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# Methods for Detecting Microbial Pathogens in Food and Water

CHARLES W. KASPAR\* AND CARMEN TARTERA\*\*

*University of Maryland, Department of Microbiology, College Park, MD, USA*

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## I. Introduction

Newly developed methods for the detection of bacteria and viruses have provided microbiologists with the means to rapidly identify and monitor specific microorganisms in food and water. Traditional methods of testing involve culture techniques to increase numbers of the organism to a detectable level; followed by isolation and biochemical identification. Methods of propagating some enteropathogenic viruses (i.e., Norwalk) are lacking and reports of viable but non-culturable bacteria (Colwell *et al.*, 1985) suggest direct methods of detection are warranted.

\* Present address: San Labs, 405 Eighth Avenue S.E., Cedar Rapids, Iowa 52401, USA; \*\* Present address: Department of Microbiology, Faculty of Biology, University of Barcelona, 08071-Barcelona, Spain.

The literature is replete with culture methodologies for the isolation and identification of food- and waterborne pathogens. Emphasis here will be on methodologies to detect pathogens and indicator organisms; however, the methods described are applicable to most bacteria. This chapter will focus on the use of nucleic acid and antibody probes which have the potential to circumvent the need to culture the organism prior to identification. It is beyond the scope of this chapter to cover all of the rapid methods and culture media described for the isolation and detection of specific bacteria, or groups of bacteria. For reviews of rapid methods, see Tilton (1981), Pierson and Stern (1986) and Lányi (1987).

## II. Food- and waterborne pathogens

### A. Bacterial pathogens

As detection and isolation methods have improved, a growing number of pathogens have been identified as important food- and waterborne pathogens (Table I). Several of the bacterial pathogens are widely distributed in soil, marine and estuarine waters, the intestinal tract of warm-blooded animals, or water contaminated with faecal matter. The challenge to microbiologists has been to develop effective monitoring procedures and control measures for a variety of samples and pathogens.

Both water and food have served as vehicles for bacterial pathogens. In the United States from 1946 to 1980, 672 waterborne outbreaks were reported (Lippy and Waltrip, 1984). Although the causative agent for one-half of the outbreaks was never established, *Salmonella* was identified as the aetiological agent in 75 of the outbreaks, *Shigella* in 61, *Escherichia coli* in 5 and *Campylobacter* in 2 (Lippy and Waltrip, 1984). A variety of foods have served as vehicles for bacterial pathogens (Table I). Bryan (1988) analysed 1586 cases of foodborne illness reported from 1977 to 1984 in the United States. The most frequently identified vehicles were seafoods (24.8%), meats (23.2%), poultry (9.8%), salads (8.8%), and others (<5%). Other foods implicated included: raw clams, fried rice, and Mexican-style foods. *Salmonella* has been the most common aetiological agent identified in foodborne cases, followed by *Campylobacter jejuni*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Staphylococcus aureus* and *Bacillus cereus* (Beckers, 1988; Todd, 1988). A number of studies of foodborne outbreaks and the vehicles and pathogens involved have been reported by others (Blake *et al.*, 1980; Hauschild and Bryan, 1980; Sours and Smith, 1980; Remis *et al.*, 1984; Archer, 1988; Archer and Young, 1988; Beckers, 1988; Bryan, 1988; Hackney and Dicharry, 1988).

**TABLE I**  
Habitat, pathogenic characteristics, and vehicles of major foodborne bacterial pathogens

Organism	Characteristics	Foods
<i>Bacillus cereus</i>	Common to soil, vegetation and water; produce one of two enterotoxin types, causing either diarrhoea or a vomiting response; infective dose $10^6$ – $10^8$ cells $g^{-1}$ of food	Diarrhoeal-type; cereals, potatoes, vegetables, meat products, and puddings Vomiting-type: fried and boiled rice
<i>Campylobacter jejuni</i>	Habitat is the intestinal tract of animals, widely distributed in nature; infective dose $10^2$ cells	Meats, poultry, poultry products, unpasteurized dairy products, mushrooms
<i>Clostridium botulinum</i>	Indigenous to soil, widely distributed; produces potent exotoxin	Meats, poultry, fish, home-canned vegetables and fruits
<i>Clostridium perfringens</i>	Indigenous to soil, widely distributed; produces exotoxin; infective dose $>10^6$ cells	Meats, poultry, fish; some frozen foods, fruits, vegetables
<i>Escherichia coli</i>	Habitat is the intestinal tract of animals and man, widely disseminated in nature; enteropathogenic, enterotoxigenic, enteroinvasive and haemorrhagic strains	Meats, fish, poultry, milk, dairy products, vegetables, rice
<i>Listeria monocytogenes</i>	Widely distributed in soil, vegetation, water and the intestinal tract of animals; haemolytic strains are pathogenic	Vegetables, dairy products, raw and pasteurized milk, soft cheeses, poultry, meats
<i>Salmonella</i>	Habitat is the intestinal tract of animals and man; widely disseminated in nature	Eggs, egg products, unpasteurized milk, poultry, meat and meat products
<i>Shigella</i>	Habitat is the intestinal tract of man and primates; invasive, shiga toxin may be produced; infective dose $10^1$ – $10^2$ cells	Salads, seafoods, Mexican foods

TABLE I – continued

Organism	Characteristics	Foods
<i>Staphylococcus aureus</i>	Associated with the nasal cavities and skin of man; produces exotoxin; $10^6$ – $10^8$ cells $g^{-1}$ of food necessary for sufficient toxin production	Meats, poultry, fish, shrimp, dairy products, vegetables
<i>Vibrio parahaemolyticus</i>	Widely distributed in marine and coastal waters; virulent strains generally Kanagawa positive, produce heat-stable haemolysin	Seafoods; oysters, shrimp, crabs, lobster, clams
<i>Vibrio cholerae</i>	Widely distributed in bay and estuarine waters; disease caused primarily by 01 serotype, cholera toxin produced by some	Raw and undercooked oysters and clams
<i>Yersinia enterocolitica</i>	Widely distributed in nature, man, and animals; pathogenicity associated with calcium dependency, autoagglutination, and binding of Congo Red dye	Meats, water, tofu, milk

## B. Viral pathogens

Over 100 types of viruses are disseminated from human faeces and urine into the environment. The source and physical characteristics of enteric viruses are shown in Table II. Enteric viruses are not normal inhabitants of the gastrointestinal and respiratory tracts of man and may be classified as pathogens although several produce asymptomatic infections. These viruses may multiply in the host reaching numbers between  $10^6$  and  $10^{10}$   $g^{-1}$  of faeces (Sabin, 1955). Symptomatology of enteric virus infections has been reported by Melnick (1984).

