*., 1989), crystal violet (Jasper and Dellinger, 1967), brilliant green (Moats and Kinner, 1974) and malachite green (van Schothorst and Renaud, 1985).

Surface-active agents
MacConkey first used bile in culture media in 1905. The active components of bile, bile salts, are more chemically defined and used more frequently in the medium formulations of today (Leifson, 1935). Other surface-active components include cetrimide (Brown and Lowbury, 1965), lauryl sulphate (Mallmann and Darby, 1941) and a range of proprietary surfactants such as Tergitol (Chapman, 1947).

Antibiotics
Because of the numbers of different antibiotics available and the differing spectra of activity antibiotics provide for targeted selectivity. Antibiotics as selective agents are in use as single selective agents, as mixtures with other antibiotics, and even in conjunction with the more conventional chemical selective agents listed above.

6.2.3 Differential culture medium components

In addition to selecting for microorganisms of the target species, it is also common to use components that allow the differentiation of the target species from other species using differences in their biochemistry.

Indicator dyes (e.g. phenol red, neutral red, bromocresol purple)
These dyes are added to indicate pH changes in media following growth of microorganisms, particularly when carbohydrates have been added to the formulation. Colour changes as a result of alkaline changes in pH are also used.

Specific substrate indicators (e.g. chromogens, fluorogens)
Chromogens are indicator compounds that act as substrates for specific enzymes and change colour due to the action of the enzyme. The chromogenic enzyme substrates generally comprise a specific enzyme substrate such as a sugar or amino acid linked to a chromophore. The compound remains colourless until utilised when the chromophore is released giving rise to coloration of the medium and/or cells. The ability to detect specific enzymes using the appropriate substrates has led to the development of a great number of new media for the identification of pathogens (Manafi, 2000). Fluorogens are similar in principle but, following enzyme action, a fluorophore is released that can be detected when observed under UV illumination.

The action of the targeted microbial enzyme on a single substrate can achieve much greater specificity than indicator systems based on changes in pH. The greatest gains are made, however, when combinations of chromogens and pH indicators are used, each targeting a different enzyme system. Much more information can be gained sooner regarding the identity of the microorganisms present in the original sample using media containing these combinations. Most developments have evolved around chromogenic enzyme substrates for detecting activity from β-galactosidase (Ley *et al*., 1993), β-glucuronidase

(Feng and Hartman, 1982), β-glucosidase (Manafi and Sommer, 1993), caprylate esterase (Humbert *et al.*, 1989) and phospholipase C (Restaino *et al.*, 1999).

The successful use of chromogens is dependent on the correct enzyme expression that might require the presence of the appropriate inducers in the medium. Careful medium design must also take into consideration that some released indicators diffuse freely into the medium causing coloration of neighbouring cells, or the indicator might be pH sensitive and not produce the correct colour.

Other diagnostic features (e.g. virulence factors)

Culture media have been designed to incorporate diagnostic features that detect the presence/activity of key virulence factors that can be used in the isolation and identification of the target microorganism species. Haemolysin activity has long been detected by the inclusion of blood into media (Turner and Pickard, 1980). Egg yolk emulsion (Baird-Parker, 1962) and lecithin (Chrisope *et al.*, 1976) have been included for the detection of phospholipase activity, and rabbit plasma fibrinogen (Beckers *et al.*, 1984) has been used to detect coagulase activity.

6.2.4 Incubation conditions (e.g. pH, temperature and gaseous atmosphere)

Use of buffer salts to resist adverse changes in pH in order to allow growth of the desired microorganism has been discussed above. Control of pH is important to allow other medium components to function optimally, e.g. antibiotic potency, precipitation of bile salts and metal complexes. Additionally, control of pH at low and high values can be used to selectively favour the growth of the target microorganism at the expense of competing species (van Schothorst and Renaud, 1985). Likewise, incubation temperature can be used to select for rapid growth of the target microorganism as well as restricting the growth of competing species. Selective incubation temperature can be lower (Hao *et al*., 1987), as was the case for many early isolation methods for *Listeria monocytogenes*, as well as higher (van Schothorst and Renaud, 1985). Incubation temperature may also be critical for the optimum expression of certain diagnostic features including antigens, e.g. *L. monocytogenes* and flagella expression at 30ºC but not at 37ºC (Peel *et al*., 1988).

Control of gaseous environment is also important to provide the optimum conditions for growth of the target microorganism. This is particularly relevant for the isolation of microaerophilic species such as *Campylobacter*. Control of oxygen concentration is typically achieved through the use of controlled atmosphere incubation in jars and cabinets. It can also be achieved through the use of oxygen consuming medium ingredients. In some instances the dynamic interaction of many factors can be important in the function of culture media. In *Campylobacter* isolation the control of oxygen concentration is not only affected by the external control of atmospheric conditions but also the growth of

competing microorganisms that will significantly affect the dissolved oxygen content.

Anaerobic conditions can be beneficial for the resuscitation of stressed pathogens such as *Salmonella*, *L. monocytogenes* and *Escherichia coli* O157 (see Section 6.2.5). Such conditions would also provide a selective benefit by restricting the growth of aerobic competing species such as *Pseudomonas*.

