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REVIEW

HACCP-based food quality control and rapid detection methods for microorganisms

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Foodborne disease is one of the most widespread problems of the contemporary world. Numerous microbiological hazards and risks are associated with different areas of the food industry. The Hazard Analysis Critical Control Point (HACCP) concept is a systematic approach to ensuring food safety. The implementation of an HACCP system is closely connected with microbiological quality control. Rapid microbiological methods are being extensively developed. The methods are based on several different principles of direct or indirect detection of microbes. In the HACCP system, these methods can be used when the system is developed, implemented and maintained. Successful combination of the HACCP programme and rapid microbiological methods may help the industry to find new ways of obtaining reliable results more efficiently and of ensuring food safety. Copyright © 1996 Elsevier Science Ltd.

Keywords: HACCP; food quality; microbiological hazards; rapid methods

INTRODUCTION

A quality system has been defined as an assembly of components such as the organizational structure, responsibilities, producers and processes. A quality system consists of two parts: quality control (QC) for operational techniques and activities and internal quality assurance (QA) to ensure that the intended quality is achieved (Nadkarni, 1993). From this point of view total quality management (TQM) can be divided into three quality processes: quality control, quality assurance and quality improvement. The Hazard Analysis Critical Control Point (HACCP) system is becoming increasingly accepted in food control. For example, an EC Directive on Food Hygiene (Council Directive, 1993) proposes that HACCP should be applied in the member states by the end of 1995. HACCP is a safety management tool and it can be incorporated into TQM programmes for the following reasons: to improve the efficacy of the operations and the quality of products, to satisfy a requirement from the customers or purchasers, to prove a due diligence defence in legal actions or to keep up with their competitors.

The major hazard in food production will continue to be microbiological contamination, and it is here that the HACCP approach is now being adopted internationally to ensure safety (Campbell-Platt, 1994).

An understanding of the microbiology of the foods being prepared and their ingredients is also an essential part of developing an HACCP concept. One of the basic characteristics of the HACCP concept is that, when such a system has been properly conceived, implemented, monitored, verified and reviewed, it provides a better assurance of the microbiological status of the products than when relying on end-product testing (Jouve, 1994).

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Traditional microbiological methods of detecting and enumerating foodborne microorganisms usually require several days to yield reliable results. In many cases, the products have already been used by the consumers before the analyses are completed. Therefore, alternative assays based on different microbiological methods are constantly being developed. The main requirements for a good microbiological method are reliability, sensitivity, selectivity and economy (Vasavada, 1993). These methods can be either direct or indirect, depending on the parameter to be monitored. Commercial methods in this area include impedimetry, direct epifluorescence microscopy, ATP bioluminescence, turbidometry, immunological methods and methods based on gene technology. At present, most of these methods are highly automated, thus minimizing manual work. Methods based on analysis of DNA or RNA are of particular interest, because they provide specific information about the contaminants and the results can be achieved in one working day. Immunological assays can provide information on specific bacterial or virulence types and also about microbial metabolites. In this review, the HACCP concept, major microbiological hazards in food materials and rapid methods are discussed.

THE HACCP SYSTEM AND RAPID MICROBIOLOGICAL METHODS

A complete quality system in the food industry covers acquisition of raw materials, production, storage, distribution and sale. It must also take into account the purpose for which the product is intended and the conditions under which it will be consumed. The HACCP concept has proved to be an effective aid in developing a quality safeguard system (van Schothorst, 1989). The HACCP program must be tailored to the specific individual product and processing line. A successful HACCP program is specific for a processing line and should be designed in accordance with the people, the equipment and the overall system in mind. Upper management commitment to HACCP is a very critical factor. It is also important that employees are involved in the planning and design stages of the HACCP plan (Stevenson and Bernard, 1993). An HACCP team must be assembled and properly trained in HACCP principles (NACMCF, 1992). The HACCP concept is a systematic approach to the identification, assessment of risk and severity, and the control of the different hazards associated with each segment of the food system from production to consumption.

According to Peri (1993), hazards to consumer safety may derive from biological, chemical or physical contamination. The HACCP system provides a means of controlling any microbiological hazard that may arise in food processing or handling operations and aims to identify problems before they materialize. The HACCP approach has several advantages over the traditional approach of end-product sampling for microbiological quality control: (i) it looks forward and thus does not rely on post-testing procedures to resolve problems; (ii) it is flexible and can be a part of all stages of production and distribution of foods; (iii) all operations and procedures are documented to ensure the production of food products within strict control limits; (iv) the expertise of many different areas within a food production plant is utilized (Smith *et al.*, 1990).

Notermans *et al.* (1994b) proposed an approach to identify potentially hazardous microorganisms. The first step is to establish the hazardous organisms associated with a particular food product. The hazards associated with each microorganism and the probability of their occurrence must be assessed. This establishment of hazardous microorganisms in a particular food is one step in implementing the HACCP system. Following an evaluation of raw materials, ingredients, the production and processing, contamination or recontamination after processing in packaging, transportation, preparation, storage and service areas, the microorganisms are either removed from or added to the list of microorganisms known to cause foodborne disease with a particular food product.

HACCP is a proactive strategy that identifies the Critical Control Points (CCPs) at which these hazards can be managed (NACMCF, 1994). The principles of HACCP and its seven steps are described by the Codex Alimentarius Commission (Codex, 1993). The HACCP concept contains observations and/or tests made to identify actual or potential hazards in special operations and also to identify CCPs in a process. Monitoring of the CCPs is performed to ensure that control is maintained (ICMSF, 1988).

