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Methodology for detection and typing of foodborne microorganisms

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Abstract

Over the past decade many improvements have been seen in both conventional and modern methods for the detection of pathogenic bacteria in foods. Modifications and automation of conventional methods in food microbiology include sample preparation, plating techniques, counting and identification test kits. ATP bioluminescence techniques are increasingly used for measuring the efficiency of cleaning surfaces and utensils. Cell counting methods, including flow cytometry and the direct epifluorescent filter technique are suitable techniques for rapid detection of microorganisms, especially in fluids. Automated systems based on impedimetry are able to screen high numbers of samples based on total bacterial counts within 1 day. Immunoassays in a wide range of formats make rapid detection of many pathogens possible. Recently, there have been important developments in the use of nucleic acid-based assays for the detection and subtyping of foodborne pathogens. The sensitivity of these methods has been significantly increased by the use of the polymerase chain reaction and other amplification techniques. Alternative and rapid methods must meet several requirements concerning accuracy, validation, speed, automation, sample matrix, etc. Both conventional and rapid methods are used within hazard analysis critical control point programs. Further improvements especially in immunoassays and genetic methods can be expected, including the use of biosensors and DNA chip technology.

Keywords: Rapid methods; Automation; HACCP; Foodborne pathogens

1. Introduction

The analysis of foods for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality. Conventional bacterial testing methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in foods. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of the microorganisms present in a food sample. However, conventional methods require several days to give results because they rely on the ability of microorganisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterization make these methods labour intensive. Especially in the food industry there is a need for more rapid methods to provide adequate information on the possible presence of pathogens in raw materials and finished food products, for manufacturing process control and for the monitoring of cleaning and hygiene practices. These rapid methods

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deal with the early detection and enumeration of microorganisms, but also with the characterization of isolates, by use of microbiological, chemical, biochemical, biophysical, molecular biological, immunological, and serological methods (Fung, 1995). Alternative and rapid methods can be divided into the following categories:

- modified and automated conventional methods;
- bioluminescence;
- cell counting methods;
- impedimetry;
- immunological methods;
- nucleic acid-based assays.

2. Modified and automated conventional methods

Many attempts have been made to improve laboratory efficiency by making the procedures for the traditional agar medium-based methods more convenient and user-friendly and to reduce the costs of material and labour (Table 1)

2.1. Sample preparation

Gravimetric diluters automatically add the correct amount of diluent to the test sample before homogenization. The Stomacher™ (Colworth) massages the samples in a sterile disposable bag, eliminating the need to sterilize and to use blender cups. Recently, the Pulsifier™ (Kalyx) has been introduced. This apparatus beats the outside of a sterile disposable bag at high frequency (3500 rpm) producing a combination of shock waves and intense stirring which drives the microorganisms into suspension. In a comparative study, no significant difference was found in colony counts of food samples using the Stomacher™ or the Pulsifier™ for preparation of the primary dilution (Beumer et al., 1998).

Table 1
Modification and automation of conventional methods in food microbiology

Method	Application
Sample preparation	
Gravimetric dilutor	Diluent addition
Stomacher™ (Colworth)	Homogenization
Pulsifier™ (Kalyx)	Homogenization
Plating techniques	
Spiral plater	Enumeration
Dipslides	Enumeration
Chromogenic/fluorogenic	
Culture media	Detection
Motility enrichment	Detection
Petrifilm™ (3M)	Enumeration + detection
HGMF	Enumeration + detection
Counting	
Automation	Colony counting
Confirmation/identification	
Test kits	Confirmation, characterization

2.2. Plating techniques

There are several methods of adding the sample homogenate to the agar plate. The spiral plater (Spiral Biotech) deposits a small volume onto the surface of the agar in a spiral such that there is a dilution ratio of 10^4 from the centre to the edge of the plate. The colonies appearing along the spiral pathway can be counted either manually or electronically. As the volume dispensed at any point is known, this technique eliminates the need for serial dilutions before plating and less time is required for counting colonies.

Dipslides, which were originally introduced in medical microbiology for estimating the number of microorganisms in urine samples, are also very useful for testing surfaces. The agar slides that may contain selective or nonselective media are pressed on the surface to be examined and replaced within sterile sleeves. After incubation, colonies appearing on the slides can be counted and isolated.

