

# A review of analytical methods for the detection of bacterial toxins

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## 1. INTRODUCTION

Food poisoning due to bacterial toxins can be caused by the ingestion of exotoxins (e.g. staphylococcus enterotoxin, *Bacillus cereus* and *Clostridium botulinum* food poisoning) which are preformed in the food, or by the ingestion of food containing large numbers of bacterial cells which then release endotoxins in the gastrointestinal tract (e.g. *Cl. perfringens* food poisoning).

Data from the surveillance scheme of general outbreaks of infectious intestinal disease in England and Wales (Djuretic *et al.* 1996) show that for the period 1992–1994 bacteria (other than *Salmonella*) were responsible for 613 outbreaks (45% of the total). *Clostridium perfringens* accounted for 7%, *Shigella* spp. 5% and *Escherichia coli* O157, *Bacillus* spp. and *Staphylococcus aureus* each accounted for 1% of the outbreaks.

Early methods for the assay of bacteria toxins were based on *in vivo* (i.e. animal challenge tests) or *in vitro* tests (i.e. tissue culture). Later, immunological tests were developed based on techniques such as gel diffusion, but these tests were laborious and difficult to apply to foods. There are now a number of rapid test kits available which give results within hours and are much simpler to perform and interpret than bioassays.

## 2. OVERVIEW OF ASSAY TECHNIQUES

### 2.1 Biological assays

Although modern assay methods are rapid and a convenient means of testing for bacterial toxins, they do not provide information on the biological activity of the toxin. Thus, bioassays and related tests remain the method of choice for some bacterial toxins (e.g. botulinum toxins). Many bioassay formats have been described (Jay 1996), including whole-animal tests (e.g. the mouse lethality test, monkey and kitten emesis tests, and rabbit and guinea-pig skin tests), part-animal tests (e.g. ileal loop tests) and cell culture systems (e.g. Chinese hamster ovary (CHO) cells). The range of bioassays and tissue culture tests for bacterial toxins is summarized in Table 1.

### 2.2 Immunological assays

Immunological assays are much simpler and cheaper than biological assays and have therefore been widely adopted. Table 2 lists some of the techniques for the detection of bacterial toxins reported in the literature and the commercial sources of kits based on these techniques.

**2.2.1 Gel diffusion assays.** One of the earliest immunological tests to be developed was the double gel immunodiffusion (Ouchterlony 1949). This and subsequent modifications, such

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**Table 1** Bioassay methods for bacterial toxins

Organism	Toxin	Bioassay method	Reference
<i>Bacillus cereus</i>	Diarrhoeagenic	Monkey feeding	Jordon and McBroom (1931)
	Diarrhoeagenic	Rabbit ileal loop	Genigeorgis <i>et al.</i> (1973)
	Diarrhoeagenic	Vascular permeability	Kramer <i>et al.</i> (1982)
	Diarrhoeagenic	Guinea-pig skin	Stark and Duncan (1971)
	Emetic toxin	Rhesus monkey emesis	Jordon and McBroom (1931)
	All	Human foetal intestinal cells	
<i>Clostridium botulinum</i>	A, B, E, F, G	Mouse lethality	Stark and Duncan (1971)
<i>Clostridium perfringens</i>	Enterotoxin	Mouse lethality	Stark and Duncan (1971)
	Enterotoxin	Mouse ileal loop	Punyashthiti and Finkelstein (1971)
	Enterotoxin	Rabbit ileal loop	Genigeorgis <i>et al.</i> (1973)
	Enterotoxin	Guinea-pig skin	Stark and Duncan (1971)
	Enterotoxin	Vero cells	McDonel and McLane (1981)
<i>Escherichia coli</i>	LT*	Rabbit ileal loop	Genigeorgis <i>et al.</i> (1973)
	LT	CHO monolayer	Guerrant <i>et al.</i> (1974)
	LT	Vero cells	Speirs <i>et al.</i> (1977)
	LT	Y-1 adrenal cells	Lovett and Peeler (1984)
	ST†	Suckling mouse	Dean <i>et al.</i> (1972)
	ST	Rabbit ileal loop	Genigeorgis <i>et al.</i> (1973)
	ST <sub>a</sub>	Suckling mouse	Aulisio <i>et al.</i> (1983)
	ST <sub>a</sub>	1-3-day-old piglets	Burgess <i>et al.</i> (1978)
	ST <sub>b</sub>	Pig jejunal loop	Jay (1996)
	ST <sub>b</sub>	Weaned piglets	Jay (1996)
<i>Staphylococcus aureus</i>	SEB‡	Guinea-pig skin	Stark and Duncan (1971)
	All enterotoxins	Rhesus monkey emesis	Jordon and McBroom (1931)
	SEA, SEB	Suckling kitten emesis	Dolman <i>et al.</i> (1936)
	SEA, SEB, SEE	Murine spleen cells	Jay (1996)
	SEA	Human peripheral lymphocytes	Jay (1996)

\*LT = *Escherichia coli* heat-labile enterotoxin.

†ST = *Escherichia coli* heat-stable enterotoxin.