Notermans *et al.* (1994a) also proposed an approach for establishing criteria (setting of critical limits) for microbiological (mainly bacterial) hazards associated with foodborne disease. After identification of potentially hazardous microorganisms, their numbers in raw food materials are determined. Storage tests, microbiological challenge testing or predictive microbiology can be used to obtain information about the effects of the whole food production process on the numbers of potentially hazardous microorganisms. Quantitative risk assessment is then utilized to determine whether or not these levels are acceptable. If microbial levels are acceptable, criteria can be established at specific CCPs.

Rapid microbiological methods can be used nearly at all steps of the HACCP system. Large amounts of data can be gathered quickly and easily to develop in-house data or expand an existing database to form the basis of risk assessment (Stier, 1993). Rapid microbiological methods for on-line monitoring as part of an HACCP system are under investigation, e.g. on-line biosensors for pathogens (Cirigliano, 1992, Goldschmidt, 1993). If a deviation from the

process or a critical limit is observed, rapid tests for e.g. pathogenic bacteria may be used for evaluation of the deviation. Rapid microbiological analysis can also be used with random sample collection to verify that CCPs are under control (Stier, 1993).

MICROBIOLOGICAL HAZARDS IN FOODS

The major role of microorganisms in the spoilage of food and the role of food as a vector for the transmission of microbes responsible for foodborne disease are well recognized. Results of the surveillance programme (WHO, 1991) indicate that the number of agents of foodborne disease continues to increase in Europe (Notermans *et al.*, 1994b). In fact, morbidity caused by foodborne disease is second only to respiratory diseases in Europe (Baird-Parker, 1994). The different reporting systems of different countries provide a poor reflection of the true situation of foodborne diseases. Generally it is estimated that the rate of underreporting is as much as 95–99%. Only a small percentage of individuals with gastrointestinal illness contact a physician. In addition, the causative agent is detected in only a small proportion of reported cases (Notermans and Hoogenboom-Verdegaal, 1992). Baird-Parker (1994) presented some reasons for the increased incidence of foodborne disease. The increase is probably the result of a combination of factors, including: better reporting and statistical reasons due to changes in reporting systems; changes in agricultural practices; changes in food manufacture and food consumption practices; identification of newly emerging pathogens; and susceptibility of populations to infections. Foodborne illnesses are more likely to be life-threatening for the immune-compromised, the aged, and those individuals debilitated by underlying health problems (Oblinger, 1988). Certain microorganisms are ubiquitous in nature, occurring in soil and on vegetation, in animal wastes and on animal carcasses. Some pathogens occur in the intestinal tracts of normal, healthy animals, including humans. Thus, pathogens can enter raw foods easily. The main requirement for foodborne disease of microbial origin is that the organisms concerned gain access to a food and once present, the food and its storage environment are capable of supporting growth or survival. The ability of some potentially life-threatening pathogens to survive and/or proliferate under refrigeration and in reduced oxygen atmospheres, and in some cases the low number necessary for disease production, indicate the seriousness of the potential hazards. Foodborne microbial diseases can be classified as (i) intoxications, (ii) toxin-mediated infections; and (iii) infections when microorganisms invade and multiply in the intestinal mucosa or other tissues (Anon, 1988). Intoxications are caused by ingestion of foods containing either poisonous chemicals or toxins produced by microorganisms. Bacteria that produce enterotoxins during colonization and growth in the intestinal tract are the cause of toxin-mediated infections. Infectious organisms such as *Salmonella*, *Campylobacter* and *Shigella* can cause disorders if present even in low numbers. Large numbers of *Clostridium perfringens* and *Bacillus cereus* must be ingested to produce intoxication (Notermans *et al.*, 1994b).

The causative agents of foodborne illnesses may also be such entities as 'viable non-culturable' forms of pathogenic bacteria (Baird-Parker, 1994). *Campylobacter jejuni* has the characteristic feature of degenerating into a coccoid, non-culturable form after a few days in culture (Saha *et al.*, 1991). Microbe amounts of only 500 cells are able to cause illness in man (Robinson, 1981). Studies on the recovery of the coccoid campylobacteria have yielded controversial results. Saha *et al.* (1991) isolated seven of sixteen non-culturable strains after passage through rat gut. Medema *et al.* (1992) used one day old chickens as animal models and suggested that recovery of the coccoid cells after passage through gut might be poor. Beumer *et al.* (1992) performed a wide series of studies with non-culturable *Campylobacter jejuni* cells using both simulated gastric, ileum and colon environments and oral administration of the bacteria. The presence of culturable cells was not demonstrated in any of the experiments.

ICMSF (1974) subdivided foodborne pathogens and foodborne disease-causing agents into three main groups according to the hazards they present: (i) severe hazards caused by *Clostridium botulinum*, *Salmonella cholerae-suis*, *S. typhi*, *S. paratyphi* A, *Shigella* spp., *Vibrio cholerae*, Hepatitis B virus and some mycotoxins; (ii) moderate hazards with potentially extensive spread, caused by pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp.; and (iii) moderate hazards with limited spread, with causative agents such as e.g. *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*. *Table 1* represents the predominant bacteria as causative agents of foodborne diseases. Notermans *et al.* (1994b) presented a literature review of unusual or additional bacteria that have appeared to cause foodborne diseases over the past 25 years. These bacteria are presented in *Table 2*.