6.2.5 Resuscitation conditions (e.g. supplements and medium design)

Pathogens present in food may be injured and as a result more exacting in their growth requirements. Such microorganisms can be difficult to detect because they are sensitive to the selective agents used traditionally in culture media. Stressed microorganisms can be sensitive to low levels of reactive oxygen species (ROS) that can exist in culture media, and stressed microorganisms have extended lag times during which repair of damage takes place. To ensure the detection of injured pathogens culture media can be supplemented with components to quench the toxicity from selective agents and ROS. Medium components can be chosen that are known to be low in toxicity and methods can be constructed that allow for complete resuscitation before exposure to selective agents (see Section 6.3).

Recent developments in recovering injured cells have focussed on defining the time needed for repair of individual cells within a population (Stephens *et* al., 1997) and in formulating resuscitation media and/or conditions to avoid exposing cells to oxidative stress (Stephens *et al*., 2000). Two sources of ROS exist for injured microorganisms recovering in a non-selective culture medium (Stephens *et al*., 2000). Firstly, the medium itself can be a source of ROS, primarily as a result of photo-oxidation reactions of medium components with light, and secondly as a result of auto-oxidation reactions of medium components at ambient and high temperatures used for sterilisation. Levels of ROS in culture media are very low and are usually not inhibitory to uninjured microorganisms. Reactive oxygen species can be prevented from forming by careful medium design or quenched after they have been produced through the use of specific medium components such as blood (Bolton *et al*., 1984), pyruvate (Baird-Parker, 1962), catalase (Mackey and Seymour, 1987), superoxide dismutase (Hoffman *et al*., 1979) and cysteine (Knabel and Thielen, 1995). Beneficial effects have been reported in many foodborne pathogens including *E. coli* O157 (Mizunoe *et al*., 1999), *Camp. jejuni* (Bolton *et al*., 1984), *Salm. typhimurium* (Mackey and Derrick, 1986), *Staphylococcus aureus* (Baird-Parker, 1962) and *L. monocytogenes* (Patel and Beuchat, 1995).

The second source of ROS is the aerobic metabolism of injured microorganisms. Through a variety of possible mechanisms, injured cells metabolising aerobically suffer oxidative stress as a result of increased intracellular levels of ROS (Stephens *et al*., 2000). By encouraging these microorganisms to metabolise anaerobically, as might be the case following ingestion, they are prevented from producing further undesirable ROS. The use

of anaerobic conditions to improve recovery of stressed cells has been reported widely for *Salmonella* (Xavier and Ingham, 1993), *Listeria* (Linton *et al*., 1992), *E. coli* O157 (Bromberg *et al*., 1998) and *Staph. aureus* (Ugborogho and Ingham, 1994). Strictly anaerobic conditions are usually achieved using the Hungate technique (Hungate, 1969) or by working entirely within an anaerobic cabinet. Neither of these is suitable for routine microbiological work. An alternative method of achieving anaerobiosis is to use a crude membrane preparation from *E. coli* that can be added to liquid media to scavenge oxygen with high affinity (Adler *et al*., 1981). A membrane preparation known as Oxyrase is now available commercially (Adler, 1990). By using a combination of a peptone medium low in ROS and Oxyrase, significant improvements in the detection of *Salmonella* in spiked ice cream and milk powder have been reported (Baylis *et al*., 2000a).

6.3 Culture method design

Culture methods for detecting pathogens in food fall into two categories, those for quantitative (enumeration) analysis and those for qualitative (presence/absence) analysis.

6.3.1 Quantitative methods (enumeration)

The number of viable cells of a target species in a specified sample can be counted by the spread-plate method, the pour-plate method and the most probable number method (MPN).

The spread-plate method can be used with opaque media and avoids exposing the inoculum to mild heat stress as occurs with the pour-plate method. Many differential features of media may only be displayed correctly by the spread-plate technique whereby surface colonies are growing under the appropriate gaseous conditions. Similarly, it is easier to subculture surface colonies for subsequent identification/confirmation. Only a small inoculum, typically 100 μl, can be used on a spread-plate in a standard 90 mm Petri dish. Where low counts are expected larger Petri dishes can be used to spread up to 1ml of sample. Where high counts are expected the sample must be serially diluted with each dilution used to inoculate a separate spread-plate. Alternatively the drop-plate method or Miles Misra technique can be used (Hedges *et al.*, 1978). Enumeration by spread plating can also be achieved using a spiral plater whereby a decreasing volume of inoculum is deposited in the form of a spiral over the agar medium from the centre to the edge of the plate (Donnelly *et al.*, 1976). This procedure allows a range of up to four decimal dilutions to be counted from a single plate.

With the pour-plate technique, a larger inoculum volume, typically 1 ml, can be used. The sample is added directly to an empty Petri dish and tempered medium is poured into the dish and the sample and medium mixed before the

medium solidifies. Precise cooling of the medium prior to mixing is essential to prevent loss of viability during mixing. Solidified pour-plates are often overlaid with a thin layer of the same medium to prevent the spreading of colonies on the surface of pour-plates.

Numerous strategies have been used to avoid underestimating microbial numbers in samples likely to contain stressed cells. These include formulating the medium to minimise any inhibitory effects on injured target microorganisms and performing a resuscitation treatment to allow repair of injury before inoculation on/into a selective medium. There are few examples of the first option in current use. Baird-Parker agar (Baird-Parker, 1962) supports good recovery of *Staph. aureus* cells damaged by drying, freezing or heating but there are not many other examples of such media. Where inhibitory selective media cannot be avoided, resuscitation on solid or in liquid media is needed.