Using chromogenic or fluorogenic substrates in selective media detection, enumeration and identification can be performed directly on the isolation plate, thus eliminating the use of subculture media and further biochemical tests (Manafi, 1996). These compounds yield brightly coloured or fluorescent products when reacting with specific bacterial enzymes or bacterial metabolites. Most fluorogenic enzyme substrates are derived from coumarin, such as 4-methylumbelliferone, while chromogenic enzyme compounds are mainly phenol derivatives. A wide range of chromogenic and fluorogenic substrates are now available and these compounds are applied in several commercial systems and media. Fluorogenic and chromogenic media are widely

applied for the detection and enumeration of coliforms and *Escherichia coli*. These media use β-D-glucuronidase (GUD) as an indicator for *E. coli*, as this enzyme is present in 94–96% of *E. coli* strains, but not in *E. coli* strains of serotype O157. For the detection of GUD activity, the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC or BCIG) are mostly used. Although not as sensitive for GUD as MUG, the principal advantage of X-GLUC over MUG is that typical colonies can be recognized without using ultraviolet light illumination. Indoxyl released from X-GLUC is rapidly oxidized to indigo, which is insoluble and builds up within the cells resulting in blue *E. coli* colonies. Another disadvantage of the use of MUG is the possible diffusion of fluorescence over the entire agar surface, complicating colony differentiation. However, the use of X-GLUC in routine plating media is limited practically because of the high cost of this compound. False-positive results with GUD-based *E. coli* detection methods are sometimes found because of the presence of GUD in some raw foods (de Boer, 1998).

The use of ''motility enrichment'' has been found effective for the rapid detection and isolation of some bacteria like *Campylobacter* (de Boer et al., 1993), *Arcobacter* (de Boer et al., 1996) and *Salmonella* (de Boer, 1998). As a combined enrichment–isolation medium for *Salmonella*, modified semisolid Rappaport-Vassiliadis medium compares favourably with standard Rappaport-Vassiliadis medium and gives results 1 day earlier. Diagnostic semisolid *Salmonella* (DIASALM) agar uses a saccharose/bromocresol purple indicator to observe migrating salmonellae as a pink zone within a green medium. Addition of nitrofurantoin to DIASALM agar favours the isolation of *Salmonella enteritidis* from poultry samples (de Boer, 1998).

The Petrifilm system (3M) is an alternative to agar-poured plates. This system consists of rehydratable nutrients that are embedded into a film along with a gelling agent, soluble in cold water. One ml of liquid sample is placed into the center of the film system, and the rehydrated media will then support the growth of microorganisms. After incubation, colonies can be counted directly from the film system as in conventional plates. Petrifilm products are available for yeast and mould counts, total aerobic plate counts, coliforms, *E. coli* and *E. coli* O157. Mean log counts of *E. coli* in poultry, meats and seafood were not significantly different from those for the standard most probable number (MPN) procedure (Gangar et al., 1999).

The hydrophobic grid membrane filter (HGMF) technique, including the ISO-GRID system (QA Life Sciences) works by confining colony growth to a set of 1600 grid cells. The HGMF technique has the advantages of removing inhibitors or unwanted nutrients, concentrating organisms, as well as a three-log counting range. The procedure consists of preparing homogenized food samples and then prefiltering the sample, which traps food particles larger than 5 μm. The sample is then filtered through the membrane filter, which traps target microorganisms within the grid cells. The inoculated HGMF is placed on an appropriate agar and colonies are counted after a suitable incubation period. In addition to an aerobic plate count, HGMF techniques for yeasts and moulds, *Salmonella*, coliforms and *E. coli* O157 have been described (Entis and Boleszczuk, 1991; Entis, 1998).

2.3. Colony counting

Colony counting is time consuming and several attempts have been made to automate this last step in the enumeration of microorganisms to improve efficiency and reduce labour costs. Various image analysis systems have been shown useful and cost effective in large laboratories, especially in the dairy industry. An image of the plate is stored and can be viewed, printed, or imported to other programmes. The user can set variables such as top or bottom lighting and colony size limits to exclude spreaders or background particulates.