Despite the fact that, in most countries, sewage is treated prior to discharge into the environment, enteric viruses have been found in river water (Simkova and Wallnerova, 1973; Block, 1983), lakes (Vaughn and Landrey, 1977), groundwater (Wellings *et al.*, 1974, 1975; Vaughn *et al.*, 1978), bathing and coastal waters (Metcalf and Stiles, 1967; Goyal *et al.*, 1978) and drinking water (Coin *et al.*, 1965; Mack, 1973; Deetz *et al.*, 1984). The presence of these viruses in environmental waters indicates that

TABLE II

The characteristics, source, and immunoassays for potentially pathogenic viruses excreted into the environment

Virus	Origin	Virus size/genome <sup>a</sup>	Immunoassay <sup>b</sup>	Growth in cell lines
Enteroviruses	Faeces	28 nm; ss-RNA		Yes
Poliovirus			FIA, EIA	
Echovirus			FIA	
Coxsackievirus A				
Coxsackievirus B			FIA, EIA	
New enteroviruses (type 68-71)				
Hepatitis A (type 72)			EIA, RIA	Slow
Rotavirus	Faeces	70-80 nm; ds-RNA	FIA, EIA	Slow
Reovirus	Faeces	70-80 nm; ds-RNA	FIA	Slow
Adenovirus	Faeces Urine	} 80 nm; ds-DNA		Yes
Norwalk virus	Faeces		27 nm; incomplete	No
Calicivirus	Faeces	35-40 nm; ss-RNA		No
Astrovirus	Faeces	28 nm; incomplete		No
Coronavirus	Faeces	80-180 nm; ss-RNA		No
Snow mountain hepatitis	Faeces	27 nm; incomplete		No
Non A-non B hepatitis	Faeces	27-30 nm; incomplete		No

<sup>a</sup> Nucleic acid type; ss = single stranded; ds = double stranded.

<sup>b</sup> RIA = radioimmunoassay; EIA = enzyme immunoassay; FIA = fluorescent immunoassay.

current disinfection treatments are not thoroughly effective for viruses. Enteric viruses have been found to be more resistant to chlorine treatment and to survive longer in the environment than bacteria, and these properties are accentuated in the presence of particulate material (Berg *et al.*, 1978). It is not surprising that enteric viruses have been found in waters which lack bacterial indicators and are considered microbiologically safe (Craun, 1978; Rose *et al.*, 1987). Recently, indicators more closely related to enteric viruses in structure, morphology, chemical composition, and size, like coliphage, F-male phages and *Bacteriodes fragilis* bacteriophages, have been used as indicators of enteric viruses. Bacterial and viral indicators are discussed below.

Although it is accepted that viruses are transmitted via water, few outbreaks have identified a virus as the causative agent. Outbreaks have been reported for hepatitis A (Poskanzer and Beadenkopf, 1961; Bryan *et al.*, 1974; Craun, 1981; Hejkal *et al.*, 1982), Norwalk virus (Grohmann *et al.*, 1980; Gerba *et al.*, 1985), rotavirus (Gerba *et al.*, 1985), poliovirus (Mosley,

1967), hepatitis non-A, non-B (Khuroo, 1980; Wong *et al.*, 1980), and adenovirus (contracted in swimming pools and involved in eye and respiratory infections; (Foy *et al.*, 1968; D'Angelo *et al.*, 1979; and gastroenteritis, Uhnou *et al.*, 1984).

The dissemination of viruses by food is not as well documented as by water; however, interest has increased with the development of methods that detect low numbers of virus. Poliovirus has been implicated in outbreaks involving milk (Dingman, 1916; Aycocock, 1927; Lipari, 1951) and has been isolated from raw ground beef (Sullivan *et al.*, 1970). Echovirus 4 found in coleslaw was the aetiological agent involved in an outbreak of meningitis (US Department of Health, Education, and Welfare, 1976). While, hepatitis A has been transmitted to food through food handlers (Cliver, 1971), other enteric viruses have been isolated from raw ground beef (Sullivan *et al.*, 1970), and fruits and vegetables irrigated with sewage (Bagdasar'yan, 1964; Larkin *et al.*, 1976; Katzenelson and Mills, 1984). Shellfish are perhaps the most important food vehicle for viruses because as filter feeders they concentrate viruses from surrounding waters and are frequently consumed raw or undercooked. A number of outbreaks of hepatitis A (Mason and McLean, 1961; Stille *et al.*, 1972; O'Mahony *et al.*, 1983; Gerba *et al.*, 1985; Richards, 1985) and Norwalk virus (Murphy *et al.*, 1979; Gunn *et al.*, 1982; Guzewich and Morse, 1986) have been traced to the consumption of raw shellfish. Recently, Snow Mountain agent, astrovirus and calicivirus have been incriminated in shellfish-associated gastroenteritis (Dolin *et al.*, 1987; Kurtz and Lee, 1987). Enteroviruses and hepatitis non-A, non-B viruses have been isolated from shellfish (Gerba and Goyal, 1978; Goyal *et al.*, 1979; Ellender *et al.*, 1980; Caredda *et al.*, 1981; Alter *et al.*, 1982), but have not been incriminated in any outbreaks.

### III. Indicator organisms

Because it is not feasible to test food and water for all pathogens, indicator organisms are used to signal the presence of faecal contamination and the possible presence of intestinal pathogens. Conceptually, indicators are found in samples at higher numbers and are more easily identified than pathogens. Standard indicators include coliforms, faecal coliforms, *E. coli*, and faecal streptococci. The following have been proposed as indicators: *Bacteriodes fragilis* (Fiksdal *et al.*, 1985), *Clostridium perfringens* (Bonde, 1966), *Bifidobacterium* sp. (Evison and James, 1975), and *Rhodococcus coprophilus* (Mara and Oragui, 1981). Because bacterial indicators fail to correlate with the presence of viral pathogens, coliphages (Guelin, 1948), F-male phages (Havelaar *et al.*, 1985), and phages against *B. fragilis* (Jofre *et al.*, 1986; Tartera and Jofre, 1987), which have survival properties similar to

those of viral pathogens, have been proposed as indicators of viral contamination. The use, shortcomings, advantages, and detection methods for indicator organisms have been reviewed extensively (Hoadley and Dutka, 1977; Mossel, 1978, 1982; Matches and Abeyta, 1983; Reinhold, 1983; Splittstoesser, 1983; Tompkin, 1983; Hartman *et al.*, 1986).

#### IV. DNA probes

Nucleic acid probes have become a valuable diagnostic reagent in the identification of human and animal pathogens and made possible the identification of viruses and bacteria which are difficult, if not impossible, to cultivate. DNA probes have also proved to be a useful tool for identifying and monitoring organisms in food and the environment (Moseley *et al.*, 1982; Fitts *et al.*, 1983; Hill *et al.*, 1983a,b; Pace *et al.*, 1985; Holben *et al.*, 1988; Stahl *et al.*, 1988). Nucleic acid probes to a number of important pathogens found in food and water have been generated (Table III). DNA probes to genes encoding for toxin (Moseley *et al.*, 1982; Kaper *et al.*, 1981, 1982) or hemolysin (Datta *et al.*, 1987; Morris *et al.*, 1987) have been used to identify virulent members among an innocuous population and to study the epidemiology of the pathogen (Moseley *et al.*, 1982; Kaper *et al.*, 1981, 1982). Other organisms have been identified using probes to characteristic plasmids (Totten *et al.*, 1983; Hill *et al.*, 1983b), chromosomal DNA (Grimont *et al.*, 1985; Fitts *et al.*, 1983), whole and fragments of viral genomes (Berninger *et al.*, 1982) and 16S rRNA (Stahl *et al.*, 1988).

The major limitations of nucleic acid probes have been the limited shelf life of radiolabelled probes and the time and problems associated with cultivating specific organisms to a detectable level. However, progress with non-radioactive probes and direct detection methods will generate wider use of nucleic acid probes in the detection of microorganisms in food and water in the near future.

