MODERN AND RAPID METHODS FOR TOXICOLOGICAL ANALYSIS OF FOODSTUFFS WITH BACTERIAL CONTAMINATION

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Abstract: Foodborne illnesses are a widespread problem throughout the world. Food poisoning due to bacterial toxins can be caused by the ingestion of exotoxins 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. Early methods for the assay of bacteria toxins were based on *in vivo* or *in vitro* tests. Later, immunological tests were developed based on techniques such as gel diffusion. Different types of methods have been developed for the detection of toxin genes and their toxic products. These methods include the detection of toxin genes by amplification methods and hybridization probing. 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.

Key words: modern and rapid methods, detection, bacterial toxins.

1. INTRODUCTION

Foodborne illnesses are a widespread problem throughout the world. Food poisoning due to bacterial toxins can be caused by the ingestion of exotoxins (e.g. *Staphylococcus aureus* enterotoxin, *Bacillus cereus* toxin, *Clostridium botulinum* toxins) 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. *Clostridium perfringens* food poisoning). [25]

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.

Different types of methods have been developed for the detection of toxin genes and their toxic products. These methods include the detection of toxin genes by amplification methods and hybridization probing. The toxins themselves have been detected using immunological assay, agglutination tests, and by bioassays.

1.1. Biological assays

The modern assay methods are rapid and a convenient means of testing for bacterial toxins but they do not provide information on the biological activity of the toxin. Thus, bioassays and related tests are still the method of choice for some bacterial toxins (e.g. botulinum toxins). Bioassay formats include wholeanimal tests (e.g. the mouse lethality test, monkey and kitten

emesis tests, and rabbit and guinea-pig skin tests), partanimal tests (e.g. ileal loop tests) and cell culture systems (e.g. Chinese hamster ovary (CHO) cells).

1.2. Immunological assays

Immunological assays are much simpler and cheaper than biological assays. Some of the techniques for the detection of bacterial toxins and the commercial sources of kits based on these techniques are:

1.2.1. Haemagglutination

The methods based on haemagglutination do not require the antigen to be in a precipitable form. In the passive haemagglutination test dilutions of sample are reacted with a constant amount of antibody. When there is free antibody present, toxin coated erythrocytes which are added then, agglutinate. In the reversed passive haemagglutination test, antibody is coupled to sheep erythrocytes and agglutination occurs when toxin is present.

1.2.2. Coagglutination

In the coagglutination test is used to detection the toxin of *Staphylococcus aureus*. A commercial version of this test for *Salmonella*, *Shigella* and enterotoxigenic *E.coli* is available.

1.2.3. Reverse passive latex agglutination

Reverse passive latex agglutination (RPLA) can be used to detect soluble antigens in food extracts or culture filtrates. The antibody is attached to latex particles and reacts with the soluble antigen (e.g. bacterial toxin). Positive reactions are indicated by the formation of a diffuse lattice pattern [8]. The assay is simple and rapid to perform, but it is relatively expensive and gives only semi-quantitative results. There are commercial tests available using RPLA for toxins including those of *B.cereus*, *C.perfringens*, *B.cereus* diarrhoeal toxin, Shiga toxin-producing *E.coli* verotoxin, *S.aureus* enterotoxins, and *V. cholerae*.

1.2.4. Gel diffusion assays

Gel diffusion methods include Electro-immunodiffusion, micro- Ouchterlony slide test, Oudin or single-diffusion tube test, and the microslide double diffusion test [15]. The most widely used gel diffusion test is the Crowle modification of the Ouchterlony test. Gel diffusion methods rely on diffusion of the toxin and detecting antibody in a semisolid gel (often agarose) matrix. Interaction of the toxin and antibody results in a visible precipitation band that indicating the presence of the toxin [16]. Gel diffusion assays have been used in the detection of toxins from *C. botulinum*, *C. perfringens*, and *S. aureus* enterotoxins [15].

1.2.5. 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. Most commercial kits use either limited or excess-reagent systems with a labelled analyte or antibody in the 'sandwich' format. 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 enzymelabelled 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.

1.2.6. Enzyme-linked immunofiltration assay

Enzyme-linked immunofiltration assay (ELIFA) is a rapid alternative to immunoassays. There are commercially available system which has 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.

1.2.7. 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.

1.3. Nucleic acid probes and polymerase chain reaction

Polymerase chain reaction (PCR) was first described in the mid-1980s [20]. PCR is a highly specific and sensitive method for amplifying nucleic acids sequences exponentially. The presence or absence of a gene provides insights into the toxin-producing ability of an organism. PCR methods for toxin detection have been developed for the detection of food-borne pathogens such as *Salmonella*, *V.cholerae*, *E.coli*, and *S.aureus*.