2.4. Identification systems

A variety of morphological, physiological and biochemical tests are used for the identification of microorganisms. Several commercial identification kits have been developed to simplify and automate the identification of individual microorganisms (e.g., API systems, Minitek, Enterotube, BBL-Crystal). The results are comparable to conventional tests and the saving in time (e.g., media preparation, ease of reading) is significant.

3. Bioluminescence

Microbial cells as well as cells from food ingredients contain adenosine-5′-triphosphate (ATP) which can be measured using the luciferase enzyme complex found in fireflies. The total light output of a sample is directly proportional to the amount of ATP present and can be quantitated by luminometers. At least 10^4 cells are required to produce a signal. Measurement of ATP from bacterial and nonbacterial cells can, in this way, be used for measuring the efficiency of cleaning surfaces and utensils. The rapid response time for obtaining results, ranging from seconds up to a few minutes, makes this system very suitable for on-line monitoring in hazard analysis critical control point (HACCP) programs (van der Zee and Huis in 't Veld, 1997). Several companies now produce ATP hygiene monitoring kits and some of these systems have been compared (Colquhoun et al., 1998). These test systems have some limitations since several factors including pH, temperature, the presence of luciferase inhibitors, and others may influence the reaction. When dry-cleaning systems are used or when the product contains little or no ATP, the ATP bioluminescence cannot be used for hygiene monitoring (Griffiths, 1997). A significant opportunity for the future may be the provision of pathogen specificity to the ATP assay (Stewart, 1997).

4. Cell counting

4.1. Flow cytometry

This is an optically-based method for analyzing individual cells in complex matrixes. Microorganisms suspended in a liquid pass a beam of laser light. As this occurs, the light is both scattered and absorbed by the microorganisms. The extent and the nature of the scattering, which is an intrinsic property of the microorganisms, may be analyzed by collecting the scattered light with a system of lenses and photocells and can be used to estimate the number, size, and shape of microorganisms. The sensitivity of the technique is very high: as few as 10^2 yeast cells and about 10^2–10^3 bacterial cells per ml can be detected, with results being obtained within a few minutes. Because of its high sensitivity, flow cytometry is very suitable for detecting low numbers of specific organisms in fluid or rinses. Flow cytometry was successfully used to enumerate viruses in seawater (Marie et al., 1999). One of its disadvantages for use in food microbiology is the lack of distinction between living and dead cells (van der Zee and Huis in 't Veld, 1997) and interference by the food matrix (Griffiths, 1997).

4.2. Direct epifluorescent microscopy

The direct epifluorescent filter technique (DEFT) is a direct method used for enumeration of microorganisms based on the binding properties of the fluorochrome acridine orange. In this technique, food samples are pretreated with detergents and proteolytic enzymes, filtered onto a polycarbonate membrane, stained with acridine orange, and examined under a fluorescent microscope. The number of viable bacteria is determined based on the count of orange cells on the filter and can be performed in as little as 10 min. Enumeration of viable bacteria in raw minced meat using DEFT was found to be equivalent to standard plate counts (Boisen et al., 1992). These techniques can also be very useful in hygiene monitoring (Holah et al., 1988).

5. Impedimetry

Impedimetry is based on changes in conductance in a medium where microbial growth and metabolism takes place. The time necessary for these changes to reach a threshold value, the detection time, is inversely proportional to the initial inoculum. Several automated systems based on impedimetry are commercially available. These systems are able to examine hundreds of samples at the same time. Instruments are fully automated and computer-driven to enable continuous monitoring of impedance changes in several samples simultaneously. The results are presented as an impedance curve, which is compared with a previously generated calibration curve to estimate the numbers of bacteria present. Typically, most impedance analysis of food samples can be completed in 24 h. These systems are most commonly used to estimate total bacterial counts and for screening of large numbers of samples, as this procedure saves substantial time and material (van

der Zee and Huis in 't Veld, 1997). However, the impedance technique is not suited for testing samples with low numbers of microorganisms. Another drawback is that the food matrix may influence the analysis, which necessitates the determination of calibration curves for each food matrix examined (Waverla et al., 1998). Assays have also been developed for detecting *Salmonella*, *Listeria* and *Campylobacter*. The detection of *Salmonella* is based on the metabolism of trimethylamine-N-oxide during growth of salmonellae, which results in a large conductance change (Gibson et al., 1992).

