CHAPTER 10

Methods for the Microbiological Examination of Foods

10.1 INDICATOR ORGANISMS

It is frequently necessary to conduct a microbiological examination of food to determine its quality. This may be necessary to estimate its shelf-life, its suitability for human consumption or to confirm that it meets some established microbiological criterion (see Chapter 11). The total mesophilic plate count is widely used as a broad indication of microbiological quality, although it is unsuitable for this purpose in fermented foods which contain large numbers of organisms as a natural consequence of their preparation. In other foods, knowledge of the total count may be useful but it is often of more value to obtain an estimate of the numbers of a particular component of the total flora. Examples of this could be moulds in a cereal, psychrotrophic bacteria in a chilled food, anaerobes in a vacuum-packed food, or yeasts in a fruit beverage.

A quite different reason for a microbiological examination of a food may be to identify the cause of spoilage or the presence of a pathogen where a food has been implicated in foodborne illness. The methods for determining an estimate of the total mesophilic count are very different from those required for demonstrating the presence of a pathogen, or its isolation for further study.

The isolation of specific pathogens, which may be present in very low but significant numbers in the presence of larger numbers of other organisms, often requires quite elaborate procedures, some of which are outlined in Chapter 7. It may involve enrichment in media which encourage growth of the pathogen while repressing the growth of the accompanying flora, followed by isolation on selective diagnostic media, and finally the application of confirmatory tests (see, for example, Section 7.12.4).

Though microbiological criteria or the investigation of an outbreak of foodborne illness may often require the monitoring of certain products for specific pathogens, the difficulties associated with detecting low numbers of pathogens make it impracticable as a routine procedure to be applied without good cause.

An alternative to monitoring for specific pathogens is to look for an associated organism present in much larger numbers – an indicator organism. This is a concept developed originally for pathogens spread by the faecal–oral route in water and which has since been applied to foods, often rather uncritically. A good indicator organism should always be present when the pathogen may be present, it should be present in relatively large numbers to facilitate its detection, it should not proliferate in the environment being monitored and its survival should be similar to that of the pathogen for which it is to be used as an indicator.

Escherichia coli is a natural component of the human gut flora and its presence in the environment, or in foods, generally implies some history of contamination of faecal origin. In water microbiology in temperate climates *E. coli* meets these criteria very well and has proved a useful indicator organism of faecal pollution of water used for drinking or in the preparation of foods. There are, however, limitations to its use in foods where there appears to be little or no correlation between the presence of *E. coli* and pathogens such as *Salmonella* in meat, for example. Although *E. coli* cannot usually grow in water in temperate countries, it can grow in the richer environment provided by many foods.

Testing for *E. coli* can itself be relatively involved and a number of simpler alternatives are often used. These are less specific and therefore the relationship between indicator presence and faecal contamination becomes even more tenuous. Traditionally the group chosen has been designated the coliforms – those organisms capable of fermenting lactose in the presence of bile at 37 °C. This will include most strains of *E. coli* but also includes organisms such as *Citrobacter* and *Enterobacter* which are not predominantly of faecal origin (Table 10.1). The faecal coliforms, a more restricted group of organisms, are those coliforms which can grow at higher temperatures than normal, *i.e.* 44–44.5 °C and the methods developed for their detection were intended to provide rapid, reproducible methods for demonstrating the presence of *E. coli* without having to use time-consuming confirmatory tests for this species. However, the verocytotoxigenic strain O157 : H7 (VTEC), which has caused so much concern during the late 1990s, does not grow well at 44 °C. Faecal coliforms contain a higher proportion of *E. coli* strains and the test can be made even more specific for *E. coli* type 1 by including a test for indole production from tryptophan to exclude other thermotolerant coliforms. Further specificity can be introduced by using a medium diagnostic for

Table 10.1 *Significance of genera of the Enterobacteriaceae in the monitoring of foods*

Genus	*Predominantly faecal origin*	*Usually detected in 'coliform tests'*	*Typically enteropathogenic in humans*
Citrobacter	No	Yes	No
Edwardsiella	Yes	No	No
Enterobacter	No	Yes	No
Erwinia	No	No	No
Escherichia	Yes	Yes	No
Hafnia	No	No	No
Klebsiella	No	Yes	No
Proteus	No	No	No
Salmonella	Yes	No	Yes
Serratia	No	No	No
Shigella	Yes	No	Yes
Yersinia	Yes	No	No

These comments are generalizations and there are exceptions to most of them. Adapted from 'Micro-organisms in Foods 1: Their Significance and Methods of Enumeration'. 2nd Edition ICMSF, University of Toronto Press, 1978

β-glucuronidase activity; an enzyme possessed by most, but not all, strains of *E. coli* and relatively uncommon in other bacteria.

One criticism of using coliforms and faecal coliforms is that their absence could give a false reassurance of safety when lactose-negative organisms predominate. The lactose-negative organisms include, not only *Salmonella* and *Shigella*, but also enteroinvasive strains of *E. coli* (EIEC) such as O124. For this reason, tests for the whole of the Enterobacteriaceae are increasingly being used. The Enterobacteriaceae includes even more genera of non-faecal origin than the coliforms, such as *Erwinia* and *Serratia* which are predominantly plant associated. For this reason Enterobacteriaceae counts are used more generally as an indicator of hygienic quality rather than of faecal contamination and therefore say more about general microbiological quality than possible health risks posed by the product. For instance, the presence of high numbers of Enterobacteriaceae in a pasteurized food would be cause for concern although it would not necessarily imply faecal contamination, and one would expect to find Enterobacteriaceae on fresh vegetables without the product necessarily being hazardous. The potential significance of genera of the Enterobacteriaceae is summarized in Table 10.1.

Some food microbiologists have tried to distinguish between 'indicator' organisms, which relate to general microbiological quality, and so-called 'index' organisms, which suggest that pathogens may be present. As will be apparent from the discussion above, this is not a simple distinction to make and the terminology has not been widely adopted.

10.2 DIRECT EXAMINATION

When examining foods, the possibility of detecting the presence of micro-organisms by looking at a sample directly under the microscope should not be missed. A small amount of material can be mounted and teased out in a drop of water on a slide, covered with a cover slip, and examined, first with a low magnification, and then with a ×45 objective. The condenser should be set to optimize contrast even though this may result in some loss of resolution. Alternatively dark-field illumination or phase-contrast microscopy may be used. It is usually relatively easy to see yeasts and moulds and with care and patience it is possible to see bacteria in such a preparation. The high refractive index of bacterial endospores makes them particularly easy to see with phase-contrast optics and, if the preparation is made as a hanging drop on the cover glass mounted over a cavity slide, it should also be possible to determine whether the bacteria are motile.

Since only a small sample of product is examined in this way, micro-organisms will not be seen unless present in quite large numbers, usually at least $10^6\,ml^{-1}$. In the case of some liquid commodities, such as milk, yoghurt, soups and fruit juices, it may be possible to prepare and stain a heat-fixed smear. But the food constituents often interfere with the heat fixing and care is needed to prevent the smear being washed away during staining. It may be necessary to dilute the sample with a little water, although that will reduce the concentration of micro-organisms further. The great advantage of such techniques is their rapidity, although in their simplest forms they do not distinguish between live and dead cells. The Breed smear is a quantitative version in which the field of view of the microscope is calibrated and a known volume of sample is spread over a known area of the slide.