‡SEA, SEB, SEE = staphylococcal enterotoxins A, B and E.

as the optimal sensitivity plate (Robbins *et al.* 1974) and the microslide test (Casman *et al.* 1969), are still in use. For these gel diffusion methods the antigen must be in a precipitable form. Probably the most widely used gel diffusion test is the Crowle modification of the Ouchterlony test (Casman and Bennett 1965). The microslide assembly consists of a clean microscope slide coated with gel diffusion agar onto which is placed a plastic template with a central well and four outer wells. An appropriate antitoxin is placed in the central well and a reference toxin sample in one of the outer wells. The remaining wells are filled with dilutions of the test sample. After incubation the template is removed and the gel examined for precipitation lines between the antitoxin and the test

and reference samples. This test requires only 20 µl of sample and is relatively sensitive (e.g. 0.01–0.1 µg ml<sup>-1</sup> of staphylococcal enterotoxin), but can be difficult to interpret. If the concentration of the enterotoxin in the test sample is too high, the formation of precipitation line is inhibited, so it is essential to use a range of dilutions of the test sample. Atypical non-specific reactions can also occur where other antigens present react with non-enterotoxin antibodies (Bennett 1995).

Electro-immunodiffusion (Laurell 1966) has been used to quantify staphylococcal enterotoxins A (Gasper *et al.* 1973). Under the influence of an electric current, the toxin migrates into agarose gel containing antibody and a precipitin cone forms whose height is proportional to the concentration of

**Table 2** Chemical and immunological methods for bacterial toxins

Organism	Toxin	Assay method	Commercial kit	Reference
<i>Staphylococcus aureus</i>	B	Fluorescent antibody		Peterkin and Sharpe (1984)
	A-E	Radioimmunoassay		Lindroth and Niskanen (1977)
	A, B	Microslide gel double diffusion		Casman <i>et al.</i> (1969)
	A, B	Single radial immunodiffusion		Meyer and Palmieri (1980)
	B	Haemagglutination inhibition		Jay (1996)
	B	Reverse passive haemagglutination		Jay (1996)
	A-D	Reverse passive latex agglutination	SET-RPLA (Oxoid)	Wienke (1991)
	A-E	ELISA	Bommeli SET-EIA	Wienke (1991)
	A-E	ELISA	Tecra SET-VIA (BioEnterprises Pty)	Park <i>et al.</i> (1996)
	A-E	ELISA	Ridascreen SET (R-Biopharm GmbH)	Park <i>et al.</i> (1996)
	A-E	Immunoassay	Transia	Lapeyre <i>et al.</i> (1996)
	A-E	Automated ELISA	VIDAS-SET (bioMerieux)	Lapeyre <i>et al.</i> (1994)
	B	Enzyme-linked immunofiltration		Valdivieso-Garcia <i>et al.</i> (1996)
	Nucleic acid probes		Wilson <i>et al.</i> (1991)	
	Polymerase chain reaction		Jaulthac <i>et al.</i> (1992)	
<i>Bacillus cereus</i>	Diarrhoeal	Reverse passive latex agglutination	BCET-RPLA (Oxoid)	Granum <i>et al.</i> (1993)
	Diarrhoeal	ELISA	Tecra BDE-VIA (Tecra Diagnostics)	Buchanan and Schultz (1994)
	Diarrhoeal	Microslide double diffusion		Bennett (1995)
	Diarrhoeal	Aggregate-haemagglutination		Jay (1996)
<i>Clostridium perfringens</i>	Enterotoxin	Reverse passive latex agglutination	PET-RPLA (Oxoid)	Berry <i>et al.</i> (1985)
	Enterotoxin	ELISA		Tranter <i>et al.</i> (1985)
	Enterotoxin	Microslide double diffusion		Giugliano <i>et al.</i> (1983)
	Enterotoxin	Electroimmunodiffusion		Jay (1996)
<i>Clostridium botulinum</i>	A	ELISA		Shone <i>et al.</i> (1985)
	E	ELISA		
	G	ELISA		Lewis <i>et al.</i> (1981)
	A and E	Endopeptidase		Hallis <i>et al.</i> (1996)
	A, B, E, F, G	Polymerase chain reaction		Fach <i>et al.</i> (1995)
	A-E	Electroimmunodiffusion		Jay (1996)
<i>Escherichia coli</i>	LT	Reverse passive latex agglutination	VET-RPLA (Oxoid)	Speirs <i>et al.</i> (1991)
	LT	Passive immune haemolysis		Jay (1996)
	LT	Coagglutination	Phadabect ETEC-LT (Pharmacia)	Speirs <i>et al.</i> (1991)
	ST <sub>a</sub>	Radioimmunoassay		
	VT	Reverse passive latex agglutination	VTEC-RPLA (Oxoid)	
	ST/LT	Gene probe	DNA hybridisation kits (Bresatec Ltd)	Medon <i>et al.</i> (1988)

toxin. Although the sensitivity of this method is similar to that of the microslide test, it requires large amounts of anti-serum and is therefore unsuitable for routine food analysis.

**2.2.2 Haemagglutination.** Two procedures based on haemagglutination have been described which are potentially more sensitive than immunodiffusion-based methods. Unlike the gel diffusion methods they do not require the antigen to be in a precipitable form. In the passive haemagglutination test (Johnson *et al.* 1967), dilutions of sample are reacted with a constant amount of antibody. Toxin coated erythrocytes are then added and these agglutinate when there is free antibody present. In the reversed passive haemagglutination test, antibody is coupled to sheep erythrocytes and agglutination occurs when toxin is present.