Solid medium repair is the method of choice where quantitative estimates of viable numbers are needed. This can be achieved by spreading cells on the surface of a non-selective agar medium and, after allowing for repair to occur, overlaying with a selective medium (Speck *et al*., 1975). A more satisfactory method is to incubate cells on a membrane filter on a non-selective medium before transferring the membrane filter to selective agar (Holbrook *et al*., 1980). Recently, this approach has been used for the enumeration of *E. coli* O157 in foods (McCarthy *et al*., 1998). Thin polycarbonate membranes were used that facilitated transfer of the pH indicator into the developing colony, thus allowing the fermentation reaction to be easily visualised on the surface of the membrane. Both these approaches have the advantage that should growth of uninjured cells take place during the resuscitation period this will not lead to a falsely high count as the early growth will only contribute to the development of a single colony.

Agar 'underlay' methods for the enumeration of stressed *L. monocytogenes* (Kang and Fung, 1999) and *Salm. typhimurium* (Kang and Fung, 2000) have also been developed. In this method, the selective medium is overlaid with non-selective medium immediately before the sample containing stressed cells is plated onto the surface of the upper non-selective layer. Initially the upper layer remains non-selective allowing resuscitation to take place. Selective agents then diffuse into the upper layer from the selective medium thus suppressing the growth of competing bacterial species. Some success has been reported from the addition of 'recovery supplements' to selective agars to aid the resuscitation of stressed cells in the presence of selective agents. The addition of egg yolk emulsion to Oxford and PALCAM agar formulations has improved the recovery of stressed *L. monocytogenes* (Wood *et al*., 1996).

The MPN method of enumeration is particularly useful when low counts are expected, when the amount of material to be tested is too large for the spread- and pour-plating methods, when testing particulate samples and when there is concern over the presence of injured cells in the sample material. In the MPN method, counts are estimated from examining multiple cultures prepared from multiple aliquots of a dilution series, and determining the proportions of such

cultures that show growth. The multiple tube count is the commonest form of MPN enumeration. A liquid enrichment medium is chosen that will support the recovery and growth of the target species. The determination of growth of the target species is made according to the production of an appropriate reaction in the medium, e.g. pH change, indole production, gas production, etc. In some instances an MPN procedure can involve processing each aliquot in accordance with a full qualitative test including primary enrichment, selective enrichment and plating. The number of aliquots and the range of dilutions required are dependent on the expected level of contamination and the precision required from the estimated count. Reference to probability tables or the use of probability equations obtains the MPN estimate. A disadvantage of the MPN method is that it is labour intensive and the precision is poor unless the number of replicate tubes per dilution is very large.

6.3.2 Qualitative methods (presence/absence)

Depending on the level of sensitivity required there are up to four sequential stages in a qualitative culture method for detecting pathogens in food:

- primary enrichment (pre-enrichment)
- selective enrichment
- plating
- confirmation.

Primary enrichment

Direct inoculation of injured cells into selective media can result in the loss of viability of some or all of them, the latter giving rise to a false negative result. A period of time in a non-selective, or reduced selectivity, medium allows the repair and growth of any sublethally injured microorganisms prior to their exposure to the selective components of selective enrichment. Numbers of target cells must be reached that ensure both their transfer on subculture to a selective medium and their survival in the selective medium in sufficient numbers that their growth is ensured (van Leusden *et al*., 1982). A non-selective primary enrichment culture will allow the growth of the majority of the microorganisms present in a food sample; these microorganisms may overgrow the target species. By the time repair has occurred, other uninjured or less-injured microorganisms may form the dominant species. When the dominant species enter their own stationary phase, they may inhibit the further growth of all other species present, including the target species that may still be in such small numbers that their detection after the selective enrichment stage is uncertain. Jameson (1962) first described this stationary phase inhibition. The early and/or progressive introduction of selective agents to the primary enrichment medium helps overcome the Jameson Effect.

The use of short primary enrichment incubation (e.g. six hours) is not sufficient to allow recovery and multiplication of all injured cells (Stephens *et*

al., 1997). The existing incubation times of 16 to 20 hours are a compromise between a longer incubation time that may allow better recovery of severely damaged cells, and the increased risk of overgrowth from competing microorganisms.

Certain foods require specific treatments for primary enrichment (Andrews and Hammack, 1998). When testing dehydrated products controlled rehydration of the sample is important to minimise osmotic shock to damaged cells. When testing herbs and spices their natural antimicrobial constituents require extra dilution, up to 1:1000, to provide conditions in which cells of the target species can grow. Milk solids might be added to cocoa rich samples to neutralise toxicity and thiosulphate might be added as a quenching agent to preservative-rich food samples.

Selective enrichment

The purpose of selective enrichment is to provide a ratio of target species to competing microorganisms that allows for successful detection. Selective agents and incubation conditions of the type described above are used to suppress the growth of as many competing microorganisms as possible whilst allowing growth of the target species. A 1:10 or 1:100 dilution of the primary enrichment medium is followed typically by an incubation period of 24 to 48 h.