##### A. Methods

The use of nucleic acid probes in the analysis of food and water has provided an alternative to conventional biochemical identification of pathogens and indicator organisms. Nucleic acid probes are more specific, can detect pathogenic members of a population, and results are generally obtained faster than when using standard identification methods. When testing food and water, pathogen-specific probes have been typically used in modifications of the colony hybridization procedure of Grunstein and Hogness (1975). Colony hybridization has been used to detect virulent



**TABLE III**  
Nucleic acid probes to important food- and waterborne pathogens

Organism	References
<i>Campylobacter</i> spp.	Stolzenbach <i>et al.</i> , 1988
<i>Campylobacter jejuni</i>	Stollar and Rashitchian, 1987 Korolik <i>et al.</i> , 1988
Enteroinvasive <i>E. coli</i>	Boileau <i>et al.</i> , 1984 Pal <i>et al.</i> , 1985 Sethabutr <i>et al.</i> , 1985 Venkatesan <i>et al.</i> , 1988
Enterotoxigenic <i>E. coli</i>	Moseley <i>et al.</i> , 1980, 1982 Hill <i>et al.</i> , 1983a
Haemolytic <i>Listeria monocytogenes</i>	Datta <i>et al.</i> , 1987
<i>Listeria</i> spp.	Klinger and Johnson, 1988
<i>Salmonella</i> spp.	Fitts <i>et al.</i> , 1983 Flowers <i>et al.</i> , 1987
Invasive <i>Shigella</i>	Boileau <i>et al.</i> , 1984 Sethabutr <i>et al.</i> , 1985 Venkatesan <i>et al.</i> , 1988
Toxigenic <i>Vibrio cholerae</i>	Kaper <i>et al.</i> , 1981
<i>Vibrio parahaemolyticus</i>	Nishibuchi <i>et al.</i> , 1986
<i>Vibrio vulnificus</i>	Morris <i>et al.</i> , 1987
<i>Yersinia enterocolitica</i>	Hill <i>et al.</i> , 1983b Jagow and Hill, 1986
Enteric Adenoviruses	Takiff <i>et al.</i> , 1985
Enteroviruses	Hyypä <i>et al.</i> , 1984
Hepatitis A	Berninger <i>et al.</i> , 1982 Scotto <i>et al.</i> , 1983 Jiang <i>et al.</i> , 1986, 1987
Rotavirus	Dimitrov <i>et al.</i> , 1985

*Yersinia enterocolitica* (Hill *et al.*, 1983b; Jagow and Hill, 1986) and toxigenic *E. coli* (Hill *et al.*, 1983a) in artificially contaminated food, and toxigenic *E. coli* (Echeverria *et al.*, 1982; Moseley *et al.*, 1982) and *Vibrio cholerae* (Kaper *et al.*, 1981, 1982) in water.

### 1. Colony hybridization

1. Food samples are homogenized if necessary and 0.1 ml of appropriate dilutions are spread onto sterile nitrocellulose filters previously placed on a suitable agar medium.
2. Plates are incubated for 24 h at 37°C (or other appropriate temperature).
3. Colonies are lysed on the nitrocellulose filters by placing the filters colony side upwards on filter paper saturated with 0.5 M NaOH for 10 min followed by three successive transfers to filter paper saturated with 1.0 M ammonium acetate and 0.2 N NaOH for 1 min each. The filters are then

transferred to a fourth ammonium acetate-NaOH-saturated paper for 10 min.

4. The filters are then air dried and baked in a vacuum oven at 80°C for 2 h and stored until probed.

5. Prior to hybridization, the filters are incubated for 3 h at 37°C in hybridization solution which consists of: 50% formamide, 5× SSC (1×SSC = 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1% sodium dodecyl sulphate, 1 mM EDTA, and Denhardt's solution [0.02% Ficoll (molecular weight 400 000), 0.02% polyvinyl pyrrolidone (molecular weight 360 000), and 0.02% bovine serum albumin].

6. The filters are then transferred to fresh hybridization solution containing approximately 10<sup>5</sup> cpm of heat-denatured probe DNA ml<sup>-1</sup> and 75 µg of heat-denatured calf thymus DNA ml<sup>-1</sup> and incubated overnight at 37°C.

7. The filters are then washed in 5× SSC with 0.1% sodium dodecyl sulphate for 45 min at 65°C, rinsed briefly with 2× SSC at room temperature, and air dried.

8. The membrane is then attached to a piece of Whatman 3MM paper, covered with plastic wrap, placed on X-ray film with a single intensification screen, and held at -70°C for 24 h.

9. The X-ray film is then developed as specified by the manufacturer.

In a similar procedure, *Salmonella* have been detected in foods following growth in pre-enrichment broth (Fitts *et al.*, 1983). Detection of salmonellae by DNA hybridization was faster than standard biochemical identification and serological confirmation, which required an additional 2-3 days to complete. Flowers *et al.* (1987) compared the standard culture method with the DNA hybridization assay; hybridization was conducted following both pre-enrichment and selective enrichment of samples. Results from the testing of 1600 samples of food showed that the DNA hybridization method was as effective as the standard culture procedure and was significantly better with some foods. For additional details and protocols on colony hybridization, see Grunstein and Hogness (1975), Moseley *et al.* (1980) and Maniatis *et al.* (1982).

The above procedures do not eliminate the need to culture the organism; however, rapid methods (Palva, 1983; Miller *et al.*, 1988) and advances in nucleic acid labelling and extraction will undoubtedly lead to quicker and more sensitive detection of methods.

## B. Non-radioactive labels

The most common means of labelling nucleic acid probes is with <sup>32</sup>P-tagged nucleotides which are incorporated into the probe using the nick translation procedure of Rigby *et al.* (1977). Although radiolabelled probes are

used in research laboratories with few difficulties, their application to large-scale testing of food and water samples is undesirable because of the short-shelf life of  $^{32}\text{P}$ -labelled probes, high cost, hazards, disposal problems associated with radioactive waste, and public acceptance.

The biotin-avidin system has been the most common non-radioactive means of labelling probes. Like radioactive labelling, nick translation is used to incorporate biotinylated analogues of nucleotides, rather than  $^{32}\text{P}$ -labelled nucleotides, into the DNA probe. Avidin, which has a strong affinity for biotin, is usually tagged with an enzyme (Leary *et al.*, 1983; Sethabutr *et al.*; 1985, Yokota *et al.*, 1986; Bialkowska-Hobrzanska, 1987) and used to detect hybridized, biotinylated probe. Enzyme-labelled antibodies to biotin, in place of avidin, have also been used (Langer-Safer *et al.*, 1982). Hybridized probe is detected following the addition of enzyme substrate which when cleaved results in the production of a visible end product. Biotin-labelled probes have been used in the detection of enterotoxigenic *E. coli* (Bialkowska-Hobrzanska, 1987), *Shigella* and enteroinvasive *E. coli* (Sethabutr *et al.*, 1985), and hepatitis B virus (Yokota *et al.*, 1986). For a thorough review of the applications of the biotin-avidin system, see Wilchek and Bayer (1988).

Other non-radioactive markers include haptens, such as dinitrophenol and 2-acetylaminofluorene (Vincent *et al.*, 1982; Landegent *et al.*, 1985), enzymes cross-linked to single-stranded DNA (Renz and Kurz, 1984; Seriwatana *et al.*, 1987), and antibodies specific for RNA-DNA hybrids (Rudkin and Stollar, 1977; Boguslawski *et al.*, 1986). Miller *et al.* (1988) utilized alkaline phosphatase-labelled anti-DNA-RNA antibodies to detect hybrids formed between latex immobilized DNA (probe) and samples containing rRNA complementary to the DNA probe. Hybridization was rapid, complete within 15 min, and the procedure detected as few as 500 cells. Alternatively, the anti-RNA-DNA antibodies can be immobilized on polystyrene and used to capture RNA-DNA hybrids from solution (Stollar and Rashtchian, 1987). This method produces little background and has been used to detect *Campylobacter jejuni* rRNA.