The direct epifluorescent filter technique or DEFT is a microscopy technique which has been applied to the enumeration of micro-organisms in a range of foods, although it was originally developed for estimating bacterial counts in raw milk. The technique was developed in response to the need for a rapid method for judging the hygienic quality of farm milks. It achieves a considerably increased sensitivity (10^3–10^4 bacteria ml^{-1}) over conventional microscopy techniques by concentrating bacteria from a significantly larger volume of sample by filtering it through a polycarbonate membrane filter. The retained bacteria are then stained on the membrane with acridine orange and counted directly under the epifluorescence microscope. It may be necessary to pretreat the sample to allow filtration thus, for example, milk can be pretreated with detergent and a protease enzyme. It is also essential to ensure that the bacteria are trapped in a single focal plane because of the limited depth of focus of the microscope at the magnifications required. This is achieved by using a

polycarbonate membrane where relatively uniform pores are produced following neutron bombardment of a plastic film, rather than cellulose acetate filters which have tortuous pores where bacteria will be held at different levels.

Acridine orange is a metachromatic fluorochrome, fluorescing either green or orange depending on the nature of the molecules within the cell to which it is bound. When bound to double-stranded DNA it fluoresces green but when bound to single-stranded RNA it fluoresces orange, as long as there is an adequate concentration of dye to saturate all the binding sites. Generally it is assumed that those cells which fluoresce orange are viable while those that fluoresce green are nonviable. This is certainly not always true. The actual colour of an individual cell depends on many factors but, probably the most important is the concentration of acridine orange within the cell. In many micro-organisms the integrity of the cell membrane restricts the passage of the dye into the cell and it is often the case that viable micro-organisms will fluoresce green and dead micro-organisms, in which the membrane is more leaky, will fluoresce orange. Thus, although there are limitations to the use of acridine orange as a vital stain, the method has been adapted for the enumeration of micro-organisms in a range of food commodities including fresh meat and fish, meat and fish products, beverages and water samples. Although not commonly used in DEFT, there are alternative viability stains such as cyanoditolyl tetrazolium chloride (CTC) and fluorescein diacetate (FDA). These only cause cells to fluoresce if they retain a functioning electron transport chain (CTC) or esterase activity (FDA).

In a modification of the technique, specific groups of micro-organisms can be enumerated. The membrane filter is incubated on an appropriate medium containing optical brighteners and the microcolonies that develop on the membrane enumerated using the fluorescence microscope.

10.3 CULTURAL TECHNIQUES

Although there is clearly a place for the direct examination of a food for micro-organisms, a full microbiological examination usually requires that individual viable propagules are encouraged to multiply in liquid media or on the surface, or within the matrix, of a medium solidified with agar.

Agar is a polysaccharide with several remarkable properties which is produced by species of red algae. Although it is a complex and variable material, a major component of agar is agarose which is made of alternating units of 1,4-linked 3,6-anhydro-L-galactose (or L-galactose) and 1,3-linked D-galactose (or 6-O-methyl-D-galactose). The properties of agar which make it so useful to microbiologists include the ability to form a gel at low concentrations (1.5–2%) which does not significantly influence the water potential of the medium. Such a gel is stable to quite

high temperatures and requires a boiling water bath, or autoclave temperatures, to 'melt' it. Once molten however, agar solutions remain liquid when cooled to relatively low temperatures (*ca.* 40 °C) making it possible to mix it with samples containing viable organisms before, or during, dispensing. A further convenient property of agar is its stability to microbial hydrolysis, despite being a polysaccharide. Only a relatively small group of micro-organisms are able to degrade agar, presumably due to the presence of the unusual L-form of galactose in the polymer.

A very wide range of media are available to the microbiologist and details of their formulation, and how they are used, may be found in a number of readily available books and manuals. A selection of some commonly used media is listed in Table 10.2.

The formulation of a medium will depend, not only on what group of organisms is being studied, but also on the overall purpose of the study; whether it be to encourage good growth of the widest possible range of organisms, to be selective or elective for a single species or limited group, to resuscitate damaged but viable propagules, or to provide diagnostic information.

General purpose media such as nutrient agar and plate count agar for bacteria, or malt extract agar and potato/dextrose agar for fungi, have evolved to provide adequate nutrition for the growth of non-fastidious, heterotrophic micro-organisms. They do not deliberately contain any inhibitory agents but they may nevertheless be selective because of the absence of specific nutrients required by more fastidious organisms.

Selective media contain one or more compounds which are inhibitory to the majority of organisms but significantly less so to the species, or group of species, which it is required to isolate. It must be noted that all selective media, because they are based on the presence of inhibitory reagents, will generally be inhibitory to some extent to the organisms to

Table 10.2 *A selection of media commonly used in food microbiology*

Medium	*Use*
Plate Count Agar	Aerobic mesophilic count
MacConkey Broth	MPN of coliforms in water
Brilliant Green/Lactose/Bile Broth	MPN of coliforms in food
Violet Red/Bile/Glucose Agar	Enumeration of Enterobacteriaceae
Crystal Violet/Azide/Blood Agar	Enumeration of faecal streptococci
Baird–Parker Agar	Enumeration of *Staphylococcus aureus*
Rappaport–Vassiliadis Broth	Selective enrichment of *Salmonella*
Thiosulfate/Bile/Citrate/Sucrose Agar	Isolation of vibrios
Dichloran/18% Glycerol Agar	Enumeration of moulds
Rose Bengal/Chloramphenicol Agar	Enumeration of moulds and yeasts
Cefixime/Tellurite/Sorbitol/MacConkey Agar	*E. coli* O157

be selected. If cells of the target organism have been subject to sublethal injury, then they may not be able to grow on the medium without a resuscitation step to allow them to repair.

Elective media on the other hand, are designed to encourage the more rapid growth of one species or group of micro-organisms so that they out-compete others even in the absence of inhibitory agents. Thus cooked-meat broth incubated at 43–45 °C allows rapid growth of *Clostridium perfringens* so that it may become the dominant organism after only 6–8 hours incubation.

The difference between selective and elective media must be seen from the viewpoint of the organism which it is desired to recover. By ensuring optimal growth in the elective medium for one organism, it is desirable that conditions are sub-optimal, or even inhibitory, to others. A problem in the use of elective media is that growth of the desirable species may change the medium in a manner which now encourages the growth of other species. On the other hand a selective medium, if well designed, should remain inhibitory to unwanted organisms even when the organisms required are growing.

Resuscitation media are designed to allow the recovery of propagules which are sub-lethally damaged by some previous condition such as heat treatment, refrigeration, drying or exposure to irradiation. Such damaged micro-organisms may not only be more sensitive to inhibitory agents present in selective media, but may be killed if exposed to conditions encouraging rapid growth of healthy cells. Typically resuscitation media are nutritionally weak and may contain compounds which will scavenge free radicals such as those which may be generated by the metabolism of oxygen.

Diagnostic media contain a reagent or reagents which provide a visual response to a particular reaction making it possible to recognize individual species or groups because of the presence of a specific metabolic pathway or even a single enzyme.

Many media used in practice combine selective reagents, elective components and diagnostic features. An interesting example is the Baird–Parker agar used for the presumptive isolation of *Staphylococcus aureus*. The selective agents are sodium tellurite and lithium chloride, the elective agents are sodium pyruvate and glycine and the diagnostic features are provided by the addition of egg yolk. The production of black colonies due to the reduction of tellurite is characteristic of *S. aureus* as well as several other organisms able to grow on this medium such as other species of *Staphylococcus*, *Micrococcus* and some species of *Bacillus*. The additional diagnostic feature shown by most strains of *S. aureus* is the presence of an opaque zone due to lecithinase activity surrounded by a halo of clearing due to proteolytic activity (see also Section 7.14.4).

