MICROBIAL TOXINS

Bacterial toxins are traditionally classified into exotoxins and endotoxins. The exotoxins are produced mainly by Gram-positive and some Gram-negative bacteria. They are extracellular proteins released from the bacterial cell during the exponential growth of bacteria. They are immunogenic and neutralizable by specific antibodies. They are all heat-labile, except enterotoxins of *Staphylococcus aureus*. They have variable structures and pharmacological actions.

The endotoxins are produced by Gram-negative bacteria. They are less potent and less specific than exotoxins. They are antigenic and immunogenic but do not form toxoid.

CHEMICAL AND BIOLOGICAL PROPERTIES OF EXOTOXINS

A–B Subunit Toxins

A large number of exotoxins are two-component toxins, so-called A–B subunit toxins. Usually subunit B is non-toxic and associated with binding to the susceptible cell. The A subunit is originally a single polypeptide which is proteolysed to form two polypeptide fragments, A1 and A2, that remain connected by a single disulphide bond. The A2 fragment is responsible for the movement of the toxic A1 fragment into the host cell, where it exerts its enzymatic activity and alters the normal function of the cell.

Diphtheria Toxin

The diphtheria toxin has a molecular mass of 62–63 kDa. The A1 fragment has a molecular mass of 22 kDa and is exceedingly stable, capable of withstanding short periods of boiling and extremes of pH. The A2 fragment has a molecular mass of *c.* 44 kDa, is very unstable in normal buffers and is soluble in the presence of urea, guanidine or sodium dodocylsulphate.

The diphtheria toxin is the sole pathogenic factor of an acute infection caused by *Corynebacterium diphtheriae*. The primary infection occurs in the throat or nasopharynx, where the organism multiplies and produces the toxin which is transported by the blood to remote tissues of the body causing haemorrhagic and necrotic damages in various organs, particularly the heart and nervous system. Such damage is very serious. The cytotoxic effect of diphtheria toxin is mainly due to inhibition of protein synthesis as a result of inactivation of the elongation factor 2, EF2.

Pseudomonas aeruginosa *Exotoxin A*

The *Pseudomonas aeruginosa* exotoxin A has a molecular mass of 66–70 kDa. The enzymatically active fragment has a molecular mass of 26 kDa. Like diphtheria toxin, it inhibits protein synthesis by inactivation of EF2 factor.

Botulinum Toxin

It has a diverse structure and variable molecular masses. The toxic component has a uniform molecular mass of 150 kDa (7S), while the nontoxic component has a variable molecular mass of 7S and more. Accordingly the complex (progenitor) may be medium (M, 12S), large (L, 16S) or extra-large (LL, 19S). Immunologically the toxin is classified into types A, B, C, D, E and F. Type A involves the three forms M, L, and LL. Types B, C and D involve the M and L forms. The toxic component is nicked by protease to produce a dichain of a light (55 kDa) and a heavy chain (100 kDa).

Botulinum toxin is the cause of a disease in man and animals called botulism. In man it is usually food-borne, caused by ingestion of the toxin already formed in food as a result of growth of *Clostridium botulinum* under anaerobic conditions. The most common types are A, B and E toxins. In infants, it is considered that the spores, which are ingested with food, germinate, grow and produce toxins in the intestine. Types A and B are common. Wound botulism has also been reported in man.

In animals, botulism is caused mainly by types C and D and occasionally by types A, B and E. The disease is caused most often by ingestion of preformed toxin in feed, carcasses (by cannibalism), fly maggots and drinking water (as in fish). Also, the toxin can be produced by the organism in the intestine or internal organs, e.g. in the liver.

Botulinum toxin is neurotoxic and causes paralysis; death occurs within 3–5 days from respiratory failure.

Tetanus Toxin

The single polypeptide chain of tetanus toxin is composed of three domains A, B and C, each with a molecular mass of *c.* 50 kDa. The A light chain is the toxic component. The B domain of the BC heavy chain forms the channel in the lipid membrane of the host cell, while a ganglionic binding site is located on domain C.