With refinement of current systems and the development of more efficient detection systems and labels, the use of nucleic acid probes will no longer be restricted because of limitations imposed by radioactive labels.

### C. Gene amplification and direct detection

Another impediment to the widespread use of nucleic acid probes has been the need to propagate the organism to a level sufficient for detection by hybridization. Saiki *et al.* (1988) have developed a gene amplification method (the polymerase chain reaction) whereby specific DNA sequences

are selectively amplified by a factor of  $10^5$ – $10^6$ . The procedure involves two primers which hybridize to opposite strands of DNA on each end of target DNA. The primers are oriented in opposite directions so DNA synthesis proceeds across the target DNA and between the two primers. Because the primers also bind to each of the newly synthesized strands, repeated cycles of heat denaturation, annealing, and DNA synthesis doubles the amount of target from the previous cycle. The technique has been improved by replacing the Klenow fragment of *E. coli* DNA polymerase I with heat stable *Thermus aquaticus* DNA polymerase (Taq), which eliminates the need to add fresh DNA polymerase after each denaturation, and by automation with a DNA Thermal Cycler (Perkin-Elmer Cetus Corporation, Norwalk, CT). Steffan and Atlas (1988) used the polymerase chain reaction to amplify a specific region of DNA from *Pseudomonas cepacia* which was then identified by dot-blot hybridization. Initial quantities of 0.3 pg of target DNA could be detected following amplification, an increase in sensitivity of  $10^3$  over non-amplified samples. Following extraction of DNA from sediment,  $10^2$  *P. cepacia* cells 100 per g of sediment (or 1 cell  $g^{-1}$ ) could be detected despite the presence of  $10^{11}$  other organisms.

Alternatively, methods which enhance the sensitivity of probes might also avoid culturing the organism prior to hybridization. Polymerization-enhanced and single-stranded probes have both been reported to increase the sensitivity of radiolabelled probes (Holben *et al.*, 1988; Somerville *et al.*, 1988a). Polymerization enhancement increased the sensitivity by at least two orders of magnitude. In this procedure, an oligomer probe served as a primer for DNA synthesis. Specificity and DNA synthesis were contingent upon the probe (primer) binding to the target DNA while sensitivity was based on DNA synthesis and the incorporation of labelled nucleotides downstream from the primer (Somerville *et al.*, 1988a). Holben *et al.* (1988) were capable of detecting  $4 \times 10^4$  bacteria  $g^{-1}$  of soil or 0.01–0.02 pg of DNA using  $\alpha$ - $^{32}P$ -labelled, single-stranded DNA probes. The single-stranded probe was generated from M13 containing the sequence of interest (Holben *et al.*, 1988). Advantages of the M13-generated single-stranded probe include a high specific activity, lower background, and the elimination of probe–probe hybridization in the reaction mixture.

Microbes have been detected directly, without cultivation, by extraction and isolation of nucleic acids from environmental samples, followed by hybridization with specific probes (Ogram and Saylor, 1988; Holben *et al.*, 1988; Stahl *et al.*, 1988; Jiang *et al.*, 1986). By extracting nucleic acids directly from samples, lengthy incubation times and problems associated with difficult to grow and non-culturable organisms are eliminated. Stahl *et al.* (1988) were able to monitor *Bacteriodes succinogenes* and *Lachnospira multiparus* in the bovine rumen without culturing. Detection was accom-

plished by extraction of total nucleic acids followed by hybridization with oligonucleotides to species-specific 16S rRNA segments. Because many ribosomes are present ( $10^4$  in actively growing cells) fewer numbers of bacteria are needed for a positive signal when 16S rRNA segments are used as targets. Giovannoni *et al.* (1988) have extended this procedure to the detection of a single cell by combining microautoradiography with microscopy.

Direct detection methods require an efficient means of harvesting cells and extracting nucleic acids from an environmental sample. Methods for extraction of DNA from soils (Holben *et al.*, 1988), sediments (Ogram *et al.*, 1987), and water (Fuhrman *et al.*, 1988; Sommerville *et al.*, 1988b) have been reported. Extraction from aquatic environments requires that large sample volumes be taken to accrue enough DNA for testing. Somerville *et al.* (1988b) described an inexpensive method for concentrating microorganisms from litres of water on a single cylindrical filter membrane. Cell lysis and proteolysis were executed in the filter housing yielding high molecular weight DNA/RNA solutions which could be tested immediately, concentrated, or purified. Hepatitis A has been detected in concentrated estuarine water samples (Jiang *et al.*, 1986). The RNA was extracted from the sample, purified, blotted onto nitrocellulose, and hybridized with a hepatitis A probe. This method, and others which bypass cultivation of the organism, save time and can be used to account for members of the population which resist cultivation (i.e. Norwalk virus). Additional information on the use of gene probes to study microbial communities can be found in a review by Ogram and Saylor (1988).

Although these procedures have not been applied to the detection of microbes in foods, the methods are applicable to most nucleic acid probes and samples. Certainly, these methods will be of great value in the detection of pathogens and studies on the ecology of food and water.

## V. Immunoassays

The basis for serological identification (i.e. agglutination, precipitation, etc.) of viral and bacterial pathogens is the presence of a pathogen-specific antigenic determinant(s). Because viral and bacterial pathogens are found in low numbers in food and water, immunological detection requires enrichment of the organism to obtain a sufficient number of cells for detection. The use of polyclonal antisera sometimes necessitates selective media to prevent growth of cross-reacting organisms. The specificity and sensitivity of immunological assays have been enhanced with monoclonal antibodies and new combinations of enzyme labels and substrate. These

advancements have facilitated the development of methods to detect microorganisms directly in samples without prior culture enrichment. As stated above, direct detection methods are important in light of recent reports that several bacterial pathogens are not culturable using standard culture methods yet are still metabolically active (Colwell *et al.*, 1985; Roszak and Colwell, 1987). Likewise, several groups among the enteric viruses cannot be propagated using standard tissue culture techniques, but are still of public health concern (e.g. Norwalk virus). Because it is not possible to cover all of the immunological methods used to detect pathogens, emphasis will be on the more sensitive techniques used in the detection and identification of important food- and waterborne pathogens.

#### A. Fluorescent immunoassay

In fluorescent immunoassays (FIA), fluorochrome molecules are used to label immunoglobulins. The fluorochrome absorbs short-wavelength light and then emits light at a higher wavelength which can be detected using fluorescent microscopy. Fluorescein isothiocyanate and rhodamine isothiocyanate-bovine serum albumin are the most common fluorochromes used to tag antibodies and counterstain samples, respectively. These fluorochromes emit light of different wavelengths permitting their use in the same assay.

The FIA, initially developed by Coons *et al.* (1941), has been applied to the detection and identification of microorganisms because of the specificity, sensitivity, and rapid nature of the procedure. The direct and indirect procedures are the most commonly used to test food and water samples. In both methods the antigen or organism is immobilized and either (a) stained directly with an organism-specific, fluorescein-tagged antibody, or (b) stained indirectly, by first reacting the sample with an organism-specific antibody and then a fluorescein-labelled anti-immunoglobulin species antibody.