10.4 ENUMERATION METHODS

10.4.1 Plate Counts

It has already been suggested that to count micro-organisms in a food sample by direct microscopy has a limited sensitivity because of the very small sample size in the field of view at the magnification needed to see micro-organisms, especially bacteria. In a normal routine laboratory the most sensitive method of detecting the presence of a viable bacterium is to allow it to amplify itself to form a visible colony. This forms the basis of the traditional pour plate, spread plate or Miles and Misra drop plate still widely used in microbiology laboratories. Table 10.3 compares the sample size examined and potential sensitivity of all these methods. In the pour plate method a sample (usually 1 ml) is pipetted directly into a sterile Petri dish and mixed with an appropriate volume of molten agar. Even if the molten agar is carefully tempered at 40–45 °C, the thermal shock to psychrotrophs may result in them not producing a visible colony. The spread-plate count avoids this problem and also ensures an aerobic environment but the sample size is usually limited to 0.1 ml.

In a thoroughly mixed suspension of particles such as micro-organisms, the numbers of propagules forming colonies on replicate plates is expected to have a Poisson distribution, a property of which is that the variance (standard deviation squared) is equal to the mean ($\bar{x}$), *i.e.*

$$\bar{x} = \text{var} = s^2 \tag{10.1}$$

A consequence of this is that the limiting precision of a colony count is dependent on the number of colonies counted. The 95% confidence limits (CL) can be estimated as approximately

$$2s = 2\sqrt{x} \tag{10.2}$$

Table 10.3 *A comparison of the sensitivity of methods of enumeration*

Method	*Volume of sample (ml)*	*Count (cfu g $^{-1}$) corresponding to a single organism or colony seen*[a]
Direct microscopy	5×10^{-6}	2×10^{6}
Miles and Misra	0.02	5×10^{2}
Spread plate	0.1	10^{2}
Pour plate	1.0	10
MPN	3×10.0 $+3\times1$ $+3\times0.1$	0.36

[a] Based on a 10^{-1} dilution of a sample obtained by, for example, stomaching 1g (or ml) of food with 9 ml of diluent

(if x is the count on a single plate and has to be our estimate of the mean). Thus for a plate with only 16 colonies, the 95% CL would be approximately ±50% (*i.e.* we would have 95% confidence that the count lies between 8 and 24). For a count of 30, it would be ±37% and for a count of 500 only ±9%. However, if the number of colonies on a plate was as high as 500, it would not only be difficult to count them accurately, but such a crowded plate is likely to result in many colony-forming units never forming a visible colony leading to an underestimate. Thus it is widely accepted that reasonably accurate results are obtained when plates contain between 30 and 300 colonies.

To obtain plates with this number of colonies it is often necessary to dilute a sample before enumeration. The most widely used dilution technique is the ten-fold dilution series. With a completely unfamiliar sample it is necessary to plate-out a number of dilutions to ensure that some plates are obtained with counts in the desired range, but with experience of a particular material plating only one dilution may be sufficient.

The diluent used must not cause any damage, such as osmotic shock, to the micro-organisms. Sterile distilled water is therefore unsuitable. A commonly used diluent, known as maximum recovery diluent, contains 0.1% peptone and 0.85% sodium chloride.

It is possible to increase the confidence in a plate count by plating a number of replicate samples from each dilution. From the results of a single dilution the count in the original sample can be calculated using Equation (10.3),

$$N = \frac{\bar{x}}{V.d} \tag{10.3}$$

where N = cfu g^{-1}; $\bar{x}$ = mean count per plate; V = volume of sample plated; d = dilution factor. Sometimes we can use the results from several sequential dilutions but it is a common experience that the apparent cfu g^{-1} increases the higher the dilution used in making the calculation. This may reflect the breaking up of clumps by the action of pipetting, and/or reduced competition on less crowded plates. The smaller numbers on plates at higher dilutions are associated with reduced levels of confidence but they can be used in the calculation of a weighted mean using Equation (10.4) based on a ten-fold dilution series:

$$N_w = \frac{(C_1 + C_2)}{(n_1 + n_2/10)} \cdot \frac{1}{V} \cdot \frac{1}{d_1} \tag{10.4}$$

where N_W = weighted mean; C_1 = the total count on n_1 replicates at dilution d_1; C_2 = total counts on n_2 replicates of the next dilution. The

use of this formula is best shown with a worked example:

Dilution	n	colony count/plate	total C	$\bar{x}$	cfu ml^{-1}[a]
10^{-4}	3	63, 74, 61	198	66	6.6×10^5
10^{-5}	3	5, 11, 9	25	8.3	8.3×10^5

[a] Calculated from a single dilution

$$\text{Weighted mean} = \frac{(198+25)}{(3+0.3)} \cdot \frac{1}{0.1} \cdot \frac{1}{10^{-4}} = 6.8 \times 10^5$$

Traditional plate counts are expensive in Petri dishes and agar media, especially if adequate replication is carried out, and the Miles and Misra drop count and spiral plater have been developed to reduce costs. In the Miles–Misra technique materials are conserved by culturing a smaller volume of each dilution, usually 20 μl. This way a number of dilutions can be grown on a single plate by dividing it into sectors each representing a different dilution. The spiral plater employs a mechanical system which dispenses 50 μl of a liquid sample as a spiral track on the surface of an agar plate. The system is engineered so that most of the sample is deposited near the centre of the plate with a decreasing volume applied towards the edge. This produces an effect equivalent to a dilution of the sample by a factor of 10^3 on a single plate, thus producing a two-thirds' saving on materials as well as saving the time required in preparing and plating extra dilutions. After incubation, colonies are counted using a specially designed grid which relates plate area to the volume applied, thus enabling the count to be determined. The system is not suitable for all food samples though, as particulate material can block the hollow stylus through which the sample is applied.

The limit in sensitivity of the traditional plate count arises from the small volumes used and clearly, the sensitivity can be increased by increasing the volume size and the number of replicate counts or plates. It may be possible to filter a larger volume through a membrane, which retains the viable organisms, and then lay the membrane onto an appropriate medium.

In all of these methods of enumeration it is essential to appreciate the statistical background to sampling and to recognize that extrapolations from colony counts depend on several assumptions that may not be justified. Thus a colony may not be derived from a single micro-organism, but from a clump of micro-organisms, and the material being examined may not be homogeneous so that the subsample actually studied is not representative of the whole.

10.4.2 Most Probable Number Counts

An alternative method of enumerating low numbers of viable micro-organisms is that referred to as the Most Probable Number (MPN) method. The method is usually based on inoculating replicate tubes of an appropriate liquid medium (usually 3, 4 or 5) with three different sample sizes or dilutions of the material to be studied (*e.g.* 10 g, 1.0 g and 0.1 g). The medium used has to be designed to make it possible to decide whether growth or no growth has occurred and the number of positives at each sample size or dilution is determined after incubating the tubes. The MPN is obtained by referring to a table such as that shown in Table 10.4. There are computer programmes for generating MPN values from different designs of the experiment and these programmes can also provide confidence limits for the MPN and suggest what the likelihood of particular combinations of positive results should be.

A modern variation on the MPN theme is the use of the hydrophobic grid membrane filter (HGMF). A sample is filtered through the HGMF which is divided by a hydrophobic grid into a number (normally 1600) of small cells or growth compartments. After incubation of the filter on an appropriate medium, each of these cells is scored for growth or no-growth. This can be done either manually or automatically and the count in the original sample determined as equivalent to a single dilution MPN using 1600 tubes.