Following primary enrichment it is probable that the target species will still be in the minority because of the low selectivity that exists in primary enrichment media. In a selective enrichment broth culture inoculated with a mixture of microorganisms, competitive growth occurs between all species able to tolerate and use the selective conditions. Rarely from a heterogeneously contaminated sample does any selective enrichment allow the growth of the target species alone. Diagnostic indicators might be included in the selective enrichment medium but they are unlikely to be sufficiently sensitive or specific to provide a confirmed result.

Plating

Plating forms the detection phase of a conventional culture method for pathogen detection in food. Theoretically, plating is one of the most sensitive detection techniques in routine use. Typically a 10 μl aliquot is transferred to the plating medium. The presence of one typical colony is sufficient to suggest contamination of the initial sample being analysed. Assuming no loss of viability upon plating and complete inhibition of competing micro-organisms, then plating could detect as few as 100 cells/ml of the enrichment broth. Realistically there is some loss of viability upon plating, and growth of competing microorganisms is likely, reducing the sensitivity by up to 100-fold. If competing microorganism growth outnumbers the target microorganism by >1000:1 then the presence of the target microorganisms will be masked entirely and the sample falsely recorded as negative. To maximise the chance of isolating the target microorganism from an enrichment broth upon subculture to selective agar, an appropriate streaking

technique must be employed. The principle is that ever-decreasing numbers of cells are spread over an increasing surface area of the medium to obtain discrete, well-separated colonies that fully exhibit the diagnostic features of the medium. Semisolid plating media exist and they use the migration of motile target bacteria through or over the medium as an additional selection process (De Smedt *et al.*, 1986). The concentration of the gelling agent is critical to the performance of such media

Confirmation

Few selective and differential plating media are wholly specific. For both quantitative and qualitative methods colonies displaying the correct typical appearance must still be confirmed to be the target microorganism before the analysis could be considered complete. Confirmation can take the form of a series of biochemical tests, antigenic tests or nucleic acid analysis. Purification of the suspect colonies might be required prior to confirmation by means of further subculture onto a differential plating medium.

6.4 Examples of qualitative methods

Across the world and across trade organisations there are often many different methods of analysis for the same microorganism of concern. The International Organisation for Standardisation (ISO) technical committee 34/sub-committee 9 is responsible for compiling standardised horizontal microbiological methods of analysis for all food and animal feeding stuffs. The standardised methods published by this committee represent an international consensus of opinion on the most appropriate method for the intended purpose. The standardised methods are not necessarily the most sensitive or the simplest to operate but act as reference methods to facilitate international trade without endangering the consumer. It might be considered that as *Salmonella*, *L. monocytogenes*, *Campylobacter* and *E. coli* O157 are pathogens that can contaminate food that their methods of analysis would be similar. However, their standardised methods of analysis differ not only in specific media, as would be expected, but differ considerably in their structure and design.

The first attempts to compile an international standard method for the detection of *Salmonella* in foods were made by ISO more than three decades ago. The method has had several technical changes made to it since then but the method in place today is similar in structure to the very first standard method (Fig. 6.1) (Anon, 2002). There is more performance data available for culture methods for *Salmonella* isolation than for any other pathogen. For this reason, and the ubiquitous nature of *Salmonella* contamination of food, the standardised method for its detection is the most thorough of all the pathogen methods. Samples are first incubated in an entirely non-selective primary enrichment (pre-enrichment) medium to facilitate resuscitation of stressed cells. This is then subcultured into two selective enrichment media. Two