Food samples tested by FIA are typically from enrichment cultures because the number of bacteria in the original sample is insufficient to be detected directly and the food particulates, which are diluted in the medium, can produce background fluorescence. Water samples have been analysed directly by concentrating bacteria using membrane filtration. Polycarbonate filters, previously stained with Irgalan Black to reduce background and improve contrast, are commonly used in this procedure (Hobbie *et al.*, 1977).

A number of bacterial pathogens have been detected in food and water (Table IV) since Thomason *et al.* (1957) first applied FIA to the detection

**TABLE IV**  
Microbial pathogens detected by immunofluorescence in food and water samples

Organism	Source	Reference
<i>Listeria</i>	Food	Donnelly and Baigent, 1986
<i>Salmonella</i>	Food	Insalata <i>et al.</i> , 1972
	Food/water	Cherry <i>et al.</i> , 1975
	Water	Thomason <i>et al.</i> , 1975
	Food	Barrell and Paton, 1979
<i>Vibrio cholerae</i> 01	Water	Brayton <i>et al.</i> , 1987
	Water	Brayton and Colwell, 1987
Poliovirus	Water	Katzenelson, 1976
	Water	Guttman-Bass <i>et al.</i> , 1981
Coxsackievirus B5	Water	Guttman-Bass <i>et al.</i> , 1981
Echovirus 7	Water	Guttman-Bass <i>et al.</i> , 1981
Reovirus	Water	Ridinger <i>et al.</i> , 1982
Rotavirus	Water	Hejkal <i>et al.</i> , 1982, 1984
	Water	Smith and Gerba, 1982
	Water	Bates <i>et al.</i> , 1984
	Water	Deetz <i>et al.</i> , 1984
	Water	Metcalf <i>et al.</i> , 1984
	Water	Guttman-Bass <i>et al.</i> , 1987
	Water	Bosch <i>et al.</i> , 1988

of *Salmonella* in foods. A shortcoming of this technique is the presence of cross-reacting antibodies which cannot be removed from antisera without a subsequent drop in titre to the organism of interest. Monoclonal antibodies may provide a solution to this problem.

Monoclonal antibodies specific for *Vibrio cholerae* 01 (Brayton *et al.*, 1987) have been used to detect this pathogen in water by FIA. Another shortcoming of FIA is the inability to differentiate viable from non-viable cells; both appear fluorescent. Brayton and Colwell *et al.* (1987) described an FIA procedure for the enumeration of viable *V. cholerae* 01 in environmental samples. Samples were incubated with a small quantity of yeast extract to supply nutrients and nalidixic acid which prevents replication by inhibition of DNA gyrase. Metabolically active cells elongate because they are unable to divide in the presence of nalidixic acid. Viable (elongated) cells are then distinguished from non-viable (unelongated) cells, microscopically. When combined with plate counts, this method provided the means to determine the numbers of viable (culturable), dead, and viable but non-culturable cells present within a sample (Colwell *et al.*, 1985; Brayton and Colwell, 1987). The significance of these viable but non-culturable cells to public health has yet to be fully elucidated.

The fluorescent antibody staining technique has been described in a number of papers (Gray and Kreger, 1985; Brayton and Colwell, 1987), and the method is essentially as follows.

### *Procedure*

1. The sample or a dilution of the sample is spread on a glass slide, allowed to air dry, and then fixed with either 95% ethanol or heat.
2. A drop of rhodamine isothiocyanate-bovine serum albumin (RITC-BSA), diluted approximately 1 : 20, is placed on the sample. A coverslip is placed on top to distribute the RITC-BSA evenly over the sample.
3. Incubate at 37°C for 30 min in a dark moist chamber.
4. Rinse the slide three times in phosphate-buffered saline (PBS; per litre: NaCl, 8.5 g; Na<sub>2</sub>HPO<sub>4</sub>, 9.1 g; KH<sub>2</sub>PO<sub>4</sub>, 1.5 g; pH 7.3) and air dry.
5. A drop of appropriately diluted antiserum (titred prior to assay) is placed on the sample and covered with a coverslip. If the direct antibody-staining method is being used, the FITC-pathogen-specific antibody is added and the procedure continued at step 9.
6. Incubate at 37°C for 30 min in a dark moist chamber.
7. Rinse the sample three times in PBS and air dry.
8. Place a drop of FITC-anti-immunoglobulin (Ig of animal species from which pathogen-specific antibody was derived) on the sample and cover with a coverslip.
9. Incubate at 37°C for 30 min in a dark moist chamber.
10. Rinse three times in PBS and air dry.
11. Place a small drop of mounting fluid (pH 9) on the sample and place a coverslip on top.
12. Examine using an epifluorescent microscope and a 450–490 nm band pass filter. The antigen (or pathogen) if present will exhibit a green fluorescence and can be graded according to intensity.

FIA has also been applied to the detection of enteric viruses present in water. Detection involves the concentration of virus from the sample, inoculation into a suitable cell line, incubation to allow for adsorption, followed by the addition of new media and incubation for 20–24 h. The cells are then dried, fixed, and stained with a fluorescein-labelled virus-specific antibody. Fluorescent cells are then observed and quantified using fluorescent microscopy. A number of enteric viruses have been isolated from waters and enumerated using this technique (Tables II and IV). The FIA requires only 6–9 h or less to complete after infection, and is equal to or more sensitive than the plaque assay (Kedmi and Katzenelson, 1978; Ridinger *et al.*, 1982). The technique can also detect viruses



that multiply poorly in cell lines (i.e. human rotavirus; Smith and Gerba, 1982).

Alternative fluorescent labels to those mentioned above, although not commonly used in microbiology, include lanthanide elements like europium and terbium chelates (Soini and Hemmilä, 1979; Soini and Kojola, 1983). The advantages of using these compounds are the high quantum yield and narrow emission peaks produced upon excitation. These labels have a short lifespan, in the microsecond range, that allows measurements to be made after the background emission has decayed. The procedure, called timed-resolved fluoroimmunoassay, couples the rare-earth elements to the antibody molecule in a non-fluorescent state. Following antibody attachment, the lanthanide element is released and it then combines with a chelating agent and emits an intense fluorescence that can be quantitated using a fluorometer. This technique has been used to detect antigens of hepatitis B, rotavirus and adenovirus in clinical samples (Siitar *et al.*, 1983; Soini, 1985), but has not been applied to the detection of food- and waterborne pathogens.

The major shortcomings of FIA are the limited availability of pathogen-specific antibody, the inability to differentiate viable from non-viable pathogens, the tedious nature of the procedure, and the expense of automation.

Flow cytometry has been applied to the detection of *Listeria* in milk (Donnelly and Baigent, 1986). In this technique, *Listeria* identification was based upon morphology, nucleic acid content, and surface antigens, detected with fluorescein-labelled antibodies. The different parameters were analysed simultaneously with a laser cytofluorograf. Flow cytometry has not been used extensively in the analysis of food and water because of the high cost of the equipment involved.