One application of the MPN, which allows one to calculate the maximum number of organisms in a batch of material, is based on the two-class attributes sampling plan (see Section 11.2.1). If a number of equal sized samples (n) is taken from a batch of material and all shown to be negative for a particular organism then the maximum percentage (d)

Table 10.4 *A selection of MPN values*[a]

Number of positive tubes	*MPN*	*95% Confidence Limits*
0 0 0	<0.30	
1 0 0	0.36	0.02 to 1.7
2 0 0	0.92	0.15 to 3.5
2 1 0	1.5	0.4 to 3.8
3 0 0	2.3	0.5 to 9.4
3 1 0	4.3	0.9 to 18.1
3 1 1	7.5	1.7 to 19.9
3 2 0	9.3	1.8 to 36
3 2 1	15	3.0 to 38
3 3 0	24	4.0 to 99
3 3 1	46	9.0 to 198
3 3 2	110	20.0 to 400
3 3 3	>110	

[a] Based on 3×1 g(ml) + 3×0.1 g(ml) + 3×0.01 g(ml) samples (expressed as organisms per 1 g)

of such samples containing at least one viable propagule at a required probability P is given by Equation (10.5).

$$d = 100[1 - \sqrt[n]{(1 - P)}] \tag{10.5}$$

Thus, if 10 samples of 25 g from a batch of material are all found to be negative then, with 95% confidence, the maximum percentage of 25g samples containing at least one viable organism would be:

$$d = 100[1 - \sqrt[10]{(1 - 0.95)}] = 26\%$$

i.e. there would be less than 10 organisms kg^{-1} ($1\ kg = 40 \times 25\ g$; 26% of $40 = ca.\ 10$).

The enumeration of micro-organisms assumes that there are distinctive propagules to be counted. This is acceptable for single-celled organisms such as the majority of bacteria or yeasts but in the case of filamentous fungi there may be a problem in interpreting the significance of numbers of colony-forming units. To assess the quantity of fungal biomass in a food commodity may require quite different techniques. One possibility is to make a chemical analysis for a constituent which is specifically associated with fungi, such as chitin, which is a constituent of the cell walls of zygomycetes, ascomycetes, basidiomycetes and deuteromycetes (but also present in insect exoskeleton), or ergosterol which is a major constituent of the membranes of these groups of fungi. Some moulds, such as species of *Aspergillus* and *Penicillium* associated with the spoilage of cereals, produce volatile metabolites such as methylfuran, 2-methylpropanol, 3-methylbutanol and oct-1-en-3-ol (this last compound having a strong mushroomy smell). It would be possible to detect and analyse these compounds in the head-space gases of storage facilities using gas–liquid chromatography (glc).

Equipment known as an artificial nose is now being developed in which a sample of head space gas from, for example, a grain silo is taken directly into a glc, the data read into a computer, and compared with a memory bank of previously recorded patterns. The pattern of peaks is often diagnostic for a particular mould species and it may even be possible to recognize patterns formed by mixtures of species.

10.5 ALTERNATIVE METHODS

Cultural methods are relatively labour intensive and require time for adequate growth to occur. Many food microbiologists also consider that the traditional enumeration methods are not only too slow but lead to an overdependence on the significance of numbers of colony-forming units. Food manufacturers require information about the microbiological quality of commodities and raw materials rapidly and it could be argued

that an assessment of microbial activity is as important as a knowledge of numbers.

A number of methods have been developed which aim to give answers more quickly and hence are often referred to as 'Rapid Methods'.

10.5.1 Dye-reduction Tests

A group of tests which have been used for some time in the dairy industry depend on the response of a number of redox dyes to the presence of metabolically active micro-organisms. They are relatively simple and rapid to carry out at low cost. The redox dyes are able to take up electrons from an active biological system and this results in a change of colour. Usually the oxidized form is coloured and the reduced form colourless but the triphenyltetrazolium salts are an important exception. Figure 10.1 shows the structures of the oxidized and reduced forms of the three most widely used redox dyes, methylene blue, resazurin and triphenyltetrazolium chloride.

From 1937, and until relatively recently, the methylene blue test was a statutory test for grading the quality of milk in England and Wales. Changes in the technology of handling bulk milk, especially refrigeration have made this test less reliable and it is no longer a statutory test because results show little correlation with the numbers of psychrotrophic bacteria. Since the reduction of resazurin takes place in two stages, from blue to pink to colourless, there is a wider range of colour that can be scored using a comparator disc and the ten-minute resazurin test is still useful for assessing the quality of raw milk at the farm or dairy before it is bulked with other milk.

Triphenyltetrazolium salts and their derivatives are initially colourless and become intensely coloured, and usually insoluble, after reduction to formazans. Triphenyltetrazolium chloride itself is most widely used as a component of diagnostic and selective agar media on which some bacterial colonies will become dark red to maroon as formazan becomes precipitated within the colony. The crystals of the formazan produced from 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) are so intensely coloured that they are readily seen in individual microbial cells under the microscope and their presence may be used to assess the viability of cells.

10.5.2 Electrical Methods

When micro-organisms grow, their activity changes the chemical composition of the growth medium and this may also lead to changes in its electrical properties. Measuring this effect has become the basis of one of the most widely used alternative techniques of microbiological analysis.

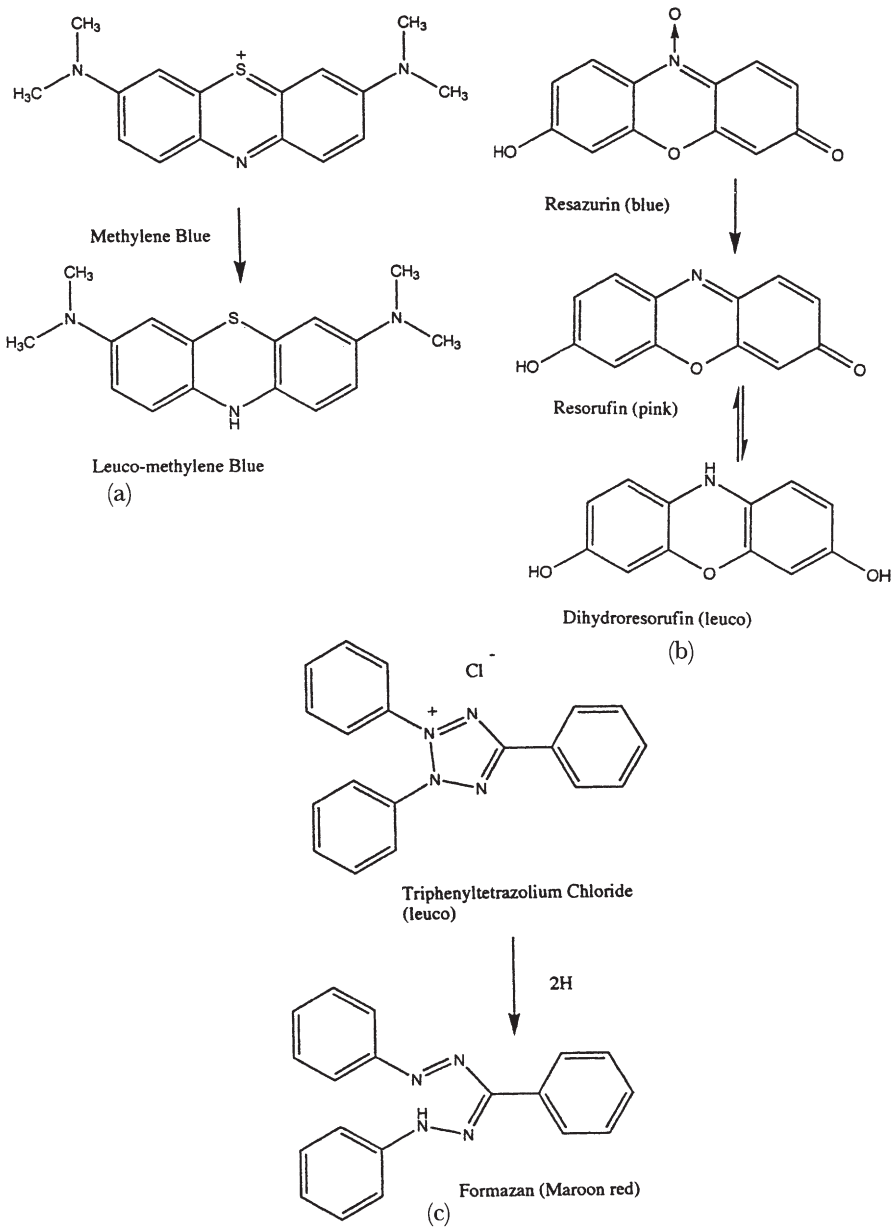


Figure 10.1 *Structures of redox dyes used in food microbiology. (a) Methylene blue. (b) resazurin. (c) triphenyltetrazolium chloride*