Moulds present in certain raw materials and processed foods can produce mycotoxins when growing under favourable conditions (Notermans *et al.*, 1992). Most mycotoxins are heat stable and orally toxic, mutagenic or carcinogenic, causing different illnesses in man and animals (WHO, 1976). A summary of the predominant mycotoxins is presented in *Table 3*.

Viruses are obligate intracellular parasites that cannot multiply in food. The majority of foodborne viruses significant to human health are present in the human intestine and are often transmitted by food or water contaminated with fecal material. Seafood

taken from water polluted with human wastes is one of the most common vehicles of human viral disease. Hepatitis A virus has been the causative agent in outbreaks of infectious hepatitis transmitted by oysters, clams, salads, sandwiches, cold meats and to a lesser extent milk and milk products. Hepatitis A belongs to the same virus family, *Picornaviridae*, as do the polio, enteric cytopathogenic human orphans (ECHO) and coxsackie viruses, which are also documented as causative agents of foodborne viral infections (Fries, 1994). An emerging viral pathogen is the Norwalk and Norwalk-like viral agent that has caused outbreaks of gastroenteritis called Norwalk virus illness. Foods implicated have been raw vegetables, salads, raw shellfish and contaminated water (WHO, 1976, Anon, 1986). The existence of viruses in raw seafood is often estimated indirectly by the determination of fecal coliforms, but it has been shown that this is not an adequate tool for the enumeration of these viruses (Atmar *et al.*, 1993).

RAPID DETECTION METHODS FOR MICROBES

The development of more rapid methods for microbiological quality control has been in the interest of scientists ever since routine microbiological analysis

Table 1 The predominant bacteria as causative agents of foodborne diseases (WHO, 1976; Anon, 1986; Oblinger, 1988; Jay, 1992; Lechowich, 1988; Notermans and Hoogenboom-Verdegaal, 1992)

Pathogen	Origin	Foods implicated (examples)	Action in foods	Foodborne disease
Gram-negative bacteria				
Campylobacter (*C. jejuni*, *C. coli*)	Intestinal tract of farm animals (e.g. poultry, pigs, sheep, cattle), wild birds and rodents	Raw vegetables, poultry, raw beef, pork, lamb, unpasteurized milk and dairy products	Generally unable to multiply in foods Produces a heat-labile enterotoxin	Acute gastroenteritis (campylobacteriosis)
Escherichia coli 0157:H7	Feces of dairy cows and other farm animals, human intestinal tract, polluted water	Raw and under-cooked beef and other red meats, unpasteurized milk, cheeses	May multiply in foods during storage and distribution Produces cytotoxin	Hemorrhagic colitis
Salmonella (*S. enteritidis*, *S. typhimurium*)	Feces of almost all food animals	Meat and poultry, unpasteurized milk, dairy and egg products	Can multiply in foods	Salmonellosis infection
Shigella (*S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*)	Intestinal tract of humans and other primates	Salads, seafoods	May survive for extended periods and may grow under certain conditions in foods Shiga toxin may be produced	Shigellosis infection, dysentery
Vibrio parahaemolyticus	Naturally occurring in the marine environment	Raw shellfish	Virulent strains produce thermostable and / or thermolabile direct hemolysin	Acute gastroenteritis
Yersinia enterocolitica	Wide variety of animals (particularly swine), water	Raw milk, meat, poultry, shellfish, vegetables	Capable of growth in food, psychrotroph	Gastroenteritis yersiniosis
Gram-positive bacteria				
Bacillus cereus	Widely distributed in soil and on vegetation	Rice and rice dishes, custards, puddings, sauces, vegetable dishes, milk, meat, spices	Produces and secretes a thermolabile, diarrhogenic or a thermostable, emetic enterotoxin during exponential growth in foods	*Bacillus cereus* intoxication
Clostridium botulinum	Widely distributed in nature (soil, water)	Improperly processed canned foods	Grows and produces neurotoxin in foods, can grow in vacuum packs/ 'sous vide'/'nouvelle carte' products	Botulism intoxication
Clostridium perfringens	Soil, dust, water, human and animal intestinal tract	Inadequately heated and cooled meats, poultry, gravy, beans	Grows and produces thermolabile enterotoxin	*C. perfingens* toxin mediated infection
Listeria monocytogenes	Soil, animal feces, sewage, silage, decaying vegetation	Raw milk, pork, poultry, ground beef, soft cheeses	Psychrophilic, produces listeriolysin	Listeriosis (meningitis, sepsis, abortions)
Staphylococcus aureus	Skin, nose, throat, infected sores of man, hides of animals	Different proteinaceous foods (warmed foods, ham, other meats, dairy products, custards, potato salad, cream-filled pastries)	May grow and produce thermostable enterotoxins in foods	Staphylococcal intoxication

was applied to foods. The recent advances in biotechnology have simplified many procedures and reduced assay time (Swaminathan and Feng, 1994).

The development of alternative methods is a continuous process leading from modifications of the classical Standard Plate Count (SPC) through more rapid detection of cells to the more advanced methods based on immunology or gene technology. A summary of the alternative assay methods described in the following sections is presented in *Table 4*.