**2.2.3 Coagglutination.** In the coagglutination test (Christensen *et al.* 1973) antibody to the target toxin is coupled to the Protein A of non-viable *Staph. aureus*. The Protein A binds to the Fc portion of the antibody, allowing the antigenic Fab sites to be freely exposed on the surface of the *Staph. aureus* cells. The sensitized *Staph. aureus* cells are mixed with the test sample on a slide and agglutinate within 10 min if the target toxin is present. A commercial version of this test for *Salmonella*, *Shigella* and enterotoxigenic *E. coli* is available (Karo Bio Diagnostics AB, Huddinge, Sweden).

**2.2.4 Reverse passive latex agglutination.** Reverse passive latex agglutination (RPLA) can be used to detect soluble antigens in food extracts or culture filtrates by a simple latex agglutination assay. In a conventional agglutination test, soluble antibody is reacted with the antigen (e.g. bacterial cells), whereas in the RPLA test, the antibody is attached to latex particles and reacts with the soluble antigen (e.g. bacterial toxin). In the assay procedure, twofold dilutions of the sample are prepared across two rows on a 96-well microtitre plate. Twenty-five microlitre volumes of sensitized (antienterotoxin) latex and control latex are added to the first and second rows, respectively. Positive and negative controls are included using a purified enterotoxin preparation with the control and latex beads. The plate is covered, shaken on a plate shaker at room temperature for 20–24 h. If the antigen (bacterial toxin) is present agglutination occurs due to the formation of a molecular lattice and a diffuse layer is formed at the base of the well. If no antigen is present the latex particles sediment and form a tight button in the bottom of the well. Results are recorded visually and classified from negative (–) to strongly positive (+ + +) depending on the degree of agglutination. The assay is simple and rapid to perform, but it is relatively expensive and gives only semi-quantitative results. A range of RPLA kits for the detection of bacterial toxins is available

from Oxoid Ltd, (Basingstoke, UK) including kits for *Cl. perfringens* (PET-RPLA), *B. cereus* diarrhoeal toxin (BCET-RPLA), *Staphylococcal* enterotoxins (SET-RPLA) and *E. coli* verotoxin (VET-RPLA). The procedure for the extraction of toxin varies with the toxin and sample type (see below).

**2.2.5 Enzyme-linked immunosorbent assay.** The quantitative enzyme-linked immunosorbent assay (ELISA) method was developed by Van Weemen and Schuurs (1971). Since then many immunoassays have been described, but the standard ELISA based on a 96-well microtitre plate is probably the most widely used. A variety of ELISA configurations have been devised, but most commercial kits use either limited- or excess-reagent systems with a labelled analyte or antibody in the 'sandwich' format (Wyatt 1992). In the direct or double antibody sandwich ELISA, target antigen is captured by incubating the test sample in specific antibody-coated wells. The bound antigen is detected by reacting with another enzyme-labelled specific antibody and an enzyme-specific substrate to form a coloured or fluorescent product. The amount of label present at completion of the assay (and hence colour) is directly proportional to the target analyte concentration.

**2.2.6 Enzyme-linked immunofiltration assay.** A rapid alternative to immunoassays is the enzyme-linked immunofiltration assay (ELIFA). A commercially available system (EASY-TITRE ELIFA, Pierce, Rockford IL, USA), is claimed to have equivalent or better sensitivity than the conventional ELISA. It consists of a nitrocellulose membrane placed between two chambers and connected to a peristaltic pump. Filtration of the test sample through the high affinity membrane accelerates the reaction between the analyte and the ligand immobilized on the membrane, reducing the total assay time to 1 h, compared with 4 h for a typical ELISA.

**2.2.7 Radioimmunoassay.** In radioimmunoassay (RIA), a radioactive label is coupled to the antigen and the antigen is reacted with the specific antibody. The amount of antigen combined with the antibody is determined with a radioactivity counter. This technique is more sensitive than gel diffusion methods, but has the disadvantage that special facilities are required to handle and dispose of radioactive materials.

## 2.3 Nucleic acid probes and polymerase chain reaction

Polymerase chain reaction (PCR) was first described in the mid-1980s (Mullis *et al.* 1986) and is a highly specific and sensitive method for amplifying nucleic acid sequences exponentially. The basic mechanism and subsequent enhancements have been reviewed by other authors (Arnheim and

Levenson 1990; Wolcott 1992). The rapid expansion of PCR techniques has been due to the development of DNA sequencing methods, the establishment of computer databases which can be searched for DNA sequences and the automation of oligonucleotide synthesis. Early problems with contamination, inhibition and primer design have now been largely overcome and commercial PCR systems for the detection of food-borne pathogens such as *Salmonella* are now available. PCR assays for the detection of toxin encoding genes in bacteria have been developed, but none have yet been commercialized (Hill and Olsvik 1994).