The electrical properties most frequently monitored are conductance (G), capacitance (C) and impedance (Z), the latter being influenced by both capacitance and resistance (R) as well as the frequency of the alternating current applied (f). The conductance is simply the reciprocal

of resistance, *i.e.*

$$G = 1/R \tag{10.6}$$

The relationship between impedance, resistance and capacitance is given by:

$$Z^2 = R^2 + (1/2\pi f C)^2 \tag{10.7}$$

It is possible to take frequent measurements of the electrical properties of a growth medium by growing organisms in cells supplied with two metal electrodes. By saving the data obtained for subsequent analysis on a computer, large numbers of samples can be monitored at the same time. Central to the successful application of the method is the choice or design of a medium which will both support rapid growth of the micro-organisms to be monitored, and will change its electrical properties as a result of their growth.

With a suitable medium, the traces obtained resemble the bacterial growth curve, although this analogy can be misleading as the curves are not superimposable. In practice it requires quite a large number of bacteria to initiate a signal, usually about 10^6–10^7 cfu ml^{-1} in the cell. The time it takes to reach this number and produce a signal (referred to as the detection time) will therefore include any lag phase plus a period of exponential growth and will depend on both the initial number and the growth rate. Thus, for a particular organism/medium/ temperature combination, the detection time will be inversely related to the logarithm of the number and activity of the organisms in the original sample. Figure 10.2 shows typical traces of samples taken from a ten-fold

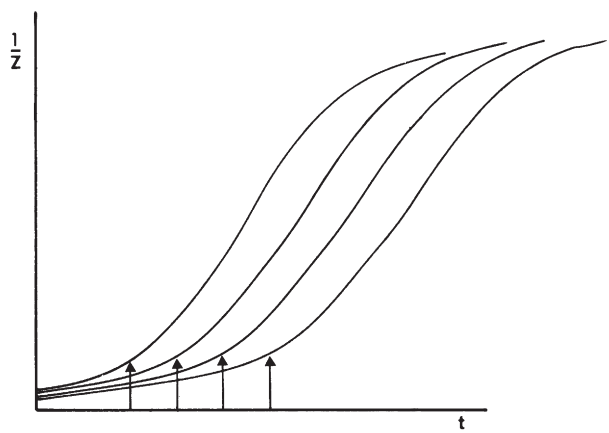


Figure 10.2 *Traces of 1/Z against time of ten-fold dilution series of Escherichia coli growing in brain/heart infusion broth at 37 °C. The detection times are marked with arrows*

dilution series of a pure culture. In the case of *Escherichia coli* growing in brain/heart infusion broth, incubated at 37 °C, it is possible to detect the presence of one or two viable cells in five or six hours.

By obtaining detection times using samples where the microbial population is known, calibration curves relating detection time and microbial numbers can be drawn so that count data can be derived from detection times. One such example for *Salmonella* Enteritidis is presented in Figure 10.3. Some claim that the only value in converting detection times to counts is that the food microbiologist derives a sense of security from having data in a familiar form. Since electrical methods measure microbial activity directly, detection time may be a more appropriate measure of the potential to cause spoilage than a viable plate count.

In the food industry the potential for simultaneously testing many samples makes electrical methods a useful means for assessing the quality of raw materials and products. They have the additional advantage that the worse the microbiological quality, the shorter is the detection time, and the sooner the manufacturer knows that there may be a problem. In modern instruments, which can accommodate more than 500 samples, the results can be displayed using an unambiguous quality colour code of acceptable (green), marginally acceptable (orange), and unacceptable (red).

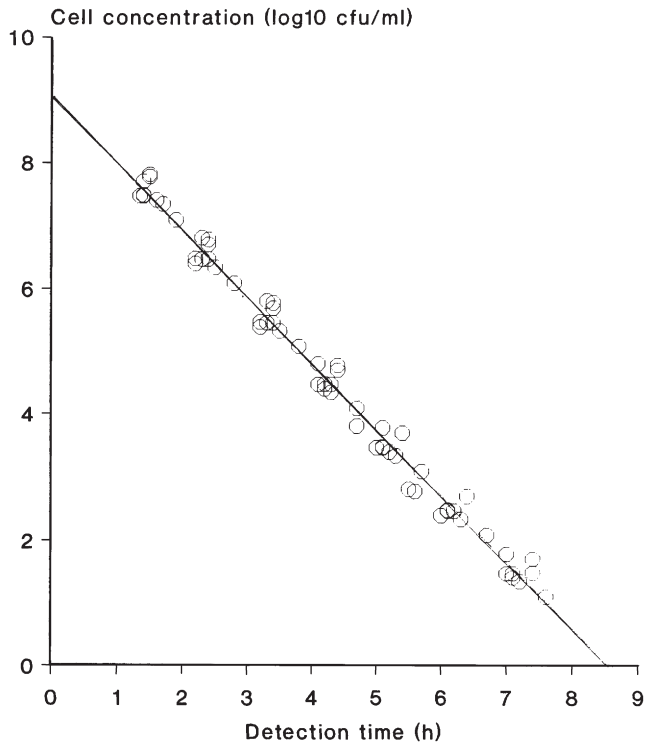
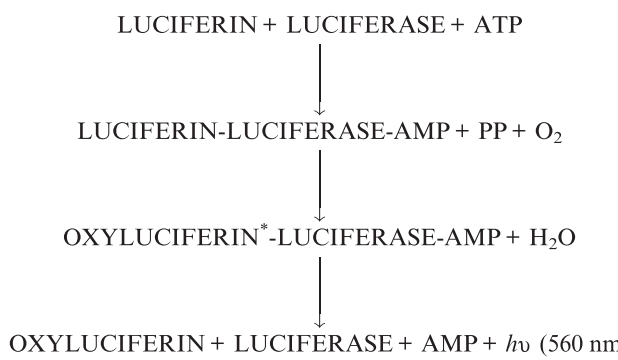


Figure 10.3 *A calibration curve for* Salmonella *Enteritidis using a Bactometer*

By carefully designing the medium to contain selective agents, and diagnostic compounds which will give a strong signal when they are metabolized, it is possible to use electrical methods to estimate the activity (and hence, by calibration, numbers) of specific groups of organisms. Thus the incorporation of trimethylamine oxide (TMAO) into a selective enrichment broth can be used as a pre-screening for the presence of *Salmonella*. The presence of the enzyme TMAO reductase converts this neutral molecule into the strongly charged trimethylammonium ion which has a considerable effect on the conductance of the medium. Absence of a detection time in control sample cells inoculated with a *Salmonella*-specific phage act as further confirmation of the presence of *Salmonella*.