6. Immunological methods

Immunological methods rely on the specific binding of an antibody to an antigen. For the detection of specific microorganisms and microbial toxins a variety of antibodies which are employed in different assay types have been described (Märtlbauer and Becker, 1995). The suitability of these antibodies depends mainly on their specificity. Polyclonal antisera contain an assortment of antibodies having different cellular origins and, therefore, somewhat different specificities. Most polyclonal antibodies used in immunoassays are derived from either rabbit or goat serum. One of the disadvantages of using polyclonal antisera in immunoassays is the variability found in the animal's immune response. The development of monoclonal antibodies greatly enhanced the field of immunoassays by providing a consistent and reliable source of characterized antibodies (Barbour and Tice, 1997). Immunoassays can be classified as homogeneous or heterogeneous.

6.1. Homogeneous immunoassays

In a homogeneous or ''marker-free'' assay there is no need to separate the bound from the unbound antibody; the antigen–antibody complex formed is directly visible or measurable. Incubation times are usually very short. Examples of homogeneous assays are agglutination reactions, immunodiffusion and turbidimetry. Both qualitative ''clumping reactions'' and quantitative turbidimetric methods are used. In latex agglutination tests, latex beads are coated with antibodies that agglutinate specific antigens and form a more easily visible precipitate. These tests are available for most pathogens.

6.2. Heterogeneous immunoassays

In a heterogeneous assay, the unbound antibody must be separated from the bound antibody using labelled reagents. In a typical sandwich assay, capture antibody is immobilized on a solid support, usually polystyrene tubes or microtiter plates. After addition of the test sample and an incubation period, the unbound sample is removed by washing and a reporter antibody conjugate is added. After a period of time, the unbound reporter conjugate is washed away and a reporting substrate is added. The amount of reporter signal is thus related to the amount of target antigen in the sample. The reporter antibody can be labeled with a fluorescent molecule and measured directly by the amount of fluorescence. Reporter antibodies are also often conjugated with enzymes including horseradish peroxidase, alkaline phosphatase and β-galactosidase (enzyme-linked immunosorbent assays — ELISA). With antibody–enzyme conjugates, the reporter antibody is detected by adding the substrate specific for the reporter enzyme in the reaction well. If the enzyme is present, the substrate will be modified, resulting in a product that can be detected by colorimetric or fluorometric techniques.

Commercial immunoassays for microorganisms and microbial toxins use a variety of supports and reporting systems. These test systems usually do not require equipment such as plate washers and readers and can be performed in minimally equipped laboratories. ELISAs for pathogens have detection limits ranging from 10^3 to 10^5 colony forming units (cfu)/ml. Therefore direct detection of pathogens in foods is not possible and enrichments are required for at least 16–24 h. Kits for the detection of bacterial toxins are based mainly on immunoassay systems. The use of these kits does not give any information on the biological activity of the toxins (Brett, 1998).

6.3. Immunocapture

Immunocapture-based separation and concentration techniques include immunological binding (capture), followed by physical separation of the target

organisms from a mixed enrichment culture. This results in concentration of the target organism. One application is immunomagnetic separation (IMS) in which samples are mixed with beads coated with antibodies for the target organism. The target organisms in the sample bind to the immunomagnetic beads, which are then isolated from other sample material and microorganisms in a magnetic field. The beads are then plated on medium and incubated overnight. The use of IMS has been especially useful for the detection of *E. coli* O157 in foods (Heuvelink et al., 1997).

6.4. Automated immunoassays

Antibody techniques are easily automated and cost-effective. An example of this is the Vitek Immuno Diagnostic Assay System (VIDAS, bioMérieux), an automated qualitative enzyme-linked fluorescent immunoassay for the detection of pathogens in foods. In this system, an aliquot of boiled enrichment medium is placed into a reagent strip which is coated with antibodies. The strip contains all the ready-to-use reagents (wash solution, conjugate and substrate) required. All assay steps are performed automatically by the instrument. Finally, the fluorescence is measured by the optical scanner in the apparatus and analyzed automatically by the computer.