### 3. DETECTION OF BACTERIAL TOXINS

#### 3.1 *Staphylococcus aureus*

Staphylococcal food poisoning is caused by the ingestion of foods containing enterotoxins produced by some strains of *Staph. aureus*. To date, seven serologically distinct enterotoxins have been identified and characterized (A, B, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, D and E). The three C enterotoxins cross-react and are therefore not usually analysed separately (Reiser *et al.* 1984). They are simple, low molecular weight (27 500–30 000) single chain proteins (Tranter 1991). The physical and chemical properties of the enterotoxins have been reviewed by Bergdoll (1991). According to Evenson *et al.* (1988) the ingestion of 100–200 ng of enterotoxin can induce symptoms of food poisoning and foods implicated in staphylococcal food poisoning typically contain about 0.5–10 µg of toxin 100 g<sup>-1</sup>. Thus, the sensitivity of any detection methods needs to be below this level, preferably in the region of 0.1–0.2 µg 100 g<sup>-1</sup>. Early immunological tests for staphylococcal enterotoxins involved a lengthy and cumbersome extraction procedure followed by detection by immunodiffusion in agarose gel, and took 5–11 days to complete (Holbrook and Baird Parker 1975). The gel diffusion assays are capable of detecting down to 0.01 µg of staphylococcal enterotoxin. A solid-phase RIA method was reported to be 100-fold more sensitive than immunodiffusion techniques (Collins *et al.* 1973). However, there is now a range of commercial kits available for the rapid detection of staphylococcal enterotoxins in foods which have equivalent or better sensitivity than RIA and are much simpler to perform.

The SET-RPLA kit (Oxoid) is a latex-based immunological test for the simultaneous detection of staphylococcal enterotoxins A, B, C and D in food extracts and culture filtrates. Haines and Stannard (1987) reported non-specific reactions with some foods, e.g. cheese and prawns, with this kit. Rose *et al.* (1989) found that non-specific reactions could be reduced by the addition of 10 nmol l<sup>-1</sup> hexametaphosphate to the diluent. Wienke (1991) compared four immunological test kits for the detection of staphylococcal enterotoxin in food and concluded that no single method was superior,

although the SET-RPLA was the cheapest and easiest to perform.

The Bommeli SET-EIA test is a solid-phase ELISA that uses polystyrene beads coated with antibodies against staphylococcal enterotoxins A–D. Wienke (1991) compared the Bommeli kit with three other commercial kits and found it was the most sensitive for the detection of staphylococcal enterotoxin in foods from food poisoning outbreaks. The kit detected enterotoxin in 14 of 18 foods, compared with nine for RPLA test and 10 for Transia ELISA. The kit had the longest test time (24 h) due to the need for overnight incubation with the antibody-coated beads.

The TECRA Staphylococcal Enterotoxin Visual Immunoassay (BioEnterprises Pty Ltd, Roseville, Australia) is a rapid microtitre plate-based screening test which uses polyvalent antisera to detect staphylococcal enterotoxins A–E at concentrations down to 1 ng ml<sup>-1</sup> in foods and culture filtrates. Results can be read visually with a colour card or by measuring the absorbance at 414 nm in a plate reader. Analysis time is typically 3.6–4 h for most samples, although for thermally processed foods 21 h is required, because a urea treatment step is specified to renature the enterotoxin. In an evaluation, the kit was equivalent in sensitivity to an enzyme immunoassay and the RPLA test. However, non-specific reactions were observed with samples contaminated by microorganisms other than *Staph. aureus*, such as *Enterobacter agglomerans*, *Ent. cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia marcescens*. The problem of false positive results was resolved by heat-treating the sample for 2 min at 100 °C (Park *et al.* 1993). The specificity, sensitivity and reproducibility of the test has also been assessed in a collaborative trial involving 13 laboratories. In this study, low levels of enterotoxin were added to meat, meat products, dairy products and seafood and in all cases the TECRA kit detected the enterotoxin. False positive results were obtained with samples of salami and mussels, but these were overcome by treating the sample with rabbit serum (Park *et al.* 1996).

The Ridascreen<sup>TM</sup> SET (R-Biopharm GmbH, Darmstadt, Germany) is a sandwich ELISA for the qualitative analysis of staphylococcal enterotoxins A–E that uses monovalent antibodies. In an evaluation, the kit was found to be highly specific, sensitive (0.2–0.25 ng ml<sup>-1</sup>) for a range of artificially contaminated food types, and relatively simple to perform, taking less than 3 h to complete. When tested with foods inoculated with *Staph. aureus*, non-toxicogenic staphylococci and *Ser. marcescens*, only samples containing toxicogenic *Staph. aureus* gave positive results, whereas the TECRA VIA yielded false-positive results with samples containing *Ser. marcescens* and with extracts of fresh raw mussel. False-positive results with the TECRA kit were eliminated by treating the extract with animal serum. Both the Ridascreen and TECRA kits gave false-positive results with naturally occurring peroxidase, because they use a peroxidase-coupled detection system.

The peroxidase was removed with sodium azide, but this reduced the recovery of enterotoxin by 20–30% (Park *et al.* 1992). A collaborative study found sensitivities of 1 ng g<sup>-1</sup> for ham, cheese, salami and turkey and 2.0 ng g<sup>-1</sup> for other samples, with no false negative results. Unlike the TECRA kit, the RIDASCREEN was not found to give false-positive results with mussels (Park *et al.* 1996).