2. DETECTION OF BACTERIAL TOXINS

2.1. Staphylococcus aureus

The ingestion of foods containing sufficient amounts of one (or more) preformed enterotoxin, produced by some strains of *S.aureus* leads to Staphylococcal food poisoning (SFP). 14 different staphylococcal enterotoxins have been described (designated A-O, with several gaps in the sequence). The commonest toxin type in food poisoning is type A. SFP have a rapid onset – 2-8 h. Symptoms include nausea, violent vomiting, abdominal cramping, with or without diarrhoea. The disease is usually self-limiting but can be severe enough to warrant hospitalization, when infants, elderly or debilitated people are concerned.

2.1.1. Staphylococcal enterotoxins (SEs) identification using molecular biological methods

- These methods usually detect genes encoding enterotoxins in strains of *S.aureus* isolated from contaminated foods. Polymerase chain reaction (PCR) approach is a specific, highly sensitive, rapid method that can characterize the *S.aureus* strains involved in SFPOs, thereby providing highly valuable information. PCR also can be combined with other techniques, such as most probable number (MPN-PCR) [22], and PCR-enzyme linked immunosorbent assay (PCR-ELISA) [14], which can provide sensitive results.

- Multiplex PCR assays, allowing rapid and simultaneous detection of staphylococcal enterotoxigenic strains and toxin genes.
- To evaluate the toxic potential of strains isolated from SFPOs, various authors [19, 1, 7] have recently designed primers to perform PCR and reverse transcription PCR (RT-PCR) for SE genes.
- ELISA technology - commercial kits may be in microplate or tube format and sometimes include immunoaffinity columns (IAC) for concentration of toxins from foods. ELISA kits for identification of specific SEs are available.
- Automated test based on Enzyme-Linked Fluorescence Assays (EFLA) are available.
- Biomolecular interaction analysis mass spectrometry (BIA-MS) to detection of bacterial toxins in food samples. This approach utilizes surface plasmon resonance (SPR) to detect the binding of the toxin(s) to antibodies immobilized on a surface of a sensor chip. SPR detection is then followed by identification of the bound toxin(s) by matrix-assisted laser desorption / ionization time-of-flight mass spectrometry. The method is used for identification of Staphylococcal enterotoxin B (SEB) at levels of 1 ng/ ml.

2.1.2. Staphylococcal enterotoxins (SEs) identification using immunoassays

- Optical-based immunoassays have been widely applied for SE detection in food. Optical-based detection methods have good application prospects in biomolecular analysis. They exploit light absorption, fluorescence/luminescence, chemiluminescence, Raman scattering and refractive index for change detection. Optical-based detection methods provide fast, highly sensitive, real-time, and high-frequency monitoring which can make the detection of SEs less time-consuming, lower sample concentration, as well as easier sample pre-treatment.
- Colorimetric Immunoassays: Enzyme-linked immunosorbent assay (ELISA) is a fundamental and widely used colorimetric method.
- Fluorescent Immunoassays are the most popular approach for the detection of SEs. A variety of labels have been used in the development of SE immunoassays.
- Surface Plasmon Resonance (SPR) Immunoassays is surface-sensitive methods for SE detection. In the immunoassay, antibodies were coated onto the sensor chip and the reflection intensity changed when the target bound with the antibodies. SPR features surface-sensitive response, label-free detection, and real-time measurement capability. The presence of the analyte can be determined directly without the use of labeled molecules, which makes continuous real-time detection of biomolecular analytes possible.

2.1.3. Immunological methods

The most commonly used method for detecting SEs in food is based on the use of anti-enterotoxin polyclonal or monoclonal antibodies. Commercially available kits have been developed according to enzyme immunoassay (EIA) comprising ELISA and enzyme-linked fluorescent assay (ELFA) principles; and RPLA principles.

- The Ridascreen™ SET is a sandwich ELISA for the qualitative analysis of staphylococcal enterotoxins A–E that uses monovalent antibodies. The kit was found to be highly specific, sensitive for a range of artificially contaminated food types, and relatively simple to perform, taking less than 3 h to complete.
- The TRANSIA immunoassay for staphylococcal enterotoxins A–E is a tube-based sandwich assay which uses a mixture of monoclonal and polyclonal antibodies.

- The VIDAS staphylococcal enterotoxin assay is an automated enzyme-linked fluorescent immunoassay (ELFA) for the simultaneous detection of staphylococcal enterotoxins A, B, C1, C2 C3, D, E in foods. VIDAS had good sensitivity for enterotoxins A, B, D and E, but was less sensitive for the C enterotoxins.