## **B. Enzyme immunoassay**

In enzyme immunoassays (EIA), enzyme-antibody conjugates are used to identify and quantitate antibody-antigen complexes. The enzyme catalyses the conversion of substrate into a quantifiable end product, amplifying the 'signal' over time, whereas, with labels such as fluorescein and  $^{125}\text{I}$ , the quantity of label or 'signal' is fixed. Thus, the enzyme tag grants immunoassays a sensitive means of detecting small quantities of antigen (Engvall and Perlmann, 1972; Yolken, 1980). The assay, developed in 1971 by Engvall and Perlmann, has been extensively used because of its specificity, sensitivity, use of non-hazardous reagents, the stability of the reagents, and low cost. Preparation of enzyme-antibody conjugates is typically carried out as described by Engvall and Perlmann (1972).

A variety of EIAs have been developed and are shown in Table V. Details and descriptions of the assays can be found in the references provided. EIAs can be divided into two types; homogeneous and heterogeneous (solid phase). In homogeneous EIAs, neither antibody nor antigen is immobilized to a solid matrix eliminating the need to remove unbound reactants with repetitive washing steps. Homogeneous EIAs are normally used to quantitate small molecules but have been used in the detection of macromolecules (Tan *et al.*, 1981). Heterogeneous EIAs involve a solid matrix to which one of the immunoreactants (antibody or antigen) is immobilized. Unbound or weakly bound material must be removed between steps.

**TABLE V**  
The various classes of enzyme immunoassay

EIA <sup>a</sup>	Homogeneous	Competitive		Rubenstein <i>et al.</i> , 1972 Carrico <i>et al.</i> , 1976 Schroeder <i>et al.</i> , 1976 Burd <i>et al.</i> , 1977 Wei and Riebe, 1977 Gibbons <i>et al.</i> , 1980 Ngo <i>et al.</i> , 1981 Bacquet and Twumasi, 1984
		Non-competitive		Ngo and Lenhoff, 1981
	Heterogeneous	Competitive	Ag binded	Voller <i>et al.</i> , 1979 Tijssen and Kurstak, 1981 Friguet <i>et al.</i> , 1983
			Ab binded	Engvall and Perlmann, 1971 Van Weemen and Schuurs, 1971 Belanger <i>et al.</i> , 1976 Van Weemen <i>et al.</i> , 1978
		Non-competitive	Ag binded	Guesdon <i>et al.</i> , 1979 Butler <i>et al.</i> , 1980 Guesdon and Avrameas, 1980 Yolken and Leister, 1981 Madri and Barwick, 1983
			Ab binded	Crook and Payne, 1980 Barbara and Clark, 1982 Koenig and Paul, 1982 De Jong, 1983

<sup>a</sup> Enzyme immunoassay.

Homogeneous and heterogeneous EIAs can be subclassified into non-competitive and competitive assays. In non-competitive EIAs, the antigen (microbe or substance of interest) within a sample is detected using complementary enzyme-labelled antibody. The antigen concentration is directly proportional to the amount of enzyme-labelled antibody bound and end product formed. In competitive assays, the antigen to be detected within a sample is usually purified and labelled with an enzyme. The enzyme-labelled antigen is added to the sample and incubated with an immobilized antigen-specific antibody. Absence of the antigen will result in the production of end product due to the binding of the enzyme-labelled antigen to the antigen-specific antibody. The presence of antigen within the sample (unlabelled) reduces the amount of end product formed because of the competition with the enzyme-labelled antigen for antibody binding sites. Thus, in competitive EIAs, the quantity of antigen within a sample is inversely proportional to the amount of end product formed. Competitive assays are faster than non-competitive EIAs, but require purification and labelling of the antigen of interest. Although non-competitive EIAs are slower, requiring several incubation and washing steps to avoid non-specific binding and background, it is more commonly used for the detection of microbial antigens.

Although several protocols have been described (Polin and Kennett, 1980; Robison *et al.*, 1983; Berdal *et al.*, 1981; Minnich *et al.*, 1982), a typical non-competitive EIA would be conducted as follows.

### *Procedure*

1. Enrichment broth is inoculated with the food or water sample and incubated. The growth is centrifuged and resuspended in coating buffer (per litre:  $\text{NaCO}_3$ , 1.7 g;  $\text{Na}_2\text{CO}_3$ , 1.7 g;  $\text{NaHCO}_3$ , 2.9 g; pH 9.6). One hundred  $\mu\text{l}$  are added to each of duplicate wells of a microtitration plate and incubated overnight at 4°C.
2. The wells are then washed three times with washing buffer (per litre:  $\text{NaCl}$ , 7.6 g;  $\text{Na}_2\text{HPO}_4$ , 0.7 g;  $\text{KH}_2\text{PO}_4$ , 0.2 g; Tween 20, 0.5 ml; pH 7.4).
3. The wells are blocked with 250 ml of a 2% solution of bovine serum albumin (BSA) in washing buffer and incubated for 1 h at 37°C to block non specific binding sites.
4. Wash the wells three times with washing buffer.
5. One hundred  $\mu\text{l}$  of pathogen-specific antibody diluted appropriately in washing buffer containing 2% BSA is added per well and incubated at 37°C for 1 h. In a direct EIA, an enzyme-labelled antibody would be added above and the procedure continued at step 8.
6. Wash the wells three times with washing buffer.

7. Enzyme-labelled anti-immunoglobulin (Ig of animal species from which pathogen-specific antibody was derived), diluted in washing buffer plus 2% BSA is added to each well ( $100\ \mu\text{l well}^{-1}$ ) and incubated at  $37^\circ\text{C}$  for 1 h.
8. Wash the wells three times with washing buffer.
9. Enzyme substrate in an appropriate buffer is added ( $100\ \mu\text{l well}^{-1}$ ), and after incubating for a specified period of time (usually 30 min), the reaction is stopped.

For alkaline phosphatase-conjugated antibodies, *p*-nitrophenylphosphate at  $1\ \text{mg ml}^{-1}$  of diethanolamine buffer (97 ml diethanolamine, 100 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 800 ml distilled water; adjusted to pH 9.8 and the final volume brought to 1 l with distilled water) is used as substrate. After incubation at  $37^\circ\text{C}$  for 30 min, the reaction is stopped with  $30\ \mu\text{l}$  of 3 N NaOH and the colour intensity quantified spectrophotometrically. With horseradish peroxidase-conjugated antibodies, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), 5 mg, in 5 ml of 0.05 M citric acid and  $15\ \mu\text{l}$  of hydrogen peroxide (prepared immediately before use) is used as substrate. After incubation at  $37^\circ\text{C}$  for 30 min the reaction is stopped and the colour intensity quantified spectrophotometrically.

EIAs have been used extensively in the detection of foodborne pathogens and their toxins (Table VI). Early difficulties with cross-reactions between closely related pathogens have been somewhat alleviated with the advent of monoclonal antibodies, but development of specific monoclonal antibodies which react with members of a particular genus or species can be challenging (Kaspar and Hartman, 1987). Another impediment to testing food and water for pathogens using EIA is the requirement for a minimum of  $10^4$ – $10^5$  organisms  $\text{ml}^{-1}$ . Rarely do pathogens reach this number in food and water. In most cases, the organism to be detected must first be grown in enrichment culture, to increase numbers, and then identified using EIA. Membrane filtration coupled with EIA has been used to detect a number of enterotoxigenic bacteria (Table VI). Following filtration of the sample onto a nitrocellulose filter and incubation, the bacterial colonies formed on the surface of the filter are screened by EIA for a particular pathogen or toxin. Using an assay similar to the immunofluorescence assay, virus-infected cells have been detected using a peroxidase-labelled antibody. If bound, peroxidase catalyses the production of a stain which enables foci to be quantitated microscopically.