Table 2 Unusual or additional bacteria as causative agents of foodborne diseases (modified from Notermans *et al.* (1994b))

Gram-positive	Gram-negative
Bacillus anthracis	*Actobacter melanogenus*
Bacillus brevis	*Aeromonas* spp. (e.g. *A. hydrophila, A. sobria*)
Bacillus licheniformis	*Alcaligenes faecalis*
Bacillus subtilis	*Citrobacter* spp.
Brucella	*Enterobacter cloacae*
Clostridium bifermentans	*Hafnia alvei*
Corynebacterium	*Klebsiella* spp.
Coxiella burnetii	*Proteus* spp. (e.g. *P. penneri*)
Erysipelothrix spp.	*Pseudomonas aeruginosa*
Flavobacterium farinofermentans	*Pseudomonas cocovenenans*
Francisella tularensis	*Vibrio* spp. (*V. cholerae, V. vulnificus, V. mimicus*)
Leptospira	
Mycobacterium spp. (e.g. *M. bovis*)	
Pasteurella multocida	
Plesiomonas shigelloides	
Providencia spp.	
Streptobacillus moniliformis	
Enterococci of group A (e.g. *Enterococcus pyogenes, E. zooepidemicus*)	

Rapid detection methods based on the detection of whole cells or their metabolites can be divided into two main classes: direct methods are based on the detection of cells with or without incubation and indirect methods are based on the measurement of metabolic products or other changes caused by the cell growth (Karwoski, 1994). Direct epifluorescent filter technique, DEFT (Pettipher, 1983) and flow cytometry (MacKenzie and Pinder, 1987) can be considered as the most important direct techniques.

Direct epifluorescence filter technique (DEFT)

In this technique, membrane filtration and epifluorescence microscopy are used for enumeration (Pettipher, 1983). To improve the filterability of food samples, enzyme-surfactant treatments are often needed. Solid particles are removed by prefiltration. The filtered sample is stained, most commonly with Acridine Orange (AO) dye. Orange fluorescing microbial cells are counted as aggregates in order to correlate with SPC (Pettipher *et al.*, 1980). The method can also be automated by linking the micro-

Table 3 Major mycotoxins and their toxic effects (Jay, 1992; Morgan and Lee, 1990; Pestka *et al.*, 1995)

Mycotoxin	Moulds	Origin	Properties
Aflatoxin B_1, B_2, G_1, G_2 Aflatoxin M_1	*Aspergillus flavus, A. parasiticus*	Corn, cottonseed peanuts, wheat	Hepatocarcinogenic
Citrinin	*Penicillium*	Rice, wheat, oats, rye	Carcinogenic
Ochratoxin	*Aspergillus, Penicillium*	Oats, barley, swine blood, citrus fruit	Kidney toxicity, carcinogenic
Patulin	*Penicillium, Aspergillus clavatus, A. terreus, Byssochlamus*	Apples, apple juice	Neurotoxicity, carcinogenic
Trichothecenes (incl. deoxynivalenol, nivalenol, T-2 toxin)	*Fusarium, Cephalosporium, Stachybotrys*	Corn, wheat, barley	T2-toxin causes alimentary toxic aleukia
Zearalenone	*Fusarium graminearum, F. tricinctum*	Corn, wheat, barley	Estrogenic effects, reproductive problems

Table 4 Examples of rapid methods for microbiology

Method	Principle	Instruments/Manufacturer	References
DEFT	Fluorescence staining of cells, direct microscopy	Bio-Foss/Foss Electric, Denmark COBRA/Biocom, France Autotrak A.M. Systems, UK	Buisson *et al.*, 1990; Pettipher *et al.*, 1992 Betts and Bankes, 1988
Flow cytometry	Detection and sorting of stained cells moving in a fluid stream	BactoScan/Foss Electric, Denmark Chem-Flow/Chemunex, France	Dasen *et al.*, 1991; Pettipher, 1991
Impedimetry	Detection of electrical changes in the growth medium	Bactometer/Bactomatic Inc., USA Malthus/Malthus Instrument, UK RABIT/Don Whitley Scientific, UK BacTrac	Hancock *et al.*, 1993; Gibson *et al.*, 1992
ATP bioluminescence	Detection of total or microbial ATP	Lumac/Lumac, The Netherlands Bio-Orbit/Bio-Orbit Oy, Finland Bio-Trace/UK	Dziezak, 1987; Ogden, 1993
Turbidometry	Detection of turbidity changes in the growth medium	Bioscreen/Labsystems, Finland AutoMicrobic System®/Vitek Systems, USA	Mattila, 1987; DeSimon and Ferrer, 1989

scope to an image analysing system, such as the Bio-Foss Automated System (Foss Electric, Denmark) or the COBRA system (Biocom, France). A sample throughput rate of 150 samples per hour is claimed by the manufacturer of the COBRA system. DEFT has been applied to several types of foods (Shaw *et al.*, 1987; Abgrall and Bourgeois, 1989; Shaw and Farr, 1989; Boisen *et al.*, 1992). Milk and milk products (Rodrigues and Pettipher, 1984; Betts and Bankes, 1988; McCann *et al.*, 1991) and beverages (Haikara, 1985; Rodrigues and Kroll, 1986) have also been studied. A different kind of application is the study of food contact surfaces by DEFT (Holah *et al.*, 1989). The main advantage of the DEFT technique is sensitivity. In theory, even one microbial cell caught by the filtration can be detected. In practice, however, the detection limit is around 1×10^3 bacterial cells/ml (Pettipher, 1983). At present, the main disadvantage of the method is the high capital cost of the instrumentation.