2.1.4. Staphylococcal enterotoxins (SEs) identification using chromatography methods

Mass spectrometry is among the most sensitive techniques currently available because it provides specific, rapid and reliable analytical quantification of the amount of enterotoxins [5]. SEs are basic proteins that can be enzymatically degraded to peptides, which can be subsequently separated by liquid chromatography (LC), and analyzed by collision-induced dissociation tandem mass spectrometry (MS/MS), yielding information regarding the analyte based on the molecular weights or primary sequences of amino acids. LC-ESI/MS is usually used for the detection of SEs in food samples with low concentrations of soluble proteins.

2.1.5. New Trends

- Aptamer-Based Bioassays: Aptamers are short ssDNA or RNA molecules that were selected through in vitro selection or the systematic evolution of ligands by exponential enrichment (SELEX) [31, 9]. Aptamers bind to target molecules with similar affinity and specificity as antibodies, but they possess many competitive advantages over antibodies [23]. They can be easily produced by chemical synthesis at higher purity; have small molecular weights and superior stability that can bear repetitious denaturation and renaturation; they are an ideal recognition element for biosensors and aptamers have been alternative recognition elements applied to SEs detection.
- Molecularly Imprinted Polymers (MIPs)-Based Bioassay embody a new class of materials possessing high selectivity and affinity for the target molecules. MIPs possess molecular recognition properties due to the cavities produced in the polymer matrix that are complementary in size and shape to the target imprint molecule [26]. MIPs are stable and economical sensing. They are suitable for the substitution of natural receptors and antibodies in assays. SPR biosensor for the detection of SEB by employing MIPs as the sensing material was reported.

2.2. Bacillus cereus

B.cereus is a spore-forming bacterium, which can produce two different types of toxin. The commonest is the 'emetic' or vomiting toxin, a highly stable cyclic peptide also referred to as cereulide. It causes rapid onset (0.5 - 6 hours) nausea and vomiting, sometimes accompanied by diarrhoea. It is normally associated with cooked rice and is caused by preformed toxin in the food. The toxin is extremely heat-stable and will withstand cooking. The second type of *B.cereus* toxin is an enterotoxin. It is a protein causing a diarrhoeal type of food poisoning, which has an incubation period of 8-16 h. It is associated with a whole range of different food types, including rice, pasta, meat and meat products, and sauces. At least two different enterotoxins have been identified, a haemolysin and a non-haemolytic enterotoxin. An intermediate type of food poisoning can occur when enterotoxin is released in the gut, but toxin can also be pre-formed in foods.

- Rapid methods for *B.cereus* enterotoxin have been developed based on the ELISA technique. These methods are quite sensitive and usually require only a simple extraction step.

- Reversed passive latex agglutination (RPLA) methods have also been developed commercially for use with food and culture filtrates. This too requires only a simple extraction procedure before use and provides a result in 24 hours.
- Lateral flow test systems uses monoclonal, rather than polyclonal antibodies for improved specificity and is said to be suitable for the rapid (time to result 4.5 hours) screening of food samples as well as for confirmation of *B.cereus* in culture.
- 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.

2.3. *Clostridium perfringens*

Clostridium perfringens food poisoning is a food-borne intoxication commonly associated with failure to refrigerate previously cooked foods. *Clostridium perfringens* 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. The intoxication was thought to be caused by the ingestion of large numbers of vegetative cells of *C.perfringens*, which then sporulate in the gut and release an enterotoxin. Milligram quantities of the toxin are required to produce symptoms and the toxin is broken down in the digestive tract.

Assay for *C.perfringens* in foods is not carried out routinely. Most assay methods have been used to detect the toxin in faeces during the investigation of food poisoning outbreaks. Several biological methods have been described for the detection of *C.perfringens* enterotoxin but the need for improved speed and sensitivity has resulted in the development of immunological assays for the detection of *C.perfringens* enterotoxin: RPLA- and ELISA-based tests.

2.4. *Clostridium botulinum*

Botulinum neurotoxins (BoNTs) are some of the most lethal human bacterial toxins and the causative agent of botulism [2, 30]. Botulinum food poisoning is relatively rare but is severe and has high mortality rate. Seven serotypes of BoNT (A–G) have been described [4], A, B, E, and F are most frequently associated with human cases of botulism [18, 3].