Tetanus toxin is the cause of tetanus, a disease that affects man and animals through wound infection. The organism grows in the wound and produces its toxin, which acts primarily on the central reflex apparatus in the spinal cord, causing continuous excitation of the motor

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neurones and hence spastic paralysis. The most prominent symptom is the muscle spasm of the jaw (lockjaw).

Shiga Toxin

The toxic subunit of shiga toxin has a molecular mass of 30–32 kDa and is nicked by protease into enzymatic A1 fragment (27 kDa) and a carboxyl terminal A2 fragment (3–5 kDa). The B polypeptide is pentameric (five subunits) with a total molecular mass of 50–65 kDa.

Shiga toxin, produced mainly by the invasive strains of *Shigella dysentriae* type 1 and other species causing dysentry in man, is cytotoxic, enterotoxic and neurotoxic. It causes death of cells in tissue culture, fluid accumulation in the rabbit ileal loop, and paralysis and lethality in animals after parenteral injection.

Shiga-like Toxin (SLT) or Verotoxins (VT)

These toxins consist of an A subunit (33 kDa) of a large (27 kDa) *N*-terminal, enzymatically active fragment and a small (6 kDa) *C*-terminal one. The B subunit consists of five equal fragments of 7·5 kDa each. It is involved in the binding of SLTs to glycolipid receptors.

The isoelectric points (p*I*) for the SLTs are 7·0, 4·1, 5·1 and 9·0.

Shiga-like toxins, produced by strains of *Escherichia coli*, have biological activities similar to those of shiga toxin. They are absorbed into blood and affect vascular endothelial cells in target organs. Diseases in which they are implicated include diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in humans and oedema disease in pigs.

Cholera Toxin

The cholera toxin consists also of a single subunit A (28 kDa) having two fragments A1 (21 kDa) and A2 (7 kDa) and five identical, monocovalently associated B subunits, each with a molecular mass of 11·5 kDa (av.).

The cholera enterotoxin, produced by *Vibrio cholerae*, activates adenylate cyclase, resulting in increased intracellular levels of cyclic adenosine monophosphate. This brings about the active secretion of chloride and hydrogencarbonate ions from the mucosal cells into the intestinal lumen. This leads to the secretion into the lumen of large amounts of water, resulting in watery diarrhoea. The massive loss of body fluids results in extreme dehydration.

E. coli *Enterotoxins*

There are two types of *E. coli* enterotoxins:

(1) Heat-labile enterotoxins (LT) are inactivated by holding at 60°C for 30 min. LT-I closely resembles cholera toxin in structure, antigenicity and activity. LT-II has similar activity but is distinct from LT-I antigenically.

(2) Heat-stable enterotoxins (ST) are not inactivated by treatment at 100°C for 15 min and are resistant to proteolytic enzymes and acids; they have a low molecular mass of 4·5–5 kDa.

E. coli enterotoxins, produced by enterotoxigenic *E. coli*, are the causes of traveller's diarrhoea in adults, and diarrhoea in infants and young domestic animals, particularly in neonatal calves and lambs as well as newborn and recently weaned pigs.

Pertussis Toxin

Pertussis toxin has a molecular mass of *c.* 73–77 kDa. The active part has a molecular mass of 26 kDa. Based on its biological activities, the toxin has been given several names such as histamine-sensitizing factor, lymphocytosis-promoting factor and islet-activating protein. It is the main pathogenicity factor responsible for the clinical manifestations of whooping cough.

Bacillus anthracis *Toxin*

The toxin consists of three distinct and serologically active proteins. The subunit A (89 kDa) is the lethal factor (LF), the subunit A1 (83 kDa) the oedema factor (EF) and the subunit B (82·7 kDa) the protective antigen (PA). The toxins cause the sudden death of infected animals because of their action on the central nervous system, which leads to respiratory failure and anorexia.

Enterotoxins

Enterotoxins are a heterogeneous group of toxins produced by several bacteria. They have the common character of acting on the intestine, causing diarrhoea. However, in the case of staphylococcal enterotoxin, diarrhoea can occur following ingestion of foods that contain ready-made toxins. Enterotoxins of *E. coli* and cholera toxins have already been described. The other enterotoxins to be described here consist of a single polypeptide chain. The most important ones are *Aeromonas hydrophila* enterotoxin, *Bacillus cereus* enterotoxin, *Clostridium perfringens* enterotoxin and staphylococcal enterotoxins.