A promising future development is the production of biosensors, which consist of a microchip-based system for analyzing the formation of antigen–antibody complexes (Malmqvist, 1993). The sensor chip consists of a glass support, an overlaid gold film and a dextran matrix to which antibodies can be immobilized. The antigen is injected over the chip surface. The antibody–antigen complex changes the refraction index at the chip surface, which can be measured optically (Robison, 1997).

7. Nucleic acid-based assays

The past decade has seen a significant increase in the development of genetically-based methods for the detection and characterization of pathogens in foods. Genetic detection methods are based on the hybridization of target DNA with a specific DNA probe. Depending on the desired specificity of the detection (genus-, species-, strain-specificity), different regions of the genome can be used as targets (Scheu et al., 1998).

7.1. Nucleic acid hybridization

Nucleic acid hybridization is typically between a DNA or RNA molecule present in the target organism and a probe DNA which has a sequence complementary to the target sequence. Probe DNAs usually contain 15 to 30 nucleotides. The specificity of a hybridization assay is completely controlled by the nucleotide sequence of the probe. The first step in these genetic methods usually is lysis of the cells and often also purification to free the nucleic acid, so that it can hybridize with the DNA probe. When the hybrid is formed, different detection techniques can be used. Most of these techniques are similar to those for antibody–antigen detection in immunoassays. Direct hybridization uses a labelled DNA probe to hybridize to nucleic acid within the sample. Radioactive and fluorescent probes allow direct detection of hybrids. Indirect detection is done with enzyme reporters. Removal of unbound reporter probe is easily achieved by immobilization of either the target DNA or the probe prior to hybridization. Solid supports are membranes, typically nylon or nitrocellulose, and polymer particles. The most common solid-phase formats are the Southern blot and the various dot blot formats, in which the target nucleic acids are immobilized on a membrane, either after separation in an electrophoresis gel (Southern blot) or from solution (dot blot) (Barbour and Tice, 1997). Sandwich hybridizations offer more sensitivity by using an immobilized probe to bind target nucleic acid to the solid-phase followed by hybridization of a reporter probe to an adjacent sequence on the same target DNA molecule. Detection of the reporter follows a wash step to remove unbound probe. Hybridization assays for several foodborne pathogens have been commercialized. The GeneTrak system uses pathogen-specific probes for ribosomal RNA targets in the bacteria and a colorimetric system for detecting the specific probe–target hybrids. Enrichment is necessary to obtain the required sensitivity.

7.2. Amplification methods

Because of the greater sensitivity, DNA-based methods which include an amplification step have become increasingly popular. The most popular method of amplification is the polymerase chain reaction (PCR) technique. In this method, first double-stranded DNA is denatured into single strands and specific short DNA fragments (primers) are annealed to these DNA strands, followed by extension of the primers complementary to the single stranded DNA with a thermostable DNA polymerase. Starting from a single target DNA or RNA sequence, more than one billion product sequences can routinely be synthesized by PCR. This quantity of DNA can be visualized as a band on an ethidium bromide-stained electrophoresis gel. Many PCR protocols for the detection of foodborne bacteria and viruses have been described (Scheu et al., 1998). An alternative to PCR is the isothermal amplification system NASBA®. This system selectively amplifies RNA through the concerted action of three enzymes. The NASBA® system was optimized for detection of pathogenic campylobacters and *Listeria monocytogenes* (Uyttendaele et al., 1997). The diagnosis of small round-structured viruses (SRSV) has recently been improved by the development of very sensitive reverse transcriptase (RT)-PCR (Jiang et al., 1992; Lees et al., 1995). The enzyme RT converts RNA into DNA molecules, which are the targets for PCR amplifications. The successful application of this technique for the detection of SRSV in foods depends mainly on the further development of adequate procedures for the extraction of viruses from foods.

A problem to routine use of PCR in food testing laboratories is that the procedures are rather complicated and a very clean environment is needed to perform the tests. Recent developments in PCR methodology make the technique more user-friendly for routine use. These include innovative reagent delivery systems, such as the PCR reagent and positive control tablets used in the Bax™ system (Bennett et al., 1998). The PCR Probelia™ test (Sanofi Diagnostic Pasteur) is divided into two modules: the first one includes optimized reagents for sample treatment and ready-to-use amplification mixture; the second includes microtiter plates, peroxidase-labeled probes and buffers needed to perform sandwich hybridization of amplified products onto microtiter plates. The availability of PCR test kits is also an important factor in the standardization of the PCR techniques.