The TRANSIA immunoassay for staphylococcal enterotoxins A–E is a tube-based sandwich assay which uses a mixture of monoclonal and polyclonal antibodies. Results are determined visually by comparison with a positive control or photometrically at 450 nm. This kit has been validated in an interlaboratory study involving 13 laboratories, using spiked samples of cheese, ravioli, mushrooms and mayonnaise. (Lapeyre *et al.* 1996).

The VIDAS staphylococcal enterotoxin (SET) assay (bioMérieux, Marcy-l'Étoile, France) is an automated enzyme-linked fluorescent immunoassay (ELFA) for the simultaneous detection of staphylococcal enterotoxins A, B, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, D, E in foods. In this assay, an aliquot of food extract is placed into a reagent strip which is then loaded into the VIDAS instrument. Subsequent steps in the assay are performed automatically as follows. The sample is drawn up into a solid phase receptacle (SPR) coated with anti-enterotoxin polyclonal antibodies, which acts as both the solid phase and pipetting device. After a washing stage alkaline phosphatase-conjugated antibody is drawn into the SPR, followed by a further washing stage and then the addition of a fluorescent substrate (4-methyl-umbelliferyl phosphate). The presence and concentration of staphylococcal enterotoxin is indicated by the production of a fluorescent end-product (4-methyl-umbelliferone), which is measured by an optical scanner in the instrument.

VIDAS had good sensitivity for enterotoxins A, B, D and E, but was less sensitive for the C enterotoxins. With artificially contaminated foods, the detection sensitivity ranged from <1 ng ml<sup>-1</sup>–1.5 ng ml<sup>-1</sup>, depending on the toxin and food (Lapeyre *et al.* 1994).

An enzyme-linked immunofiltration assay (ELIFA) for the detection of staphylococcal enterotoxin was described with an analysis time of ≈ 1 h and a detection limit of 1 ng ml<sup>-1</sup> of enterotoxin B in spiked cheese samples. (Valdivieso-Garcia *et al.* 1996). The sensitivity was similar to that reported for three commercial kits for the detection of staphylococcal enterotoxin (Park *et al.* 1992).

Techniques based on nucleic acid probes with (Wilson *et al.* 1991) and without (Jaulhac *et al.* 1992) PCR amplification have been described, but these are not commercially available.

### 3.2 *Bacillus cereus*

*Bacillus cereus* is reported to be responsible for between 1% and 25% of food poisoning outbreaks world-wide of known

aetiology (Kramer and Gilbert 1989). The identification and widespread incidence of psychrotrophic strains from a variety of food, including pasteurised dairy products (Van Netten *et al.* 1990) led to renewed interest in methods for the detection of *B. cereus* toxins. In a survey of dairy products, 7% of *B. cereus* isolates were both psychrotrophic and enterotoxigenic. Two distinct types of food poisoning due to *B. cereus* are recognized. The acute-onset 'emetic type' with an incubation period of 1–5 h is normally associated with cooked rice and is caused by preformed toxin in the food. The longer-onset 'diarrhoeal syndrome', which has an incubation period of 8–16 h is associated with a whole range of different food types, including rice, pasta, meat and meat products, puddings and sauces and is caused by an enterotoxin.

*Bacillus cereus* emetic toxin is a highly stable cyclic peptide of less than 10 kDa (Granum *et al.* 1993). It withstands heating to 126 °C for 90 min and is not sensitive to trypsin or pepsin treatments. The diarrhoeal toxin is a protein of M<sub>w</sub> ≈ 50 000 with an isoelectric point of 4.9. It is inactivated by heating at 56 °C for 5 min and by treatment with trypsin, pepsin and pronase. According to Beecher and Wong (1994) there are two distinct *B. cereus* diarrhoeal enterotoxins. One is haemolysin BL (HBL), a tripartite enterotoxin that requires all three components for maximal activity. The other enterotoxin is a single protein (Shinagawa *et al.* 1991). This has important implications with regard to the effectiveness of the two commercially available kits, since they are reported to detect different antigens (Christiansson 1993).

Early biological assay methods for screening for *B. cereus* emetic toxin involved feeding to rhesus monkeys and were therefore laborious and very expensive (Melling *et al.* 1976). These tests were by *in-vitro* tissue culture assays with Hep-2 (Hughes *et al.* 1988) or CHO cells (Buchanan and Shultz 1992). At present there are no commercially available rapid test kits for the detection of the emetic toxin, due to difficulties in the purification and characterization of the toxin.

Biological assays for the detection of *B. cereus* diarrhoeal toxin include the rabbit ileal loop (RIL) test and the vascular permeability reaction (VPR) test (Kramer *et al.* 1982). The microslide gel double diffusion test can be used to confirm the presence of *B. cereus* diarrhoeal toxin in culture supernates (Bennett 1995). There are also two commercial kits for the rapid detection of the *B. cereus* diarrhoeal toxin in foods and cultures. The BCET-RPLA (Oxoid Ltd) kit was evaluated by Granum *et al.* (1993) and compared with the Western immunoblot and vascular permeability reaction (VPR), 94.5% of 110 enterotoxigenic isolates were positive by both the RPLA and Western immunoblot methods and they concluded that the BCET-RPLA kit was a simple and reliable method for the detection of *B. cereus* diarrhoeal toxin. However, Buchanan and Schultz (1992) found that the BCET-RPLA failed to detect diarrhoeal enterotoxin in samples which were positive by cell culture assays, and other

workers have reported higher false-negative rates with this kit (Christiansson 1993; Notermans and Tatini 1993).