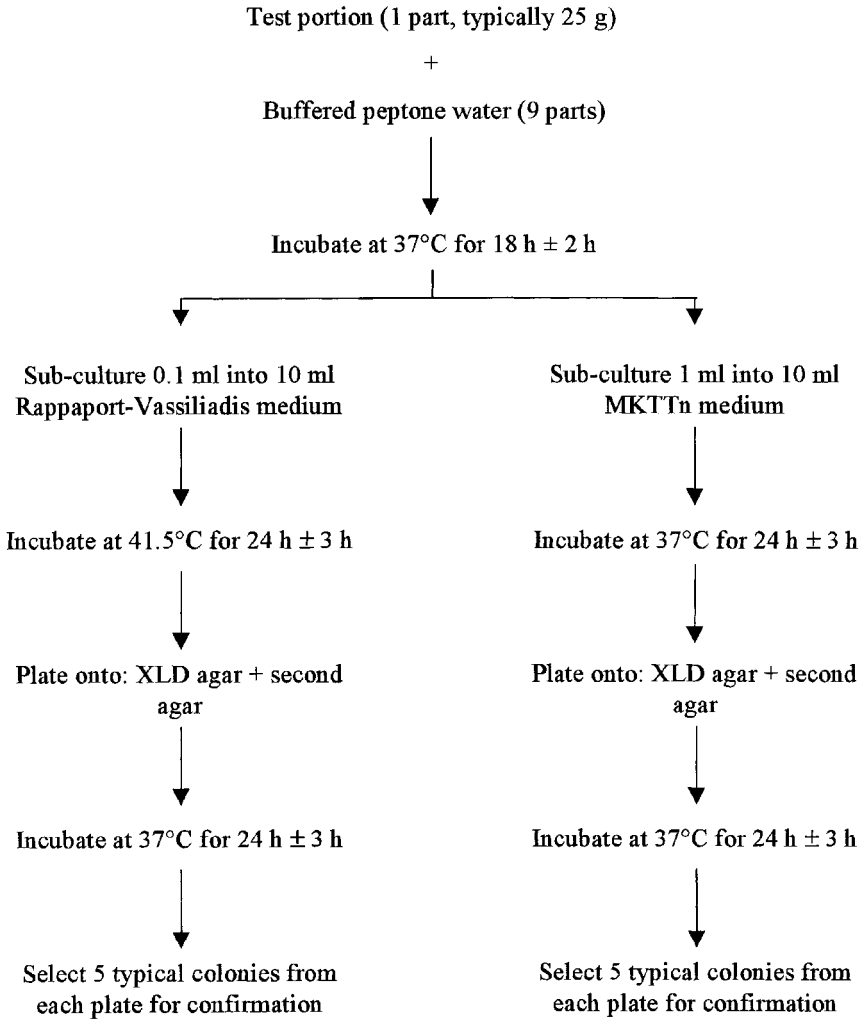

Fig. 6.1 ISO 6579:2002 (Anon, 2002). Microbiology of food and animal feeding stuffs – horizontal method for the detection of *Salmonella* spp.

media are necessary because of the biological diversity of the *Salmonella* group, and the need to maximise the chance of detecting all strains. Similarly, it is necessary to utilise two different selective plating media. Because of the close relatedness of *Salmonella* and some other species of the Enterobacteriaceae it is important that the proper plating and confirmation methods are followed.

Following much activity in the early 1990s regarding *L. monocytogenes* method development, ISO published their standardised method in 1996 (Fig. 6.2) (Anon, 1996). *Listeria monocytogenes* does not compete well in mixed

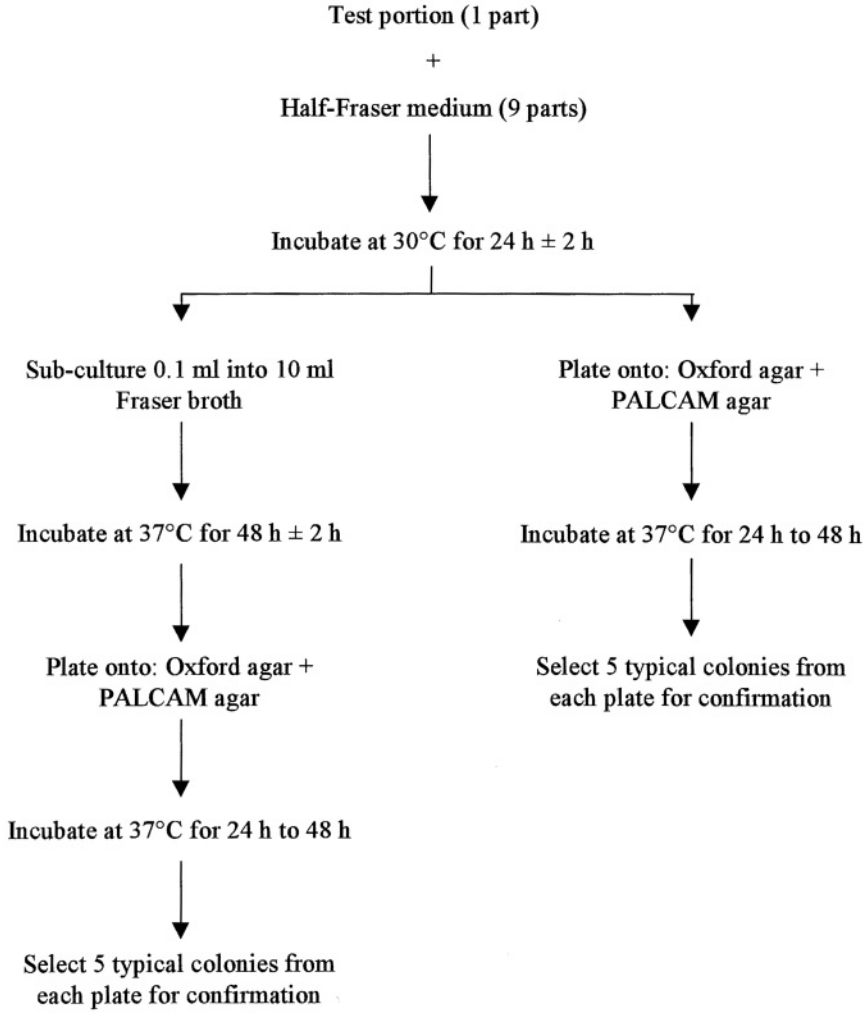

Fig. 6.2 ISO 11290-1:1996 (Anon, 1996). Microbiology of food and animal feeding stuffs – horizontal method for the detection and emuneration of *Listeria monocytogenes*. Part 1. Detection method.

cultures where the dominant microflora are Enterobacteriaceae. To reduce the risk of a false negative result due to overgrowth of competing microorganisms it is necessary to include some selective agents in the primary enrichment medium. This is at the expense of not resuscitating all stressed cells. Unlike *Salmonella*, not all species of the *Listeria* genus are of concern. However, because of their close relatedness it is difficult to devise a mix of selective agents that preferentially enriches *L. monocytogenes* and not members of the other *Listeria* species (Scotter *et al*., 2001).