### 1. Enzyme labels

Several enzymes have been used as labels in immunoassays. Important characteristics of an enzyme used to tag immunoglobulins include: (a)

**TABLE VI**  
Microbial pathogens and toxins detected by enzyme immunoassay in food and water samples

Organism	Source	Reference
Toxigenic <i>Clostridium perfringens</i>	Food	Stelma <i>et al.</i> , 1985
<i>Listeria</i>	Food	Farber and Speirs, 1987
	Food	Butman <i>et al.</i> , 1988
	Food	Mattingly <i>et al.</i> , 1988
<i>Salmonella</i>	Food	Minnich <i>et al.</i> , 1982
	Food	Robison <i>et al.</i> , 1983
	Food	Smith and Jones, 1983
	Food	Aleixo <i>et al.</i> , 1984
	Food	Mattingly, 1984
	Food/water	Rigby, 1984
	Food	Anderson and Hartman, 1985
	Food	Farber <i>et al.</i> , 1985
	Food	Mattingly <i>et al.</i> , 1985
	Food	Cerqueira-Campos <i>et al.</i> , 1986
	Food	Ibrahim and Lyons, 1987
Staphylococcal Enterotoxin	Food	Saunders and Bartlett, 1977
	Food	Morita and Woodburn, 1978
	Food	Stiffler-Rosenberg and Fey, 1978
	Food	Berdal <i>et al.</i> , 1981
	Food	Freed <i>et al.</i> , 1982
	Food	Notermans <i>et al.</i> , 1983
	Food	Fey <i>et al.</i> , 1984
	Food	Peterkin and Sharpe, 1984
	Food	Thompson <i>et al.</i> , 1986
	Food	Ocasio and Martin, 1988
<i>Vibrio cholerae</i> 01	Water	Tamplin <i>et al.</i> , 1987
Enteric viruses	Water	Payment and Trudel, 1985, 1987
Coxsackievirus B5	Food	Loh <i>et al.</i> , 1985
Hepatitis A	Water	Nasser and Metcalf, 1987
Poliovirus	Food	Loh <i>et al.</i> , 1985
Rotavirus	Water	Steinmann, 1981
	Water	Raphael <i>et al.</i> , 1985
	Water	Guttman-Bass <i>et al.</i> , 1987

attachment should produce a minimal effect on the binding properties of the antibody (Takashi and Kayoto, 1977; Chandler *et al.*, 1982; (b) a high specific activity; (c) a low molecular weight to maximize the quantity of enzyme per immunoglobulin molecule; (d) the enzyme should be stable;

and (e) a substrate which yields a quantifiable product should be available. Enzymes commonly used in the detection of foodborne pathogens are shown in Table VII.

The avidin–biotin system is an important tool for EIAs. Avidin, a glycoprotein, has a very high affinity for biotin, a vitamin (Bayer and Wilcher, 1980; Guesdon *et al.*, 1979). Biotin is generally used to tag immunoglobulins, and an avidin–enzyme conjugate used to detect bound biotin–labelled antibody. The biotin–avidin system acquires its high degree of sensitivity from the avidin molecule that has two pairs of binding sites acting as a bridge between biotinylated molecules.

Alternative assays include thermometric (Mattiason *et al.*, 1977) and cyclic EIAs (Harper and Orengo, 1981). In thermometric EIAs, the heat generated by an enzyme, such as catalase, is measured rather than a visible end product. In cycling EIAs, a portion of the substrate which has been converted into end product is immediately regenerated into substrate (Harper and Orengo, 1981). Neither of these assays has been applied to the detection of foodborne pathogens.

## 2. Enzyme substrates

The most commonly used enzyme substrates (chromogenic) in EIAs release a visible end product that can be measured spectrophotometrically. New methods (e.g. avidin–biotin system) and new enzyme–substrate combinations have enhanced the sensitivity of EIA. For example, enzyme substrates which release a fluorogenic portion when cleaved are available for a number of enzymes (Table VII; Guilbault *et al.*, 1968). The sensitivity with fluorogenic substrates has been reported to be 10–100-fold greater than chromogenic substrates (Yolken and Leister, 1982; Swaminathan *et al.*, 1985).

EIAs which utilize radioactive substrates are highly sensitive, but find limited use in the food industry due to the hazards and disposal problems associated with the use of radioactive materials. Radioactive substrates have been used with several enzymes to detect toxins and viral antigens (Harris *et al.*, 1979); however, the radioactive end product must be isolated from the reaction mixture, generally by ion-exchange methods, prior to measurement (Yolken, 1980). Fields *et al.* (1981) eliminated the need for separation steps by using glutamate decarboxylase which cleaves  $^{14}\text{CO}_2$  from L- $^{14}\text{C}$ -glutamic acid; the  $^{14}\text{CO}_2$  is captured from the atmosphere above the reaction mixture and measured. The sensitivity of this assay was reported to be 100 times higher than radioimmunoassay.

Chemiluminescent substrates, like luminol, 3-aminophthalhydrazide (Puget *et al.*, 1977) and its derivatives (Cheng *et al.*, 1982) generate light

**TABLE VII**  
Enzymes and substrates commonly used in enzyme immunoassay

Enzyme	Substrate			
	Chromogenic	Fluorogenic	Radioactive	Luminescent
Alkaline phosphatase	<i>p</i> -Nitrophenyl phosphate Disodium phenyl phosphate + aminoantiprene	4-Methylumbelliferyl phosphate Fluorescein methyl phosphate NADH 3-( <i>p</i> -Hydroxyphenyl) propionic acid	<sup>3</sup> H-adenosin monophosphate <sup>3</sup> H-nitrophenyl phosphate <sup>14</sup> C-nitrophenyl phosphate	
Peroxidase	5-Aminosalicylic acid + H <sub>2</sub> O <sub>2</sub> <i>o</i> -Dianisidine + H <sub>2</sub> O <sub>2</sub> 2,2-Azino-di(3-ethyl benzothiazolin sulfone-6) 3,3'-Diaminobenzidine + H <sub>2</sub> O <sub>2</sub> <i>o</i> -Toluidine + H <sub>2</sub> O <sub>2</sub> <i>o</i> -Phenylendiamine + H <sub>2</sub> O <sub>2</sub>			Isoluminol + H <sub>2</sub> O <sub>2</sub> D-Luciferin + luminol + H <sub>2</sub> O <sub>2</sub> D-Luciferin + 7-dimethylamino-naphthalene 1,2 dicarbonic acid hydrazide + H <sub>2</sub> O <sub>2</sub> Pyrogallol + H <sub>2</sub> O <sub>2</sub>
β-Galactosidase	<i>o</i> -Nitrophenyl β-D-galactopyranoside	4-Methylumbelliferyl-β-D-galactopyranoside	<sup>3</sup> H-β-galactose phosphate	

**TABLE VII – continued**

Glucose oxidase	Glu <sup>a</sup> + 5-aminosalicylic acid Glu + p-nitro blue tetrazolium chloride Glu + thiazolyl Sodium, 3,5-dichloro-2 hydrobenzene sulfonate 4-Aminoantipyrine glucose peroxidase o-Dianisidine + horseradish peroxidase	NADH β-Hydroxyphenylacetic acid	<sup>3</sup> H-Glucose	Glu + peroxidase + luminol
Urease	Bromocresol purple + urea			
β-Lactamase	Starch + iodine + penicillin G			
Catalase	H <sub>2</sub> O <sub>2</sub>			
Glutamate decarboxylase			<sup>14</sup> C-glutamate	
Glucose 6-phosphate dehydrogenase	Glu 6-phosphate + NAD <sup>+</sup>			Glu 6-phosphate + NADP + luciferase

<sup>a</sup> Glucose.



when enzymatically degraded. The reaction is quantitated by measuring the emitted light. The sensitivity of EIAs using chemiluminescent substrates is not as high as with other assays. Similarly, bioluminescent assays utilize enzyme-substrate combinations which shuttle electrons to luciferase and result in the production of bioluminescence. Bioluminescent EIAs have sensitivities comparable to radioimmunoassays and require shorter reaction times than assays using chromogenic substrates. A number of excellent reviews of enzyme substrates have been published (Ishikawa *et al.*, 1983; Yolken, 1984; Swaminathan and Konger, 1986).