Flow cytometry

Flow cytometry is a promising technology which utilizes both microscopic and biochemical analyses in order to enumerate microorganisms in liquids (Nader *et al.*, 1991). The cells are introduced into a rapidly moving fluid stream through the beam of a laser, xenon or mercury arc lamp. The illuminating light is scattered by the passing cells and its intensity at different angles provides the possibility of multiparametric analysis on a cell-by-cell basis. The cells can be labelled with fluorochromes (Reseland, 1990; Patchett *et al.*, 1991; Jespersen and Jakobsen, 1994). Fluorescent antibodies or DNA staining can also be used to label the target cells (Donnelly and Baigent, 1986; Amman *et al.*, 1990; Bertin *et al.*, 1990). Immunomagnetic separation by antibody-coated paramagnetic beads is an efficient tool for separating the target cells from background material (Reseland *et al.*, 1992). The flow cytometric analysis is rapid, taking typically less than 30 minutes from sampling to results (MacKenzie *et al.*, 1987).

An important advantage of flow cytometry is the high number of observations that can be collected within minutes. The high throughput rate guarantees statistically significant results (Hutter, 1993). Similarly to DEFT, flow cytometry has been applied mainly to raw milk (Kæreby and Asmussen, 1987; Heddeghem *et al.*, 1990; Dasen *et al.*, 1991), but also to meat (Patchett *et al.*, 1991) and other foods (Pettipher, 1991). The detection limit depends on the food matrix and has been reported to be 10^4 colony forming units (CFU)/ ml of raw milk (Dasen *et al.*, 1991), 10^5 CFU/ml of meat homogenate, about 10^2 CFU/ml of yogurt (Easter and Prentice, 1989) and 5×10^1–1.4×10^4 CFU/ml of soft drinks (Pettipher, 1991).

Impedimetry

Impedance microbiology is based on the monitoring of electrical changes caused by the growth of microorganisms. Impedance is a vector parameter consisting of two components: conductance (G) and capacitance (C). The changes in conductivity can be measured either directly or indirectly. In direct impedance measurement, nutrient macromolecules are broken down into smaller high-charged units as a result of microbial metabolism. The conductivity change of the medium is measured. The bacterial population must reach a threshold level of 10^5–10^6 CFU/ml before the conductivity change can be monitored. The time point at which the change can be visualized is usually called the detection time (Fung, 1994). The primary result of the measurement is a curve similar to a classical growth curve. However, the conductance curve is different from a true growth curve, because it starts from a level of around one million bacteria per milliliter. Specific growth media are usually needed in direct impedimetry.

Indirect impedance is a more recent application in the field (Owens *et al.*, 1989). This method is based on the production of carbon dioxide by growing microorganisms. The carbon dioxide is absorbed into an alkaline solution and the reduction of conductivity of the solution is measured. The main difference in the case of indirect impedance is that the formation of CO_2 can be detected much earlier than the conductance change caused by the breakdown of nutrients, thus reducing the time needed for the analysis and enhancing the sensitivity of the method (Deak and Beuchat, 1993). The indirect measurement can be carried out without specific growth media. Impedance measurement has been applied to many fields of food microbiology. The Malthus *Salmonella* system has been given Official First Action Approval by the AOAC (Association of Official Analytical Chemists). Procedures and media for other foodborne pathogens such as *Staphylococcus aureus* (Prentice and Neaves, 1987, Bolton 1990a), *Clostridium botulinum* (Gibson, 1987), *Listeria monocytogenes, Yersinia enterocolitica* (Walker, 1989; Easter and Kyriakides, 1991; Hancock *et al.*, 1993; Capell *et al.*, 1995) and *Campylobacter* (Bolton, 1990b) have also been developed. Indirect impedance measurement has been applied mainly to food products with low contamination levels, such as juices and beverages. Deak and Beuchat (1995) concluded that a lower detection limit of one cell/10 ml is possible if the samples are pre-incubated.

ATP bioluminescence

Detection of the high-energy molecule adenosine triphosphate (ATP) extracted from cells is a widely used indirect assay method. The ATP amount is measured as the light energy released by the

luciferin-luciferase system in the presence of magnesium ions (Stanley, 1989). The assay is rapid, only a few seconds in hygiene monitoring applications and less than an hour for most other samples. The limitation of this technology lies in the fact that ATP is present in all viable cells. Therefore, intrinsic ATP in the sample material interferes with the ATP originating from the target cells and must be removed enzymatically before the assay.

In addition, the ATP analysis provides only an estimation of the total bacterial count, but cannot differentiate between bacteria (Baumgart, 1993). Theoretically, ATP amounts as low as 100 fg (10^{-13} g) can be measured, corresponding to about 100 bacterial cells. Under practical conditions the sensitivity is about 1000 fg (10^{-12} g), which corresponds to about 1000 bacterial cells or one to two yeast cells (Heeschen *et al.*, 1991). Stressed cells and cells in the stationary growth phase contain less ATP, which also affects the results (Bülte and Reuter, 1985). On the other hand, however, the amount of ATP in a sample provides an estimate of the active microbial population, which is important when considering the shelf life of the product. Stressed cells can also be allowed to resuscitate before the ATP assay (Graumlich, 1985).

ATP technology is widely used in the on-site hygiene control of surfaces in industrial plants. The results are available within a few minutes and represent the overall hygienic status of the plant (Simpson *et al.*, 1989).

Other indirect methods

Colorimetry is a method based on the measurement of colour changes caused by metabolic activities. The method has been approved for class b inclusion in the 16th edition of *Standard Methods for the Examination of Dairy Products*, published by American Public Health Association (APHA) (Andrews, 1991). Theoretically, the sensitivity of the method is *ca* 5 CFU/ml (Karwoski, 1994).