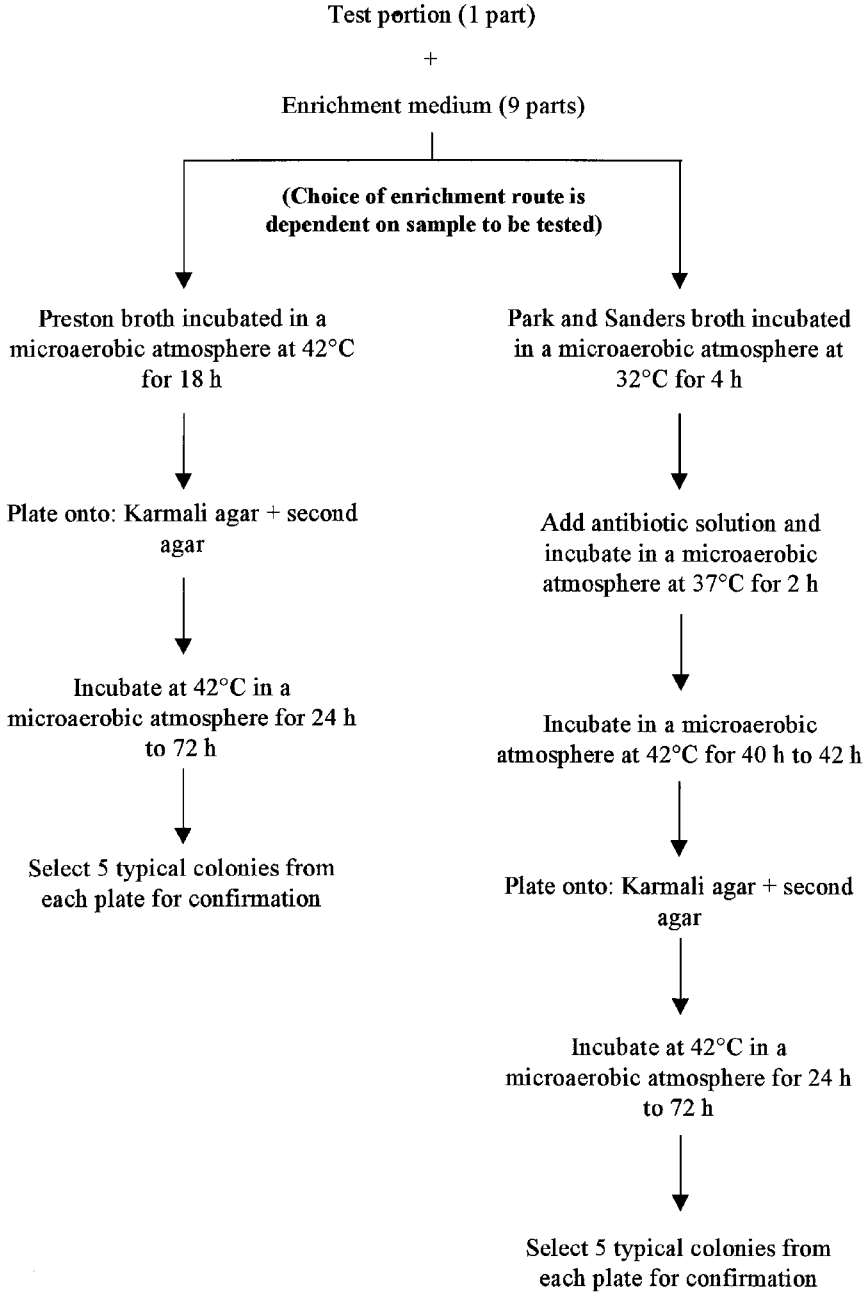

Fig. 6.3 ISO 10272:1995 (Anon, 1995). Microbiology of food and animal feeding stuffs – horizontal method for the detection of thermotolerant *Campylobacter*.

The method is due for modification with changes proposed to the selective plating media. The first-choice medium of Oxford agar, which is unable to differentiate the pathogenic *L. monocytogenes* from the other *Listeria* spp. is to be replaced with a more differential chromogenic medium.

The ISO standardised method for *Campylobacter* detection has two different protocols depending on whether it is to be used for detection in foods heavily contaminated with competing microorganism species or detection of low levels of stressed cells (Fig. 6.3) (Anon, 1995). The protocol for heavily contaminated foods involves direct selective enrichment in a medium containing four antimicrobial compounds and incubation at 42ºC. The protocol for use with foods, where the detection of low levels of stressed cells is of concern, involves a delayed introduction of antimicrobial compounds and a gradual increase in incubation temperature. The method is due for modification with changes proposed to the enrichment protocols, with the inclusion of Bolton Broth (Baylis *et al*., 2000b), and the choice of plating media.

The first ISO standardised method for the detection of *E. coli* O157 was published in 2001 (Fig. 6.4) (Anon, 2001). The method differs to the other three described above in that it does not include a non-selective or reduced selectivity primary enrichment step. Although reflecting the need to produce a prompt standardised method, following the rapid emergence of this pathogen, and the fact that the main food type likely to be contaminated is raw meat, it is still seen as a weakness of the method that it is unsuitable for the detection of low levels of stressed cells (Stephens and Joynson, 1998). A second difference of the method is the incorporation of an immunomagnetic separation step (Chapman *et al*., 1994). In the three methods described above the main selective challenges have been to distinguish between the target species and microorganisms from other species, albeit closely-related species. In the detection of *E. coli* O157 it is necessary to distinguish a specific serogroup of *E. coli* from all other *E. coli* as well as numerous closely-related species. This is aided by exploiting the antigenic differences within the *E. coli* spp.