Further drawbacks of PCR are the inability to distinguish between live and dead cells, the presence of polymerase inhibitors in food samples leading to false-negative results, and the accessibility of the target organisms. Pre-enrichment prior to PCR analysis overcomes most of these problems. PCR inhibition can be prevented by separating bacteria from the food matrix prior to DNA extraction by differential centrifugation, IMS, dilution and addition of bovine serum albumin (Lindqvist, 1997; Al-Soud et al., 1998; Scheu et al., 1998). The efficiency of cell lysis can be improved by enzymatic treatment of the cells before purification of the target DNA (Abolmaaty et al., 1998).

A limitation in the use of nucleic acid-based assays is that they indicate only the genetic potential to produce toxin or to express virulence and do not give any information on toxins already present in foods or expressed virulence. They also do not lead to the isolation of the organism and so no further characterization can be done. On the other hand, molecular-based techniques may be more reliable for the detection of viable but nonculturable bacterial cells (Colwell, 1997).

7.3. Molecular subtyping methods

Molecular subtyping methods can identify different strains within a species, generating data useful for taxonomic or epidemiologic purposes. Nucleic acid-based methods have the advantage over phenotypic identification methods of not being influenced by environmental conditions of the cells, because the nucleotide sequence of the DNA does not change during growth. DNA fingerprinting methods like restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis are suitable for grouping of isolates in transmission studies or outbreak investigations. In the RFLP technique, DNA is cleaved by restriction enzymes and the resulting fragments are separated by gel electrophoresis. Different binding patterns (polymorphism) may be observed after transferring the DNA from the gel to hybridization filters by blotting and hybridization with labeled probes (van der Vossen and Hofstra, 1996). In the RAPD assay,

patterns are generated by the amplification of arbitrary nucleotide sequence and a subsequent gel electrophoresis of the amplified DNA (Williams et al., 1990). A frequently observed shortcoming of these fingerprinting techniques is their lack of reproducibility among or even within laboratories. Sources of variability are the primer concentration, DNA template concentration and quality, concentration and source of polymerase, $MgCl_2$, the thermocycler and the number of thermocycles (Barrett, 1997). Pulsed-field gel electrophoresis (PFGE) is considered to have both good reproducibility and sufficient resolving power for the epidemiological typing of bacterial isolates. In this technique, restriction enzymes digest the complete genome and large DNA molecules are resolved by continuous reorientation of the electric field during gel electrophoresis (Tenover et al., 1995). AFLP® (amplified fragment length polymorphism) is a recently developed method for genotyping based on selective amplification of restriction fragments of digested total genomic DNA. This technique combines the reliability of RFLP with the advantages of PCR (Vos et al., 1995). An automated ribotyping system (RiboPrinter®, duPont de Nemours) has recently been introduced. DNA is extracted from cells and fragmented by a restriction enzyme. The DNA fragments are separated by electrophoresis and transferred to a membrane. Pattern detection occurs after hybridization with a DNA probe. First, a chemiluminescent label is introduced and the emission of light from the hybridized fragments is captured by a camera. This image pattern can be compared to others in a database and the identification of the organism is based on a pattern match (van der Plas et al., 1998).

The reproducibility of molecular typing procedures will depend on the methods used, the organism to be typed and the experience of the analyst. A problem in interpreting fingerprinting data is the genetic changes that may occur naturally over time. This ‘‘genetic drift’’ causes changes in fingerprinting patterns (Barrett, 1997). However, single band differences between strains will not always be epidemiologically significant. PFGE patterns may change due to loss of genes in the course of maintenance or subcultivation of strains (Murase et al., 1999). When it is difficult to decide about differences probably caused by genetic changes, the use of more than one subtyping method is advised.