The Tecra Bacillus Diarrhoeal Enterotoxin (BDE) VIA is a microtitre plate-based immunoassay which can be used to test all types of foods and culture filtrates for BDE in 4 h. Buchanan and Schultz (1994) compared this test with the BCET-RPLA and CHO assays and concluded that overall the tissue culture method was the more accurate, but that the Tecra kit produced similar results and was more convenient for the rapid detection of *B. cereus* diarrhoeal enterotoxin.

Although both the BDE-VIA and BCET-RPLA kits are in general use their usefulness has been questioned. Beecher and Wong (1994) found that the BDE-VIA kit detected two apparently non-toxic proteins and the BCET-RPLA detects the L<sub>2</sub> component from haemolysin BL. They therefore recommended caution when drawing conclusions based on data from these kits. Slade and Davies (1995) found that a diarrhoeagenic toxin-producing strain gave a positive result with both kits, whereas an emetic toxin-producing strain gave a positive result with the BDE-VIA and a negative result with the BCET-RPLA.

### 3.3 *Clostridium perfringens*

*Clostridium perfringens* food poisoning is a food-borne intoxication commonly associated with failure to refrigerate previously cooked foods. It is a spore-forming bacterium that can survive heat treatment and multiply rapidly under favourable conditions. The species is classified into five serotypes (A–E) based on the major extracellular toxins produced, but only types A and C cause significant disease in man and type A is the strain normally responsible for food-borne intoxication and infectious diarrhoea in man. Although the toxin can be preformed in foods, the amount ingested by this route is unlikely to cause intoxication, since milligram quantities of the toxin are required to produce symptoms and the toxin is broken down in the digestive tract. The intoxication was thought to be caused by the ingestion of large numbers of vegetative cells of *Cl. perfringens* ( $1 \times 10^8$  cfu g<sup>-1</sup>), which then sporulate in the gut and release an enterotoxin. However, enterotoxin synthesis has been reported in non-sporulating cultures of *Cl. perfringens* and it has been suggested that sporulation and enterotoxin production are coincidental events which occur under similar environmental conditions (Ely 1996). Thus, assay for *Cl. perfringens* in foods is not carried out routinely, and most assay methods have been used to detect the toxin in faeces during the investigation of food poisoning outbreaks. The toxin is a heat-labile protein with a molecular weight of around 35 000 which is believed to act on the epithelial cells in the intestine, causing leakage of intracellular components, leading to diarrhoea (Stark and Duncan 1971).

Several biological methods have been described for the

detection of *Cl. perfringens* enterotoxin (Genigeorgis *et al.* 1973; Stringer *et al.* 1982), including the rabbit ileal loop assay, erythematous activity and Vero cell tissue culture assay. However, with the exception of the Vero cell assay (McDonel and McClane 1981; Mohony *et al.* 1989) the biological tests lack sensitivity and are too time-consuming to be used for the routine analysis of foods.

The need for improved speed and sensitivity has resulted in the development of immunological assays for the detection of *Cl. perfringens* enterotoxin. Earlier methods such as gel diffusion assays (Giugliano *et al.* 1983) have been replaced by RPLA- and ELISA-based tests. At present there appear to be no commercial ELISA-based kits for the detection of *Cl. perfringens* enterotoxin; but Tranter *et al.* (1986) described an ELISA for *Cl. perfringens* type A enterotoxin with a sensitivity of 1–2 ng ml<sup>-1</sup>, and a commercial test (PET-RPLA, Oxoid Ltd) for the detection of *Cl. perfringens* type A enterotoxin in faecal samples and culture filtrates with a sensitivity of around 2 ng ml<sup>-1</sup> is also available. Berry *et al.* (1986) found this kit to be as specific and sensitive as a plate ELISA developed by Bartholomew *et al.* (1985).

### 3.4 *Clostridium botulinum*

Although botulinum food poisoning is relatively rare its severity and high mortality rate has resulted in considerable efforts to prevent its occurrence. *Clostridium botulinum* neurotoxin is the most lethal known to man, with the lethal dose in humans thought to be around 0.2–2.0 µg. Strains of *Cl. botulinum* are classified into seven types (A–G) based on the antigenically distinct neurotoxins that they produce. Types A, B, E and F are responsible for the majority of cases of human botulism, whereas type C and D are usually associated with animals and birds. The toxins are proteins with a molecular weight of  $\approx 150\,000$  and are thought to block the release of acetyl-choline from nerve endings, causing the symptoms of blurred vision and muscle weakness. Botulism is normally confirmed by the detection of botulinum toxin in suspected food and faeces and blood of the patient, since isolation of the bacterium alone is of limited significance.

The mouse lethality test is the most sensitive and reliable and the most widely applied test for botulinum toxins at the present time. However, the mouse assay is expensive to perform, lacks specificity and requires specialized laboratory and animal house facilities. With the increasing concern over animal welfare in general, there is an urgent need for the replacement of the mouse test with one of equivalent sensitivity and reliability.