10.5.3 ATP Determination

Adenosine triphosphate is found in all living cells and is the universal agent for the transfer of free energy from catabolic processes to anabolic processes. A number of quite different living organisms have evolved a mechanism for producing light by the activity of enzymes known as luciferases on substrates known as luciferins. These reactions require the presence of ATP and magnesium ions and produce one photon of light at the expense of the hydrolysis of one molecule of ATP through a series of intermediates. An ATP molecule facilitates the formation of an enzyme–substrate complex which is oxidized by molecular oxygen to an electronically excited state. The excited state of the molecule returns to the lower energy ground state with the release of a photon of light and dissociates to release the enzyme luciferase again. These reactions are summarized below:-



Because instruments are now available which can accurately measure low levels of light emission and pure luciferin and luciferase from, for example fireflies, can be manufactured, the reaction can be used as a very sensitive assay for ATP. Sensitive instruments using photomultiplier

tubes can detect as little as 10^2–10^3 fg (fg = femtogram = 10^{-15} g) which corresponds to as few as 10^2–10^3 bacterial cells.

Although the method gives very good results with pure cultures, when applied to foods it is essential to ensure that non-microbial ATP, which will be present in foods in considerably larger quantities than the microbial ATP, has been destroyed or that the micro-organisms have been separated from interfering food components. Non-microbial ATP can be selectively removed by treating with an ATPase after disrupting the somatic cells of animal and plant origin with a mild surfactant. The next stage is to destroy the ATPase activity and then extract the microbial ATP using a more powerful surfactant. The alternative, of removing microbial cells from the food before the ATP assay, can be achieved by centrifugation or filtration of liquid foods but is very much more difficult from suspensions of solid foods. Even when they are successfully separated there are problems arising from the different amounts of ATP in different microbial cells. Thus yeast cells may contain 100 times more ATP than bacterial cells and sub-lethally stressed micro-organisms may contain very low levels of ATP and yet be capable of recovering and growing on a food during long-term storage.

Immunomagnetic separation is a versatile method for removing specific groups of bacteria from a complex matrix of food and other organisms. It has been successfully applied prior to ATP determination as well as in a number of other contexts to extract and purify micro-organisms. Antibodies specific to the target organism or lectins, plant proteins which recognize and bind to specific carbohydrate residues exposed on the outer surface of a micro-organism, are attached to magnetisable beads. The bacteria adhere to the beads *via* the antibody–antigen, or lectin–carbohydrate, reaction and can be removed from the food suspension by a powerful magnet acting through the walls of the container. After the food materials have been poured away and the cells washed they can be released into suspension for assay by removing the magnet.

The need for often complex sample preparation has meant that, rapid and sensitive though it is, ATP measurement is not widely used for routine monitoring of microbial contamination of foods. It is however being increasingly used to monitor hygiene in food processing plant. Instruments are available where a swab taken from equipment can be assayed directly for ATP giving a virtually immediate measure of surface contamination. In these cases it is not necessary to distinguish between microbial and non-microbial ATP since the presence of either at high levels would indicate poor hygiene. This speed and simplicity make ATP determination the most overtly microbiological test that can be applied for the routine-monitoring of critical control points as part of the HACCP technique of quality assurance (see Section 11.6).

10.6 RAPID METHODS FOR THE DETECTION OF SPECIFIC ORGANISMS AND TOXINS

10.6.1 Immunological Methods

Because of the potential specificity of immunoassays using polyclonal or monoclonal antibodies, there has been considerable effort devoted to developing their application in food microbiology. Commercial immunoassay kits are now available for detecting a variety of foodborne micro-organisms and their toxins, including mycotoxins.

Raising antibodies to specific surface antigens of micro-organisms, or to macromolecules such as staphylococcal or botulinum toxins, is relatively straightforward and can be achieved directly. Mycotoxins, however, belong to a class of molecules known as haptens which can bind to an appropriate antibody but are of relatively low molecular weight and are not themselves immunogenic. Haptens can be made immunogenic by binding them chemically to a carrier protein molecule, and antibodies have now been raised using this technique to a wide range of mycotoxins including the aflatoxins, trichothecenes, ochratoxin and fumonisins.

Although a number of different formats are used in immunoassays, their essential feature is the binding of antibody to antigen. A commonly used protocol is that of the sandwich ELISA (enzyme linked immunosorbent assay) in which a capture antibody is immobilized on a solid surface of say a microtitre plate well. The sample containing antigen is then added to the well, mixed and removed leaving any antigens present attached to the antibodies. These are then detected by adding second antibody which is coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. This antibody will also bind to the antigen producing an antibody sandwich. Binding is detected by addition of a chromogenic substrate for the enzyme attached to the second antibody and measuring the colour developed (Figure 10.4). Alternative detection systems are used, such as attachment of antibodies to latex and looking for agglutination in the presence of the antigen and fluorescence-labelled antibodies which can be used to detect target organisms using a fluorescence microscope or flow cytometry.

Commercial ELISAs are available for such organisms as *Salmonella* and *Listeria monocytogenes* but they still require the presence of at least 10^5–10^6 organisms. Detection of smaller numbers therefore depends on some form of enrichment or concentration by one of the separation methods briefly mentioned above, so that although the immunoassay itself may be rapid the whole analytical protocol may take almost as long as conventional procedures. Some advantage can be gained from the automation of the assay and a number of instruments are commercially available. There may also be some concern over the specificity of

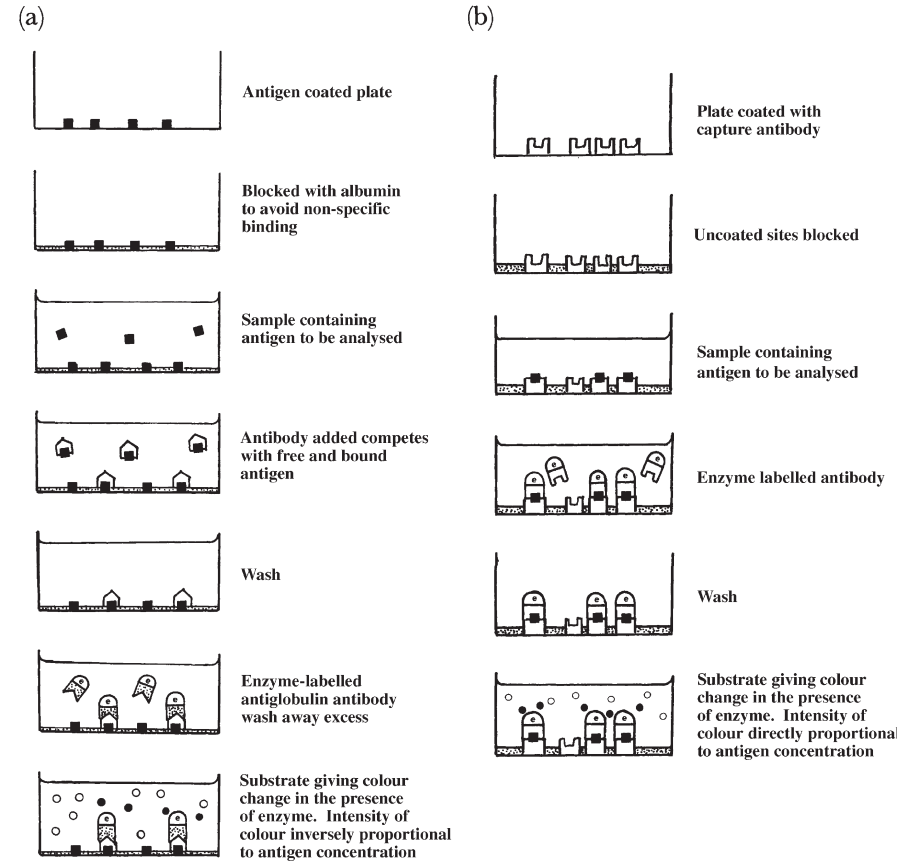


Figure 10.4 *Sandwich ELISAs. (a) Competitive sandwich ELISA, (b) direct sandwich ELISA*

immunoassays. While striving for antibodies that are sufficiently broad in their specificity to recognize all strains of the desired target organism, it is difficult to avoid the problem of cross reactivity with organisms other than those under investigation.