8. Requirements for alternative and rapid methods

There are several factors which must be considered before adapting a new alternative or rapid method (Swaminathan and Feng, 1994; Fung, 1995; van der Zee and Huis in ’t Veld, 1997; Notermans et al., 1997):

1. Accuracy — false-positive and false-negative results must be minimal or preferably zero. The method must be as sensitive as possible and the detection limit as low as possible. In many cases, the demand is less than one cell per 25 g of food, as small numbers of some pathogens may cause disease. Analytical tests for these agents need only be qualitative (presence/absence). For rapid screening methods, a higher false-positive frequency may be acceptable, as positive screening tests are followed by confirmation tests.

   The sensitivity of a method is the proportion of target organisms that can be detected; it can be calculated with the following equation:

   $$\text{Sensitivity (\%)} = \frac{\text{number of true positives } (p)}{p + \text{number of false negatives}} \times 100$$

   A failure to detect the target when present is a false-negative result and will lower the sensitivity of a test. In food microbiology, only a very low frequency of false-negative results can be tolerated for safety reasons.

   The specificity of a method is the ability to discriminate between the target organism or toxin and other organisms or substances; it can be calculated with the formula:

   $$\text{Specificity (\%)} = \frac{\text{number of true negatives } (n)}{n + \text{number of false positives}} \times 100$$

   A positive result in the absence of the target is a false-positive result and will lower the specificity of a method. Table 2 shows an example of sensitivities and specificities found for some test systems for *Listeria*.
2. Validation — the alternative test should be validated against standard tests and evaluated by collaborative studies. In these studies, preference

Table 2
Sensitivity and specificity of some rapid methods for detection of *Listeria* (Beumer, 1997)

Type	% Sensitivity	% Specificity
Immunological method		
VIDAS-LMO (bioMérieux)	93	100
Listeria Rapid test (Oxoid)	70	100
Listeria Visual Immunoassay (Tecra)	97	100
DNA assay		
Gene-Trak	100	100

should be given to naturally contaminated food specimens; the tests are then performed under conditions in which users will apply them. Results obtained with samples containing a low contamination level should be emphasized, since there is sufficient evidence that in most cases high numbers of target cells in samples will lead to positive test results (Beumer, 1997). In the US, the evaluation programs of Association of Analytical Chemists (AOAC) International are most widely accepted. In France Association Française de Normalisation (AFNOR) has a similar position. To deal with these problems, the European project MICROVAL was initiated by the European Community.

3. Speed — rapid tests for the detection of pathogens or toxins should give an accurate result within hours or at the utmost 1 day. However, many detection systems need an overnight enrichment for resuscitation and amplification of the target pathogens, as they rely on the presence of at least 10^4–10^5 organism/ml for results to be reliable. Table 3 gives (for some methods) information on the time necessary to obtain a positive result, the amount of cells necessary for a positive reaction and the possibility of detecting specific groups of microorganisms.
4. Automation and computerization — the ability to test many samples at the same time. Many systems utilizing the microtiter plate format can handle 96 samples at one time. However, for smaller laboratories, the availability of single-unit tests is also very important.
5. Sample matrix — new systems should give a good performance of the matrices to be tested. Baseline extinction values may depend on the type of food being tested. Background flora, natural substances or debris can interfere with the test method and invalidate the test result.
6. Costs — purchasing, reagents, supply, operational costs, upkeeping. The initial financial investment for rapid methods may be high, because many systems require expensive instruments. Operating costs of many commercial rapid test kits are also high.
7. Simplicity — methods should be user-friendly, which means easy to operate and manipulate.
8. Reagents and supply should be rapidly available.
9. Training, technical service and company support is essential.

Table 3
Characteristics of some alternative and rapid methods

Method	Detection limit (cfu/ml or g)	Time before result	Specificity[a]
Plating techniques	1	1–3 days	Good
Bioluminescence	10^4	$\frac{1}{2}$ hour	No
Flow cytometry	10^2–10^3	$\frac{1}{2}$ hour	Good
DEFT[b]	10^3–10^4	$\frac{1}{2}$ hour	No
Impedimetry	1	6–24 h	Moderate/good
Immunological methods	10^5	1–2 h[c]	Moderate/good
Nucleic acid-based assays	10^3	6–12 h[c]	Excellent

[a] Possibility of detecting specific groups of microorganisms.
[b] Direct epifluorescent filter technique.
[c] After enrichment procedure.