A single antibody ELISA with a monoclonal capture antibody and a polyclonal antibody-enzyme conjugate to detect botulinum type A toxin has been described (Shone *et al.* 1985). This test was capable of detecting 5–10 mouse 50% lethal doses ml<sup>-1</sup> of type A neurotoxin (one MLD<sub>50</sub> is equi-

valent to  $\approx 5$  pg of pure neurotoxin). No cross-reaction was observed with other botulinum toxins and the assay did not appear to be affected by food constituents. The test was used to identify the type and concentration of toxin in the outbreak of botulism attributed to contaminated hazelnut yoghurt in 1989. The same workers have also developed equally sensitive ELISAs for types B and F neurotoxin. These tests are not commercially available and have the disadvantage that they do not measure the biological activity. Also, ELISAs that use monoclonal antibodies in the solid phase capture may give false negative results, because of the antigenic variations within botulinum toxins of the same type (Minton 1995).

To address these problems, a rapid *in-vitro* assay based on the biological activity (endopeptidase activities) of the botulinum neurotoxins was developed and successfully applied to the detection of type A and type B toxins (Hallis *et al.* 1996).

An amplified enzyme-linked immunosorbent assay-enzyme-linked coagulation assay (ELISA-ELCA) was developed for the detection of botulinal toxin E in fish (Roman *et al.* 1994). The ELISA-ELCA was reported to have several advantages over the mouse bioassay, including earlier detection and no pretreatment of the sample. Fach *et al.* (1995) used PCR and five individual gene probes to identify botulinal neurotoxin A-, B-, E-, F- and G-producing *Clostridia* spp. in foods. After enrichment, the method detected down to 10 bacteria per g of sample and showed good correlation with the mouse bioassay. A PCR-based method for the detection of the botulinal type E neurotoxin gene in smoked fish was reported to be more sensitive than the mouse bioassay (Sciacchitano and Hirschfield 1996).

### 3.5 Enteropathogenic *Escherichia coli*

The diarrhoeagenic *E. coli* are currently grouped according to their virulence factors, clinical symptoms, epidemiology and serology. The five major classes are verotoxigenic *E. coli* (VTEC), also known as enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAaggEC). To date, toxin production is only believed to be important for VTEC and ETEC.

ETEC are not reported to be a significant cause of diarrhoea in developed countries, although several outbreaks have been reported in the UK and North America. It is a major cause of diarrhoea in infants in developing countries (Doyle and Padhye 1989) and is probably the most important cause of diarrhoea in travellers to these countries. ETEC produce two heat-stable toxins, known as STI (ST<sub>A</sub>) and STII (ST<sub>B</sub>), and two heat-labile enterotoxins, known as (LTI and LTII). Two related forms of STI have been described, STIa (ST<sub>A1</sub> or STp) and STIb (ST<sub>A2</sub> or STTh). The heat-stable toxin

STI and the heat-labile toxin LTI, which closely resembles cholera toxin, are of most significance in human disease.

A range of *in-vivo* tests and *in-vitro* tests have been described for the detection of ETEC ST and LT toxins (Lingood 1982) including ileal loop (Burgess *et al.* 1978), rabbit skin, CHO (Guerrant *et al.* 1974), radioimmunoassay and suckling mouse assay (Lovett and Peeler 1984), but they are not suitable for routine use. Simpler alternatives to the biological assays based on immunological techniques and nucleic acid hybridization are now available.

A simple gel immunodiffusion test (Biken test) has been used to detect LT producing strains of *E. coli*. A total of 273 strains of *E. coli* isolated from cases of traveller's diarrhoea and 516 stock cultures were screened using the Biken test and the CHO cell assay. Results showed the Biken assay to be 98% sensitive and 99% specific (Honda *et al.* 1981). A disadvantage of this test is that it takes 3–4 days to give a result.

The *E. coli* ST EIA kit (Oxoid Ltd) is a competitive enzyme immunoassay for the detection of *E. coli* heat stable toxins ST<sub>A1</sub> and ST<sub>A2</sub> in culture filtrates. The assay time in 3–4 h with a claimed sensitivity 10 ng ml<sup>-1</sup>. Scotland *et al.* (1989) used the ST-EIA kit and infant mouse test to show that 100 *E. coli* strains with the ST<sub>A1</sub> and ST<sub>A2</sub> genes produced heat-stable enterotoxin. The ELISA was easy to perform and gave results within 24 h. Stavric *et al.* (1992) also compared the ST-EIA and infant mouse assay and found that 31 of 46 strains of *E. coli* were positive by both assays, while 15 strains were negative. They reported that the sensitivity of the ST-EIA kit was up to 64-fold lower than the infant mouse assay.

Two commercial agglutination kits are available for the detection of *E. coli* heat-labile enterotoxin (LT). The VET-RPLA kit (Oxoid Ltd) and the Phadabact ETEC-LT coagglutination test (Karo Bio Diagnostics AB) can be used to test culture filtrates and bacterial suspensions for isolation plates, respectively. Chapman and Daly (1989) compared the ETEC-LT test with a Y1 mouse adrenal cell assay for detecting the heat-labile enterotoxin of *E. coli* and found that some culture media could adversely effect the results of the test. Of four culture media evaluated, only modified blood agar gave a positive result with all strains known to produce LT toxin. They concluded that the ETEC-LT kit provided a simple, sensitive and economic method for detection the heat-labile enterotoxin.