2.4.1. ELISA and ELISA-based methods of detection

ELISA based methods are fast, inexpensive and simple. Sharma and colleagues designed an amplified enzyme-linked immunosorbent assay (ELISA) for detecting toxins in food matrices [29]. Specifically, toxins for serotypes A, B, E, and F could be detected in liquids, solid, and semisolid food. Some of the ELISA-based methods which have been developed for examining food samples for botulinum toxins are:

- Method using digoxigen labelled IgGs and ELISA (DIG-ELISA) can be used to test food samples and/or cultures for toxin.
- A single antibody ELISA with a monoclonal capture antibody and a polyclonal antibody-enzyme conjugate to detect botulinum type A toxin has been described. The assay did not appear to be affected by food constituents and no cross-reaction was observed with other botulinum toxins.

- A sandwich ELISA specific for BoNT/B that is both rapid and easy to use. This assay is based on monoclonal antibodies for both the capture and detector reagents and thus represents an assay that can be consistently produced over time without the use of animals and the variability inherent in polyclonal antisera.
- A lateral flow immunoassay able to detect and distinguish BoNT/A and BoNT/B in a few minutes [12].

2.4.2. PCR methods

- The GeneSystems PCR Technology have been adapted by Fach and colleagues to amplify *C.botulinum* genes encoding BoNT serotypes A, B, E, and F on different microchambers [11]. This technology allows the simultaneous amplification of multiple targets along with a number of different internal controls.
- Real-time PCR methods for detection that examine expression of the NTNH (non-toxic, non-hemagglutinin) genes of clostridia have been developed, as well as methods to study toxin gene expression in *C.botulinum* serotypes A, B, E, and F [10].

2.4.3. Affinity immunochromatography column-based methods

Affinity immunochromatography column-based methods are accurate and sensitive detection of contaminated food and other biological samples. Brunt and colleagues [6] have developed a number of rapid affinity immunochromatography column (AICC) assays for the detection of BoNT serotypes A, B, E, and F in food matrices.

2.4.4. Immuno-polymerase chain reaction (I-PCR)

Immuno-polymerase chain reaction (I-PCR) is an innovative approach for toxin detection combines antibodies with the amplification power of PCR in an assay called immuno-PCR (I-PCR). Template DNA is conjugated to the antibody and upon binding of antigen by the antibody, an indirect test for the presence of the BoNT is carried out using PCR.

2.5. Salmonella

Optical biosensors have shown great potential for rapid detection of foodborne pathogens. Surface plasmon resonance (SPR) sensors have been used to detect various biological agents including *Salmonella* group B, D, and E, *Salmonella Paratyphi* [4], *Listeria monocytogenes* [20], *Salmonella Enteritidis* [3,20], *Salmonella Typhimurium* [3, 21], *Escherichia coli* O157:H7 [13].

2.6. Enteropathogenic Escherichia coli

The diarrhoeagenic *E.coli* are 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). Toxin production is only believed to be important for VTEC and ETEC.

ETEC produce two heat-stable toxins, known as STI (STa) and STII (STb), and two heat-labile enterotoxins, known as (LTI and LTII). Two related forms of STI have been described, STIa (STA1 or STp) and STIb (STA2 or STb). The heat-stable toxin STI and the heat-labile toxin LTI, which closely resembles cholera toxin, are of most significance in human disease.

- 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*. The Biken assay is sensitive and specific but 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 STA1 and STA2 in culture filtrates. The assay time is 3–4 h.
- The ELISA is easy to perform and gave results within 24 h.
- Commercial agglutination kits are available for the detection of *E.coli* heat-labile enterotoxin (LT). The VETRPLA kit (Oxoid Ltd) and the Phadabact ETEC-LT cooagglutination test (Karo Bio Diagnostics AB) can be used to test culture filtrates and bacterial suspensions for isolation plates, respectively.
- 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* STA1, STA2, STII and LTI toxins, respectively.

VTEC, including *E. coli* O157:H7, are an important cause of human diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in North America and Europe. VTEC produce one or more cytotoxins, known as verotoxins (VT), because of their cytotoxic effect on Vero cells [17]. 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 [26]. The classical test for VT toxins is the Vero cell culture assay. 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.
- 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. [30] 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. PCR method might be suitable for screening *E.coli* isolates from foods and faeces for VT production.

3. 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.

Cultural enrichment methods for detection of bacterial toxins are most widely used, but time-consuming and therefore inadequate for rapid pathogen detection from food samples. Modern and rapid detection of bacterial pathogens and toxins in foods provides real-time results to mitigate foodborne illness outbreaks and ensures public health safety and security. New technologies for detection of bacterial toxins in food such as immunosensors combined with optical, electrical and mass transducer detection techniques, are reliable options. The bioassays based on aptamers, peptides, and other have superior characteristics of sensitivity, selectivity, rapidity and reliability.

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