6.5 Examples of commercial kits

6.5.1 Cold water setting gels

The rehydratable film product (e.g. Petrifilm™, 3M; CLONdisc™, BD Biosciences) is a simple to use version of the traditional pour-plate method (Ginn *et al*., 1986). It comprises two plastic films coated with adhesive, powdered culture medium constituents and a dehydrated cold water soluble gelling agent. In use the top film of the plate is lifted and a 1 ml sample is added to the centre of the bottom film. The top film is released and a spreading device is used to press down the top film. Rehydratable film products are available for total viable counts and enumeration of indicator organisms with the only pathogen enumeration product being for *Staphylococcus aureus*.

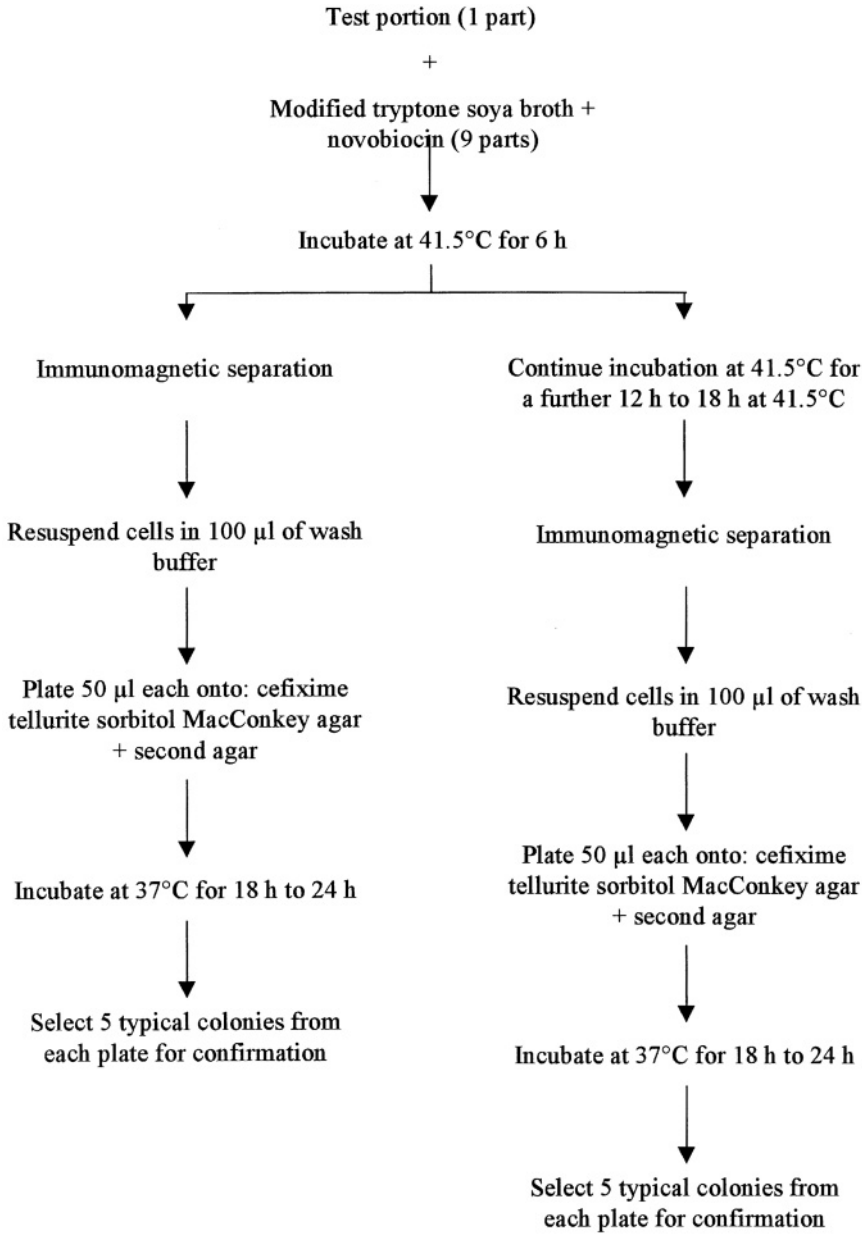

Fig. 6.4 ISO 16654:2001 (Anon, 2001). Microbiology of food and animal feeding stuffs – horizontal method for the detection of *Escherichia coli* O157.

Products similar in principle are available as Compact Dry (Scil Diagnostics) (Ellis and Meldrum, 2002) and SANITA-KUN (Chisso).

The Redigel™ product (3M) is a pectin gel method similar to agar pour-plate techniques (Roth, 1988). Pre-sterilised medium containing the pectin gel is added to specially treated Petri dishes whereby the base of the dish has been coated with divalent cations to cause solidification of the gelling agent. The Redigel™ products can also be used in the spread-plate technique. At present there are no Redigel™ products available for pathogen detection.

6.5.2 Selective motility

The Oxoid Salmonella Rapid Test combines liquid and semi-solid enrichment stages and liquid indicator media (Holbrook *et al*., 1989). Motile salmonellae migrate through the selective medium under the influence of chemoattractants. The test is performed in a disposable culture vessel containing two tubes with porous bases. Each of the tubes contains two culture media separated by a further porous disc. The media in the bottom halves of the tubes are selective for *Salmonella* and the media in the top halves contain indicators for *Salmonella*.