Turbidometric measurement has been used for preliminary studies of food and beverage samples. The sensitivity of the method has been reported to correspond to about 10^2 CFU/ml (Mattila, 1987, Haikara *et al.*, 1987, 1990). However, turbidometric methods can be problematic in food microbiology because of high background turbidity or non-homogeneity of the sample. Fluorometric methods have been used to monitor the resazurin reduction test with contaminated milk samples and heat-processed foods (Tiusanen *et al.*, 1989; Ali-Vehmas *et al.*, 1991, Mattila-Sandholm *et al.*, 1991). The fluorometric method was found to increase the sensitivity, rapidity and throughput capacity of the reductase test and to be insensitive to turbidity of the samples. Microcalorimetry is a method hitherto employed mainly as a research tool to study various foodborne bacteria (Lampi *et al.*, 1974) and the microbial loads of minced beef samples (Gram and Sægaard, 1985) and milk (Adams, 1989). The method is based on measurement of the heat generated by growing microbes.

Immunological methods

Immunoassays are analytical techniques based on the specific and high affinity binding characteristics of a group of inducible animal-derived proteins called antibodies with particular target molecules called antigens (Rittenburg, 1990). In addition to analysis of pathogenic bacteria and their toxins, immunoassays can also be used for the detection of mycotoxins in food and feed materials.

The basis of every immunoassay is the detection and measurement of the primary antigen–antibody reaction. Immunological assays based on polyclonal or monoclonal antibodies are performed as Enzyme-Linked Immuno Sorbent Assays (ELISAs), Radio-Immuno-Assays (RIAs), fluorescent techniques or agglutination tests (Huis in't Veld and Hofstra, 1991).

Enzyme immunoassay technology employs enzyme markers as a means of amplifying and visualizing the primary antibody–antigen binding reaction and is probably the most rapidly growing and most widely used immunoassay technology employed today. Immunoassays can be divided into two basic systems: heterogeneous (separation-requiring) and homogeneous (separation-independent) assays. The methods can be further classified into six groups, of which the sandwich assay type is the most used in ELISA (Deshpande, 1994; van Poucke, 1990). ELISAs are usually carried out in a microtitre tray. Alternatively, polystyrene or ferro-metal beads can be used as solid phases. The latter have the advantage that a magnetic device can be used to collect the beads for the washing steps. Instead of the enzyme, a number of different molecules and compounds can be used to label antigen (Morris, 1985).

A major disadvantage of the ELISA assay is that every step in the assay requires a rigorous washing procedure, which is labour-intensive and difficult to automate. Attempts have been made to develop more simple homogeneous immunoassays in which no separation steps are required. New types of homogeneous immunosensors have been described (Oh, 1993), with the aim of automated methods and data processing.

Another very rapid immuno-assay is the latex agglutination. In this technique, latex particles coated with antibodies are mixed with sample dilutions or extracts. If the antigen is present, it will agglutinate the latex particles within minutes, giving a macroscopically visible result. (Huis in't Veld and Hofstra, 1991).

Among the different methods, immunological techniques are promising because of their sensitivity and rapidity. However, even rapid detection tests normally require enrichment of the target bacteria to

the level of the assay's detection limit (van Beurden and Macintosh, 1994). Selective enrichment procedure in the case of *Salmonella* and its competitors can be replaced by rapid immunomagnetic separation (Vermunt *et al.* 1992; van Beurden and Macintosh, 1994).

Immunoassays need to select for appropriate antigens and antibodies. The antibody may react with cell surface antigens of the microbe or with bacterial toxin or mycotoxins. For gram-negative bacteria, this selection is somewhat easier because different proteins from the outer membrane are known to be common antigens of a genus and/or species. A wide range of microorganisms and bacterial or fungal toxins relevant to the food industry can now be detected and quantified by immunological techniques (Huis in't Veld and Hofstra, 1991, Labadie and Desnier, 1992).

Several rapid methods have been developed for testing foods for the presence of *Salmonella* (*Table 5*). Most commercial test kits are based on ELISA techniques, but immunodiffusion and dip-stick technology are also used. There are also some commercially available test kits for staphylococcal enterotoxins (*Table 5*). Many of the commercially available test kits have also been adopted for first action by AOAC International. The sensitivity of these methods corresponds to that of standard methods. In addition to the commercially available test kits, studies concerning some other microbes have also been carried out (*Table 6*). ELISA was also the most frequently used test in these studies.

Antibodies have also been made to the major known mycotoxins, and both ELISAs and RIAs have been successfully applied to the screening of mycotoxins in foods. Pestka *et al.* (1995) recently reviewed examples of reported immunoassays for aflatoxins, ochratoxins, trichothenes, fumonisins and other mycotoxins, many of which can detect picogram or nanogram levels of toxin. Many commercial test kits are also available for mycotoxins (Pestka *et al.*, 1995).

Table 5 contains some examples of the commercially available test kits for whole bacteria or their enterotoxins. *Table 6* contains examples of some non-commercial tests.