10.6.2 DNA/RNA Methodology

All biochemical, immunological and other characteristics used in the detection of micro-organisms are governed directly or indirectly by the base sequences encoded in the organism's genome. The specificity of this information can now be mobilized to provide methods capable of identifying genera, species or even strains within a species. Nucleic acid probes can be designed which recognize and bind (hybridize) to specified regions of either chromosomal or plasmid DNA or to RNA, and the region chosen to give the desired level of specificity. Thus, for example, ribosomal RNA contains both conserved and variable regions, the

former being suitable for recognition at the genus level whereas the latter may be considerably more specific. Although RNA is a more labile molecule than DNA, there are many more copies of ribosomal RNA in a cell than genomic DNA which should make methods based on this molecule more sensitive.

The nucleic acids have to be released from the cells by some form of lysis and, in the case of double-stranded DNA, it has also to be denatured, usually by heat treatment, to the single-stranded form. The denatured nucleic acid is then adsorbed onto a membrane, fixed to it by heat or alkali treatment, and the membrane is treated with some form of blocking agent to prevent non-specific binding of the probe. After incubating with the labelled probe and washing off unadsorbed probe, the presence of the hybridization product is measured using the label attached to the probe. In the earliest stages of the development of this methodology probes were directly labelled with radioactive isotopes such as ^{32}P or ^{35}S and hybridization was detected by autoradiography. This is a very sensitive method but the routine use of radioactive compounds in a food-associated environment is not usually acceptable. Probes can be labelled with an enzyme and detected with a chromogenic substrate or they can be labelled with a small molecular weight hapten for which an enzyme-linked monoclonal antibody is available. Such probes are available for the enterotoxin gene of *Staphylococcus aureus*, the haemolysin gene and rRNA of *Listeria monocytogenes*, 23S rRNA of *Salmonella*, as well as several other systems. One interesting example is a ribosomal RNA probe to detect *Listeria monocytogenes* which uses a chemiluminescent label. The single-stranded DNA probe has a chemiluminescent molecule bound to it. When the probe binds to its RNA target, the chemiluminescent molecule is protected from degradation in a subsequent step so that successful hybridization is indicated by light emission measured in a luminometer (Figure 10.5).

Like the ELISA methods, nucleic acid methods also require some enrichment of the target to produce sufficient nucleic acid to reach the threshold of sensitivity of about 10^6 copies of the target sequence. They are particularly well suited for rapid confirmation of isolated colonies on an agar plate.

The polymerase chain reaction (PCR) provides a method for amplifying specific fragments of DNA, usually less than 3kb in length, and in principle could allow detection of a single copy of the target sequence. The method uses two short oligonucleotide primer sequences (usually about 20 nucleotides long) which will hybridize to opposite strands of heat-denatured DNA at either end of the region to be amplified (Figure 10.6). A DNA polymerase then catalyses extension of the primers to produce two double-stranded copies of the region of interest.

The whole process is then repeated a number of times. In each cycle, the reaction mixture is heated to 94–98 °C to separate the double

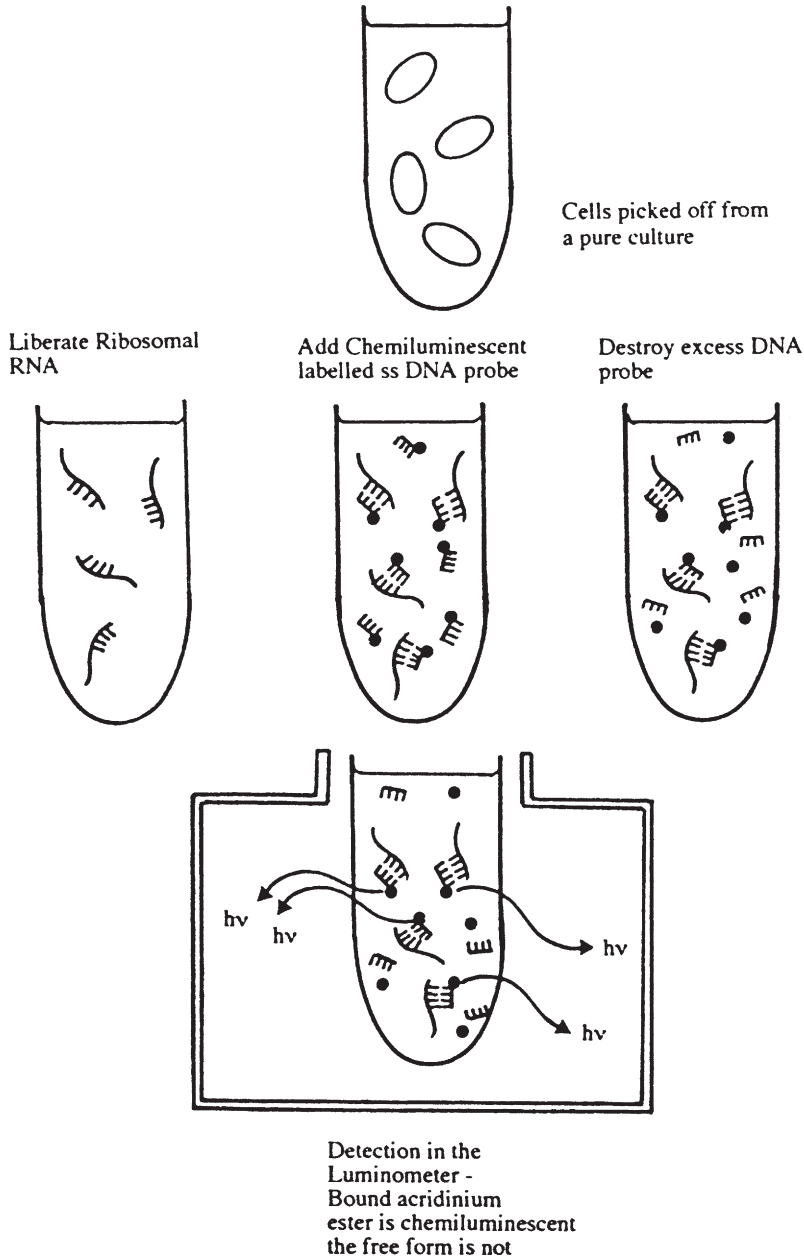


Figure 10.5 *Hybridization protection assay*

stranded DNA into single strands. The mix is then cooled to 37–65 °C to allow the primers to anneal to the single strands and then warmed to 72 °C to allow synthesis of their complementary strands. Thus as the cycles progress one double stranded segment of DNA will become two,