6.5.3 Delayed addition

Salmosyst® (Merck) is a manual delayed addition method whereby selective agents are added to a primary enrichment sample in the form of a tablet after a pre-determined time period (six to eight hours) (Pignato *et al*., 1995). SPRINT Salmonella is a combined primary enrichment and secondary enrichment method whereby selective agents are automatically released directly into the primary enrichment medium after a pre-determined time (4-6 hours) from delayed-release capsules (Baylis *et al*., 2000a). The primary enrichment medium is optimised for the rapid resuscitation of stressed cells so that by the time the medium is turned selective all stressed cells have begun growing. Selective agents are packaged in capsules that are sealed with a hydrogel plug. The plugs rehydrate in the primary enrichment medium, swelling at a controlled rate until a point where they are forced from the end of the capsules releasing selective agents into the medium. Both methods are only available for *Salmonella* detection.

6.5.4 MPN

The hydrophobic grid membrane filter (HGMF) (e.g. ISO-GRID™, Neogen) method uses a membrane filter imprinted with a hydrophobic grid that divides the filter surface into 1600 equal compartments (Sharpe and Michaud, 1974). When a sample is filtered through the HGMF, microorganisms are randomly distributed into the filter compartments. The filter is placed onto a selective differential agar medium to enable identification of colonies of the target

species. The number of compartments occupied by colonies is determined and an MPN estimate is calculated. The HGMF method can also be used as a qualitative method by filtering selective enrichment broths and using the appropriate selective differential plating medium. The hydrophobic grid is then used to separate colonies to maximise the chances of detecting the target species in a mixed culture. The HGMF method is available for *Salmonella*, *L. monocytogenes*, *E. coli* O157 and *Staph. aureus*.

The SimPlate® system (BioControl) is a pre-moulded circular plastic dish with microwells (Beuchat *et al*., 1998). The sample is mixed with the culture medium prior to pouring into the dish. Fluorescent enzyme substrates in the medium are hydrolysed by the target bacteria during the incubation period. After incubation the number of fluorescent microwells is noted and the MPN estimated. The product is available for performing total viable counts, enumeration of indicator microorganisms and *Campylobacter* enumeration.

Colilert® and Quanti-Tray® (IDEXX Laboratories) are proprietary products for broth-based MPN enumeration (Yakub *et al*., 2002). Each product comprises a moulded device with multiple self-contained chambers into which a mixture of sample and culture medium is poured. Chromogens, fluorogens and gas production are used as indicators for the presence of the target microorganism. After incubation the number of positive chambers is noted and the MPN estimated. The products are available for the enumeration of indicator microorganisms in water samples but could be used on diluted samples of food although no products are available for pathogen detection. Similar products, such as ColiTrak® (BioControl) are available for qualitative testing.

6.5.5 Specific substrate media

Patent protection for culture media formulations was relatively unknown until the advent of specific substrate media. There are many proprietary products for enrichment and plating that contain the novel use of an existing chromogenic or fluorogenic substrate, or a novel compound in itself (Manafi, 2000). Many products are available for the detection of *E. coli* O157, *Salmonella*, *L. monocytogenes*, *Bacillus cereus*, *Staph. aureus* and *Clostridium perfringens*.

6.6 Future trends

Despite the long existence of culture methods there is still great potential for further significant improvements. Products with increased specificity and sensitivity will be brought about by improvements in medium components, such as new chromogens that reduce the requirement for confirmation and chromogens with enhanced properties such as strength of colour thus enabling faster visualisation. New medium components will include new types of selective agent that act in a more targeted fashion based on enzyme expression without toxicity to stressed cells of the target species. New medium components

for improved resuscitation of stressed cells are likely.

Products will be made simpler enabled by improvements in presentation and design of products. The emergence of new pathogens, e.g. detection of all VTEC in foods, will bring about the development of new culture methods. Changes in testing requirements, e.g. enumeration of *Campylobacter* spp. in poultry products, will also lead to the development of new culture methods. Culture media will be specifically designed that are compatible with rapid end-point technologies. As in the past where media have been designed with impedance techniques in mind (Druggan *et al*., 1993), media in the future will be designed that are optimised for techniques such as PCR (Knutsson *et al*., 2002). New diagnostic features will be built into culture methods. New principles for culture detection will be designed, e.g. bacteriophage amplification, targeted responsive liposomes. Finally, there will be increased harmonisation of standardised culture methods with more cross-border recognition of national and international standardised culture methods.

Much is now understood about the complex interactions of culture medium components. Commercially produced media in dehydrated and ready-to-use forms are of very high standard often giving more reliable and reproducible performance than media prepared from individual components in the user's laboratory. Continuing innovations in medium components will lead to faster, simpler to use and more sensitive products. Although, in comparison with newer alternative technologies, they are time-consuming and require a moderate degree of skill to operate culture methods still dominate the field of detecting pathogens in food. Culture methods have universal acceptance, are lower in cost, are highly specific and highly sensitive and often have a long history of good reliable performance. Even with the introduction of alternative technologies culture methods will continue to form the heart of the method fulfilling the essential roles of resuscitation, growth and selectivity.

6.7 Further reading

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