Methods based on gene technology

The application of molecular biological techniques to food microbiology has vastly increased during recent years. These techniques represent a new generation of rapid methods, based on the primary information contained in the nuclear acid sequences of a particular organism. This can possibly lead to entirely new and reliable methods for the detection, identification and characterization of foodborne organisms (Grant and Kroll, 1993). Methods based on nuclear acid hybridization are on the market for several organisms, e.g. *Salmonella*, *S. aureus*, *Y. enterocolitica*, *E. coli*, *Listeria* and *C. jejuni* However, the detection level of nucleic acid hybridization methods is about 10^5–10^6 CFU/ml, and enrichment steps are therefore needed for food samples (Dreher and Märtlbauer, 1995; Manafi and Holzhammer, 1994).

The new and more sophisticated methods based on amplifying specific DNA or RNA sequences *in vitro* theoretically allow the detection of a single molecule of target DNA or RNA (Swaminathan and Feng, 1994). The polymerase chain reaction (PCR) is based on repetitive cycles of DNA denaturation, primer annealing and extension by a thermostable DNA

Table 5 Some commercially available test kits for whole bacteria or their enterotoxins

Test kit	Method	Microbe or toxin	Matrix	Additional information	References
Salmonella-Tek	ELISA	*Salmonella*	Dry milk, chocolate, egg, turkey	1 organism/25 g	Eckner *et al.*, 1994; Schippers, 1991
TECRA	Immunocapture ELISA	*Salmonella typhimurium*	Dairy, meat, vegetable, fish	0.4 CFU/g in 24 h	Flint and Hartley, 1993
TECRA	ELISA	Staphylococcal enterotoxins	Seafood, mushrooms, dry milk, beef, chicken		Park *et al.*, 1993; Bennett and McClure, 1994
VIDAS	ELISA	*Salmonella*, *Listeria*, Staphylococcal enterotoxin	Food	Detection limit 1.8×10^6 salmonellas ml^{-1} (pure culture)	Anon, 1992; Blackburn *et al.*, 1994
Listeria-Tek	ELISA	*Listeria* spp.	Dairy, seafoods, meat	Negative samples excluded within 2 d	Meier and Terplan, 1993; Curiale *et al.*, 1994; Rodriquez *et al.*, 1993
Salmonella 1-2 TEST	Immuno-diffusion	*Salmonella*	Poultry, shrimp		Warburton *et al.*, 1995, Oggel *et al.*, 1995
PATH-STIK	Dip-stick technology	*Salmonella*	Food	Detection limit 10^6/ml	van Beurden, 1992
Locate test	ELISA	*Salmonella*	water		Walter *et al.*, 1993
RIDASCREEN	ELISA	Staphylococcal enterotoxins	Food	0.2 - 0.75 ng/ml in 3 h	Park *et al.*, 1994

Table 6 Studies on immunological methods other than commercial test kits

Microbe	Method	Matrix	Additional information	Reference
Pseudomonas fluorescens	ELISA	Meat, milk	10^5–10^6 CFU/cm^2	Gonzales *et al.*, 1993, 1994
Staphylococcus aureus	Latex Agglutination Test, ELISA	Processed foods	28 h	Chang and Huang, 1993, 1994
Candida intermedia and *C. parapsilosis*	ELISA	Orange juice	$>10^3$	Yoshida *et al.*, 1991
Enterobacteriaceae	ELISA	Drinking water	24 h	Hubner *et al.*, 1992
Escherichia coli O157:H7	ELISA	Ground beef	0.2 CFU/g in less than 20 h including enrichment	Padhye and Doyle, 1991
E. coli O157:H7	Immunomagnetic beads		10 CFU/ml	Fratamico *et al.*, 1992
Pediococcus	A membrane immunofluorescent antibody test	Brewery pitching yeast	0.001% <4 h	Whiting *et al.*, 1992
Legionella pneumophila	membrane immunoassay	Water		Berube *et al.*, 1989
Aspergillus, Penicillium	ELISA and Latex Agglutination Test	Wheat, rice		Schwabe *et al.*, 1993
Salmonella	ELISA	Chicken	400 cells/ml	Wyatt *et al.*, 1993
Clostridium toxins	ELISA	Food 0.1–1.0 ng/ml	Nagahama *et al.*, 1991	
Staphylococcal enterotoxin B	ELISA	Cheese	0.5–1.0 ng/ml, 1 h	Morissette *et al.*, 1991
Staphylococcal enterotoxin B	ELISA	Cheese, beef	0.1 fg/ml for purified toxin or 10 pg/g of contaminated food in 3 h	Bhatti *et al.*, 1994
Botulinal toxin	Colony immunoblot			Goodnough *et al.*, 1993

polymerase (Hill and Olsvik, 1994). The primers can be designed for different purposes: specific primers are targeted to a known virulent factor of a single species and allow simultaneous detection and identification, whereas multiple primers with a broader spectrum are suitable for plural species and produce different sizes of amplified DNA (Tsuchiya *et al.*, 1992)

The PCR reaction with an RNA probe is considered advantageous by some investigators, because RNA–DNA hybrids are more stable than DNA–DNA, there are antibodies available to recognize RNA–DNA hybrids and it is expected that higher signal-to-noise ratios can be achieved because of less nonspecific binding (Blais and Phillippe, 1993). Theoretically, several pathogens in one sample can be detected simultaneously with this technique, provided that species-specific primer sets able to function in the same conditions are available (Giesendorf *et al.*, 1992). Multiplex PCR methods have given promising results in the detection of common pathogens in poultry processing environments, e.g. *Listeria* (Lawrence and Gilmour, 1994) and *Salmonella* (Mahon *et al.*, 1994).