Speirs *et al.* (1991) compared the ETEC-LT and VET-RPLA kits with the Y-1 and Vero cell culture assays. Of 48 toxigenic strains, 23 were positive by all assays. Four LTII producing strains were positive by cell culture only. They concluded that the ETEC-LT test was the least sensitive, but was simple and rapid. The VET-RPLA was simple, sensitive and a good substitute for cell culture assays.

Commercial DNA hybridization kits (ETC-01, ETC-02, ETC-03 and ETC04, Bresatec Ltd, Adelaide, Australia) are



available for testing bacterial isolates for the presence of genes controlling the production of *E. coli* ST<sub>A1</sub>, ST<sub>A2</sub>, ST<sub>II</sub> and LTI toxins, respectively. These employ mixtures of synthetic oligonucleotide probes labelled with alkaline phosphatase and positive colonies are detected by a colour reaction. A comparison of these kits with bioassays and radiolabelled recombinant DNA probes showed good agreement between the three methods. False positive results are given by some strains of ETEC that hybridize but do not produce detectable levels of toxin (Medon *et al.* 1988).

VTEC, including *E. coli* O157:H7, are now recognized as an important cause of human diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in North America and Europe. The first confirmed outbreak of O157 VTEC infection in Britain occurred in 1983 and since then the disease has been reported with increasing frequency. VTEC produce one or more cytotoxins, known as verotoxins (VT), because of their cytotoxic effect on Vero cells (Konowalchuk *et al.* 1977). VT1 has identical immunological and biological activities to Shiga toxin and is therefore also known as Shiga-like toxin (SLT). Another group of antigenically distinct toxins is known as VT2 (SLTII). Both toxins are reported to act by blocking protein synthesis (Ogasawara *et al.* 1988).

The classical test for VT toxins is the Vero cell culture assay (Speirs *et al.* 1977). In this test differentiation of VT1 and VT2 toxins requires neutralization of the cytotoxic activity with specific antisera.

There are several commercial immunoassays for the detection of VT in pure cultures of *E. coli*. The Verotox-F kit (Denka-Seiken) and VTEC-RPLA (Oxoid Ltd) kit can detect VT1 or VT2 in culture filtrates after an overnight incubation. Beutin *et al.* (1996) combined the enterohaemolysin test and VTEC-RPLA to isolate and characterize faecal *E. coli* isolates within 72–96 h. The Verotest (Microcarb) is an ELISA that takes 3 h to perform and can detect, but does not differentiate, VT1 and VT2. The Premier EHEC (Meridian Diagnostics) is another rapid ELISA that detects VT1 and VT2 in 3 h and is intended for use with pure cultures or stool specimens.

A number of nucleic acid-based assays have been described for the detection of VTEC. Read *et al.* (1992) used a pair of oligonucleotide primers, targeting conserved sequences in the VT1, VT2 and VTE genes, to develop a PCR assay for all types of VTEC. The assay was used to test culture supernates of 223 VTEC strains and 72 non-VTEC strains. The PCR assay correctly identified all of the VT-positive strains at levels down to 17 cfu. *Shigella dysenteriae* was the only non-VTEC bacterium that gave a positive result. The authors concluded that the PCR method might be suitable for screening *E. coli* isolates from foods and faeces for VT production. Thomas *et al.* (1994) used a PCR assay to examine 50 faecal specimens for the presence of VT genes. Nineteen specimens were positive by PCR and 16 of these yielded VT-positive *E. coli* O157.

#### 4. FUTURE TRENDS

The modern food industry is an increasingly complex business, meeting consumer demand for a wide range of food types, from minimally processed to modified-atmosphere-packaged products. Commercial, legislative and hygiene concerns and other pressures, together with the move toward hazard analysis and on-line monitoring systems, will no doubt ensure that the sensitivity and specificity of techniques for bacterial toxins will continue to improve. Increasing adoption of the HACCP approach by food manufacturers can reduce the risks of bacterial toxin contamination, but cannot guarantee product safety. Testing for toxins must therefore be considered as part of properly evaluated and implemented HACCP programme. Current techniques for detecting bacterial toxins do not give results in real-time and are therefore of limited value in monitoring critical control points (CCPs). However, there are other roles for toxin analysis, include testing of sensitive raw materials, risk assessment, challenge testing and compliance with legislation or customer specifications. Epidemiology is another useful application for toxin analysis where need for real-time testing may be less critical.

This review has highlighted the significant developments in rapid and alternative techniques for the detection of bacterial toxins which have been made in recent years. It is not easy to forecast how microbiological techniques will develop, but there is clearly a need for alternatives to whole-animal assays, which have significant drawbacks, including poor reproducibility, low sensitivity and high cost. Sensitive ELISA-based tests for bacterial toxins have been developed and commercialised, but they do not measure functional activity of the toxin. Highly specific and sensitive DNA probes and PCR have been described which can detect potentially toxigenic strains of bacteria, but a positive result does not indicate that toxin gene was actually expressed. Therefore, it is likely that future effort will be focused on the development of assays which measure biological activity.

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