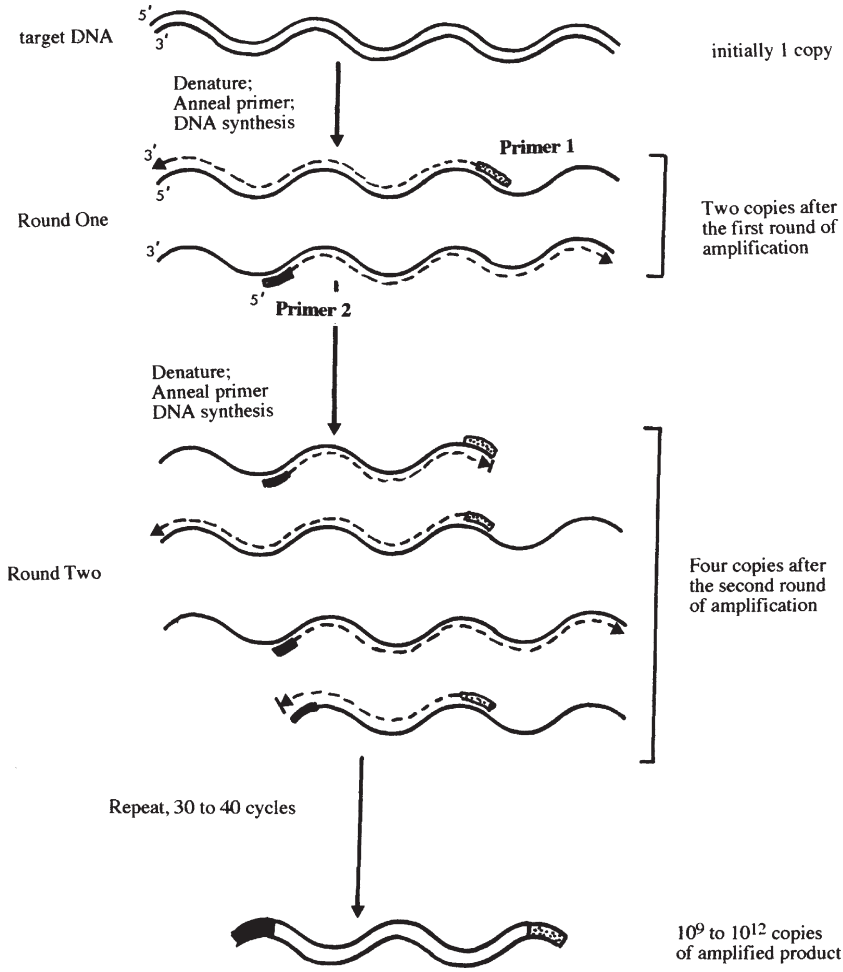


Figure 10.6 *The polymerase chain reaction*

four, eight, sixteen and so on so that after 20 cycles approximately 10^6 copies can be generated from the original.

Two key components essential to the successful application of PCR are precision automated thermal cycling equipment and a heat stable DNA polymerase which will survive the DNA denaturation step and catalyse subsequent extension of the primers. The latter, known as Taq polymerase, is obtained from the very thermophilic bacterium *Thermus aquaticus*.

There have been numerous embellishments of the basic PCR technique. These include:-

- Multiplex PCR which uses several primer pairs for the simultaneous amplification of a number of targets.
- Nested PCR which improves the sensitivity of the reaction by first amplifying a larger sequence using a pair of outer primers followed

by amplification of a shorter sequence within the amplicon using a second set of inner primers.

- Reverse transcriptase PCR which uses conventional PCR to amplify an RNA template after it has been transcribed into DNA. This can have higher sensitivity because of the multiple copies of RNA within the cell but is also essential for the detection of RNA viruses such as Norovirus.
- Real time PCR. Traditionally PCR products are detected by agarose gel electrophoresis on the basis of their size and/or their reaction with a complementary probe. An important development of the technique has been the advent of real time PCR where a fluorescent signal is produced. There are several techniques for doing this but the simplest uses a dye Sybr Green which fluoresces when it intercalates with double stranded DNA. Thus as the PCR reaction progresses more double stranded DNA is present and the more intense the fluorescence. The time taken to produce a detectable fluorescence will depend on the amount of target present initially, the more that is there the sooner the threshold level will be reached. This means that real time PCR can be both qualitative and quantitative.

The polymerase chain reaction can be inhibited by food components. This can be reduced by separation of the cells from the inhibitory food matrix by procedures such as immunomagnetic separation or by cultural enrichment to allow subsequent dilution of the sample and any inhibitory components. A cultural enrichment before PCR also helps overcome the objection that since PCR detects fragments of DNA these may not necessarily originate from a viable cell.

10.6.3 Subtyping

The ability to identify an organism to the species level is not sufficient to establish a firm link between food and clinical isolates or to identify whether a number of apparently unrelated cases have a common source. To do this it is necessary to have more highly discriminating methods that can distinguish between different strains of the same species.

Traditional subtyping procedures have been based on phenotypic characteristics. Thus biotyping employs particular biochemical activities for discrimination while phage typing and serotyping are based on the presence or absence of particular phage receptors or antigens on the cell surface. While these techniques have proved invaluable in particular circumstances they are not universally applicable due to factors such as the variability of gene expression. Genotypic subtyping methods however have broader applicability being based on an organism's underlying genetic make up rather than its phenotypic expression.

In restriction fragment length polymorphism (RFLP), a restriction enzyme is used to cut DNA into a number of fragments depending on the number and location of restriction sites present. Separation of these fragments by electrophoresis will reveal a fingerprint of that particular organism. Ribotyping, a particular type of RFLP where the probes used are specific for rRNA genes, has been automated and equipment is available commercially that can ribotype an organism in 8 hours.

A commonly encountered problem is that RFLP patterns are excessively complex with many poorly resolved bands. Use of restriction enzymes with fewer sites on the bacterial chromosome overcomes this problem but specialist electrophoretic techniques are needed to separate the large DNA fragments produced. This is pulsed field gel electrophoresis (PFGE) which uses an alternating electric field to tease apart the large DNA molecules.

10.7 LABORATORY ACCREDITATION

From what has already been said it should be clear that there can be a number of different ways of detecting the same organism in a food matrix. The choice of method used can be governed by several factors and the relative merits of different methods is a topic of constant investigation and debate. This can however lead to the situation where differences in a result reported by two laboratories simply reflect the different method used.

In addition to problems arising from intrinsic differences in the performance of different methods, the same method in different laboratories can be subject to variation introduced by factors such as differences in procedures, equipment and its calibration. Some possible examples would include autoclave temperature profile when sterilizing media, time and temperature of incubation, sources of medium components and, of course, competence and experience of laboratory personnel.

A number of approaches are adopted to avoid such potential problems. Several national and international bodies approve standard methods for conducting certain analyses and one of these should be adopted for routine work and strictly adhered to wherever possible. Testing laboratories also often participate in proficiency testing schemes where a central body distributes standard samples for analysis, often specifying the precise time this should be conducted and the method to be used. Results are reported back, collated and a report circulated to participating laboratories which can then judge their performance against that of others. Finally, laboratories can seek some form of third party, independent recognition. There are quality systems such as the Good Laboratory Practice scheme and standards such as (ISO 9000 series) which are concerned with the quality of management within the organization but

which do not set a particular level of quality or competence to be achieved (see Section 11.7). There are also schemes of laboratory accreditation more concerned with the quality of performance in specific tests. In the UK this accreditation is usually sought through the UK Accreditation Service (UKAS) which accredits laboratories over a whole range of activities, not just microbiological testing. Most countries have their own equivalent organization such as NATA (Australia), DANAK (Denmark), ILAB (Ireland) and STERLAB (The Netherlands). The accrediting body inspects the laboratory and its procedures to ensure that tests are carried out consistently and correctly using approved methods with suitable quality control measures in place. Among the features investigated are the training and qualifications of staff, the suitability of equipment and procedures for its calibration and maintenance, participation in a proficiency testing scheme and the presence of full documentation prescribing the laboratory's operating procedures.

Obtaining laboratory accreditation can be a costly exercise but, if achieved, provides independent testimony to a laboratory's proficiency and will give increased confidence to potential customers.