The main advantages of PCR technology are its sensitivity and specificity. With the automated devices now under development, it will be possible to complete a PCR assay during one working day. However, sensitivity is also the greatest potential weakness of this method. Cross-contamination with external DNA very easily gives false results (Russell *et al.*, 1995). Technical improvements under development will probably solve this problem.

At present, one major problem in PCR technology is the fact that it cannot distinguish between genetic material from dead and living cells (Wegmüller *et al.*, 1993). A new modification of PCR technology, NASBA™ (Nucleic Acid Sequence-Based Amplification), is based on the detection of messenger RNA (mRNA), which is transiently present in the cells and is thus indicative of living cells in the sample (Malek *et al.*, 1994).

In food microbiology, inhibitory compounds in the samples currently limit the application of PCR technology. Complex food samples containing high amounts of fat and protein have been shown to inhibit the reaction, as well as certain components used in selective growth media. Routine use of this technology will require the development of efficient procedures to eliminate the inhibition (Rossen *et al.*, 1992). The application of PCR technology to food microbiology usually also requires a selective enrichment step before the amplification. Thus, the entire procedure may take 2–3 days to complete (Swaminathan *et al.*, 1994). Immunomagnetic separation (IMS) has proved to be a very efficient method to separate target organisms from the food material. In addition to the reduced time for analysis, IMS technology also overcomes the problems associated with inhibition due to components of the selective media (Swaminathan and Feng, 1994).

PCR technology has mainly been applied to the detection of *Salmonella* and *Listeria* and also to non-culturable forms of organisms such as *Campylobacter* and *Legionella*. With a 3-hour preincubation, a sensitivity of 1–10 cells/ml or g in raw milk and different dairy products has been achieved. The cells can be either alive or dead, but they are always an indication of a contamination at some point (Wegmüller *et al.*, 1993).

Filtration combined with sonication before PCR assay applied for campylobacters in water gave a

sensitivity of about 2 CFU/ml (Kirk and Rowe, 1994). Giesendorf *et al.* (1992) applied PCR with 16S rRNA primers to detect *Campylobacter* spp. in naturally and artificially contaminated chickens. Correlation to the conventional method was found to be satisfactory and levels of 10^6 to 10^8 of contaminating bacteria did not affect the *Campylobacter* assay specificity in natural samples. Similar sensitivities have also been achieved for *Salmonella* and *Listeria*. Immunomagnetic separation of the target bacterial cells has proven to be very promising as means of removing interfering background compounds from the mixture (Fluit *et al.*, 1993).

Enteric viruses such as Norwalk virus have also been studied, because they cannot be detected easily by conventional methods. Atmar *et al.* (1993) studied polio viruses and Norwalk viruses seeded in oysters. The oysters contained inhibitory agents, probably polysaccharides, that had to be precipitated before the assay. In these studies, the sensitivity of PCR was found to be about 50 to 500 virus particles per ml. Usually, PCR products are detected by gel electrophoresis. For routine applications, however, more rapid and convenient methods are needed. Kapperud *et al.* (1993) developed a method for the detection of *Y. enterocolitica* by immunomagnetic separation and colorimetric detection of the PCR products. In their study, a plasmid-encoded virulence determinant was chosen as the target for the PCR, because this plasmid is necessary to cause disease. In artificially seeded food samples, a sensitivity of 10 to 30 CFU/g was obtained in the presence of a 10^6 fold excess of indigenous bacteria. In natural samples, a slightly lower sensitivity can be expected due to the presence of stressed or sublethally weakened yersiniae.

Gannon *et al.* (1992) studied a PCR technique for the enterohemorrhagic *E. coli* O157:H7. Artificial inoculation with *E. coli* followed by enrichment and PCR resulted in a total analysis time of around 9 hours and a sensitivity of 1 CFU/g. This application could be used as a screening method; positive samples had to be confirmed by conventional methods.

PCR technology has not yet been adopted as a routine tool for food industry laboratories because of the contamination problems and rather complicated test protocols. However, the enormous effort being put into studies in this area will certainly result in simple and cost-effective methods for routine purposes.

Future prospects

Traditionally, empirical data has been relied on to provide information concerning the ability of a microorganism to grow and produce toxin in a particular food. More than one factor always governs the growth of microbial populations, and in most cases there are interactive effects among the various factors (Farber, 1986).

Using mathematical models, many independent variables can be included and combinations of interactive effects can be studied to predict the probability of microbial growth in a particular food product. This concept is called predictive microbiology (McClure *et al.*, 1994)

A commercial application of this principle is the Food MicroModel (Food MicroModel Ltd, UK), which provides a range of predictive models for many major foodborne pathogens.

A different approach is provided by the development of biosensors. Biosensors consist of a sensing element and a transducer. The sensing element, also called the receptor, is composed of immobilized biologically sensitive material, such as antibodies, enzymes or DNA. The transducer interprets the intensity of signal change on the sensor by electrochemical, electric optical or thermal techniques. Theoretically, biosensors could offer a real 'on-line' control system for food processes, because they are sensitive and the analysis is completed within minutes. Biosensor systems have been applied to several types of foods. However, problems such as long term stability, reusability and sterilizability still limit the use of these devices (Goldschmidt, 1993).

CONCLUSIONS

A successful combination of an HACCP plan and rapid methods for microbiological control can form a good concept for the food manufacturer to gain better quality with less laboratory work and reduced total costs. The benefits of the combination will be available both for the industry and the consumers. However, many of the methods are at present still under evaluation and much experimental work is still needed before they can be applied to quality control in industrial laboratories.

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