10. Space requirements — instruments are preferably compact and small.

A major disadvantage of alternative and rapid methods over cultural methods is that most rapid methods include damaging of the cells and therefore, viable cells for confirmation and further characterization can only be obtained by repeat analysis using standard cultural procedures. Moreover, rapid methods usually detect only one specific pathogen, while cultural methods may simultaneously detect and isolate many pathogens by including several types of selective media in the analysis. The use of several rapid assays to do multipathogen analyses on a food sample, makes this analysis unacceptably expensive.

9. Microbiological methods in HACCP programs

In the past decade, the control of the safety of foods has been mainly carried out by product testing rather than process control. The main problem with doing end-product testing is the high number of samples to be examined before one can decide on the safety of the product batch, especially when pathogens are expected to be heterogeneously distributed in the batch. Moreover, end-product testing detects only failures and does not identify causes (van Schothorst and Jongeneel, 1994). HACCP is now generally accepted as the most effective system to ensure food safety.

For evaluating the control of microbial hazards in a HACCP-based process, both chemical and microbiological methods can be used. Main factors for the growth and survival of microorganisms are temperature, time, a_w, pH and preservatives. These parameters are easily measurable with physicochemical methods. Most chemical indicators like lactic acid, acetic acid and amines are unsuitable for monitoring for safe food production because of lack of sensitivity in respect of assessing microbial growth (Sheridan, 1995).

Microbiological methods are needed within a HACCP program for risk assessment, the control of raw materials, the control of the process line and the line environment, and for validation and verification of the HACCP program. For checking of raw materials and for monitoring of supplies, both conventional and rapid methods may be used, depending on the period that the materials can be kept in stock. Rapid methods like ATP bioluminescence are important for testing the efficiency of cleaning of the production environment. These methods allow sufficient time for surfaces to be recleaned before production begins if they were found to be contaminated (Griffiths, 1997). Verification is done by checking if the HACCP system is working, which includes microbiological end-product testing by conventional and rapid methods. For a proper risk assessment, numerous microbiological examinations of samples, of ingredients and end-products are necessary. Rapid methods can be very helpful for this data collection.

10. Future development

Improvements in the field of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient methods in food microbiology. Further development of ''on-line'' microbiology, including ATP bioluminescence and cell counting methods, is important for rapid monitoring of cleanliness in HACCP programs.

One of the most challenging problems is sample preparation. More research is needed on techniques for separating microorganisms from the food matrix and for concentrating them before detection by immunological or nucleic acid-based assays. The possibilities of combining different rapid methods, including immunological and DNA methods, should be further exploited. Antibodies can be used for capture of target cells that are then detected by a genetic method. On the other hand, amplified PCR sequences can be quantified using immunoassays (González et al., 1999). Further developments in immunoassays and PCR protocols should result in quantitative detection of microorganisms and the simultaneous detection of more than one pathogen or toxin. For immunoassays, further research on the application of sensor chips may result in multianalyte assays. As positive results of PCR tests do not indicate if the virulence or toxin gene was actually expressed, future studies will focus on the development of assays which measure biological activity (Pimbley and Patel, 1998).

Molecular detection and typing methods are largely based on gel electrophoresis, which is a labour-intensive expensive technique and difficult to automate (Southern, 1996). A new development, which combines semiconductor manufacturing technology with molecular biology to build so called ''DNA chips'', promises to be one of the major molecular diagnostic break-throughs for the future (Wallraff et al., 1997). With this technique, DNA sequences can be analyzed quickly and cheaply. DNA chips consist of large arrays of oligonucleotides on a solid support. The array is exposed to labeled sample DNA and hybridized (Ramsay, 1998). The detection of the probe–target hybrid is achieved by direct fluorescence scanning, or through enzyme-mediated detection (O'Donnell-Maloney et al., 1996). DNA chip technology also makes it possible to simultaneously detect diverse individual sequences in complex DNA samples. Therefore, it will be possible to detect and type different bacterial species in a single food sample. Development of this approach is continuing at a rapid pace and for the microbiologist, the DNA chip technology will be one of the major tools for the future. There are still many problems to solve, such as sample preparation, eliminating the effects of nonspecific binding and cross-hybridization and increasing the sensitivity of the system.

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