### C. Radioimmunoassay

Radioimmunoassay (RIA), described by Yalow and Berson (1959), can be used in all of the variations of EIA described above. RIA combines the specificity of immunoassays with the sensitivity of radioisotopic methods, detecting nanogram to picogram quantities of antigen (Yung *et al.*, 1977; Kalmakoff *et al.*, 1977). Although  $^3\text{H}$ ,  $^{14}\text{C}$  and  $^{131}\text{I}$  have been utilized as labels,  $^{125}\text{I}$  is more commonly used because of its high specific activity and short half-life. Several methods are available to prepare  $^{125}\text{I}$ -RIA reagents (Miles and Hales, 1968; Hunter, 1978; Marchalonis, 1969).

Several variations of radioimmunoassay have been used to test food and water samples (Robern *et al.*, 1975, 1978; Miller *et al.*, 1978). The method outlined below was described by Pierce and Klinman (1976).

#### *Procedure*

1. Following inoculation and incubation, cells from enrichment broth (for the organism of interest) are pelleted by centrifugation and washed in PBS (per litre: NaCl, 7.6 g;  $\text{Na}_2\text{HPO}_4$ , 0.7 g;  $\text{KH}_2\text{PO}_4$ , 0.2 g; pH 7.4).
2. To each of duplicate wells of a 96-well polyvinyl plate, 100  $\mu\text{l}$  of the cell suspension is added and incubated at 4°C (usually overnight).
3. The wells are then washed three times with RIA buffer (PBS containing 0.1% BSA).
4. To minimize non-specific binding, 250  $\mu\text{l}$  of PBS containing 2% BSA is added to each well and incubated for 1 h at 37°C.
5. Wash the wells three times with RIA buffer.
6. The organism-specific antibody is diluted appropriately in RIA buffer, and 100  $\mu\text{l}$  is added to each well and incubated for 1 h at 37°C.
7. The wells are then washed three times with RIA buffer.
8. Between 50 and 100  $\mu\text{l}$  of  $^{125}\text{I}$  anti-immunoglobulin (10  $\mu\text{Ci } \mu\text{g}^{-1}$ ) is added to each well (10 000 cpm well $^{-1}$ ) and incubated at 37°C for 1 h.
9. Unbound  $^{125}\text{I}$  anti-immunoglobulin is then removed by washing the wells three times.

10. The wells are then separated and counted in a gamma scintillation counter. The background values for controls should not exceed 100–200 cpm.

Enterotoxigenic bacteria have been detected within natural populations (Shah *et al.*, 1982) by blotting colonies onto polyvinyl membranes and probing with specific radiolabelled antibody. A radioimmunoassay (Lemon *et al.*, 1983) has been used to detect viruses following multiplication in suitable cell lines. Infected cells are detected and enumerated using  $^{125}\text{I}$ -labelled antibodies and autoradiography. In Table VIII are listed pathogens which have been detected in food and water by RIA.

**TABLE VIII**  
Pathogens and toxins detected in food and water using radioimmunoassay

Pathogen/toxin	Source	Reference
Staphylococcal Enterotoxin	Food	Collins <i>et al.</i> , 1973
	Food	Robern <i>et al.</i> , 1975
	Food	Orth, 1977
	Food	Pober and Silverman, 1977
	Food	Miller <i>et al.</i> , 1978
	Food	Robern <i>et al.</i> , 1978
<i>Cl. perfringens</i> Enterotoxin	Food	Stelma <i>et al.</i> , 1983
Hepatitis A	Water	Hejkal <i>et al.</i> , 1982

Despite the high sensitivity of the assay, RIA has not been used extensively in the food industry because of the problems associated with the use and disposal of radioactive materials.

## VI. Enzyme substrates

The ability to detect specific enzymes rapidly using chromogenic and fluorogenic substrates has led to the development of a number of rapid methods for the identification of several bacteria (Kilian and Bülow, 1976; Godsey *et al.*, 1981; Facklam *et al.*, 1982; Trepeta and Edberg, 1984; O'Brien and Colwell, 1985; Feng and Hartman, 1982; Littel and Hartman, 1983; Petzel and Hartman, 1985; Freier and Hartman, 1987). Feng and Hartman (1982) tested a variety of food, water, and milk samples using lauryl tryptose broth containing 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG). After incubation for 24 h, *E. coli* was presumptively identified and confirmed in most-probable-number tubes using lactose fermentation (gas production) and MUG (fluorescence, detected under long-wave

ultraviolet light), respectively. The lauryl tryptose broth–MUG–MPN was sensitive, produced few false-positive reactions, detected anaerogenic strains of *E. coli*, and yielded faecal coliforms from 90% of the tubes which were gas and fluorescence positive. The MUG test has compared favourably with standard MPN methods for *E. coli* (Alvarez, 1984; Robison, 1984; Koburger and Miller, 1985; Peterson *et al.*, 1987). The basis for the MUG test is the presence of  $\beta$ -glucuronidase which cleaves MUG to release the fluorescent 4-methylumbelliferyl portion.  $\beta$ -Glucuronidase is found in a number of bacterial genera; however, among the *Enterobacteriaceae* it is restricted to *E. coli* (96%), a few *Salmonella*, and *Shigella* (50%) (Kilian and Bülow, 1976; LeMinor, 1979).

The incorporation of chromogenic and/or fluorogenic substrates into a selective medium can eliminate the need for subculture and subsequent biochemical testing, saving time, supplies and money. Several substrates have been incorporated into media or detection schemes to aid in the identification of coliforms, faecal coliforms (Warren *et al.*, 1983), enterococci (Bosley *et al.*, 1983), faecal streptococci (Littel and Hartman, 1983), *E. coli* (Feng and Hartman, 1982; Ley *et al.*, 1988; Watkins *et al.*, 1988) and *Vibrio cholerae* (O'Brien and Colwell, 1985). Although not feasible at this time, these substrates may be of use in direct detection procedures in the future.

## VII. Concluding remarks

Molecular biology techniques have made it possible to develop highly specific nucleic acid and antibody probes to an organism or group of organisms. The application of these probes and methods to the detection of important food and waterborne microorganisms has increased in recent years. New gene and signal amplification techniques, as well as the use of rRNA genes, could circumvent the need to propagate these organisms prior to hybridization, saving time and allowing the detection of non-culturable organisms. Likewise, with new labels and/or enzyme substrates, immunological methods may be of even greater value in direct detection procedures in the near future.

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