Aeromonas hydrophila *Enterotoxin*

Two types are known, a cytotonic, cholera-like, but serologically unrelated enterotoxin with a molecular mass of 15 kDa and p*I* of 4·2–4·6 which is heat-stable at 56°C for 10 min, and a cytotoxic enterotoxin which has a molecular mass of 50 kDa, p*I* of 5·4–5·5 and is unstable at 56°C for 10 min.

The cytotonic toxin causes rounding of Y-1 cells but not death, stimulates cyclic AMP synthesis and steroid secretion and causes fluid accumulation in the rabbit ileal loop. The cytotoxic enterotoxin causes rounding and death of Y-1 and CHO cells and fluid accumulation in rabbit ileal loops and suckling mouse intestines.

Bacillus cereus *Enterotoxin*

Two enterotoxins are produced: (1) a diarrhoeal enterotoxin which is thermolabile, inactivated by proteolytic enzymes, has a molecular mass of 39–46 kDa and a p*I* of 5·1–5·6, and (2) an emetic enterotoxin which is thermostable, resistant to proteolytic enzymes and has a molecular mass of 10 kDa.

These enterotoxins are the causes of food poisoning characterized by vomiting that occurs about 4 h after ingestion of foods such as fried rice in which extensive multiplication of *B. cereus* occurred, and diarrhoea, which has a longer incubation period (about 17 h).

Clostridium perfringens *Enterotoxin*

The molecular mass of type A enterotoxin is about 43 kDa. It consists of a single polypeptide with a unique sequence. Its p*I* is 4·3 and it is activated by trypsin. It is the cause of a mild form of food poisoning that occurs about 8–24 h after ingestion of foods, mostly meat dishes such as roasts, poultry, fish and stews that are heavily contamined with *Cl. perfringens*. The main symptoms are diarrhoea and abdominal pain; nausea may occur but vomiting is uncommon.

Staphylococcal Enterotoxins

These are a group of heat-stable, water-soluble toxins which are resistant to the action of trypsin and pepsin. The staphylococcal enterotoxins occur in six serologically distinct types which vary in their molecular masses and p*I* values (see **Table 1**). They are the cause of food poisoning that occurs shortly (2–6 h) after ingestion of foods containing preformed toxins such as custard or cream-filled bakery products, ham, ice-cream, cottage cheese, chicken salad, etc. Symptoms consists of severe cramps, abdominal pain, nausea, vomiting and diarrhoea.

Cytolysins

These toxins were discovered initially because of the damage they cause to erythrocytes; they are therefore called haemolysins. However, some of these toxins have a much broader range of target cell membranes. These toxins are either thiol activated or not.

The thiol-activated cytolysins represent a distinct family of toxins produced by members of the genera *Streptococcus, Bacillus, Clostridium* and *Listeria*, with streptolysin O as the prototype of this family. They are immunogenic, single-chain, oxygen-labile, cytolytic toxins which are inactivated by a low level of cholesterol. They are immunologically closely related, but they differ in their amino acid sequences. The values of molecular mass and p*I* of the individual toxins reported in the literature are contradictory. Streptolysin O has a molecular mass of 53–75 kDa and a p*I* of 6·5–7·2, cereolysin has a molecular mass of 49–59 kDa and a p*I* of 6·3–6·7, and listeriolysin has a molecular mass of 58 kDa, etc. Other cytolysins of this family include pneumolysin, tetanolysin, perfringolysin, septicolysin, histolyticolysin, botulinolysin and thuringiolysin, which are produced by the respective organisms that their names indicate.

The other cytolysins which are not thiol-activated are as follows:

(1) Streptolysin S is an oxygen-labile, nonantigenic protein which consists of a single polypeptide attached to an oligonucleotide.
(2) Haemolysin II of *B. cereus* is a thermolabile, antigenic protein with a molecular mass of *c.* 29–34 kDa and a p*I* of 4·9–5·3. It is protease susceptible and is not affected by thiol, cholesterol or anti-streptolysin O.
(3) *Vibrio parahaemolyticus* haemolysin is a thermostable, trypsin-sensitive protein with a molecular mass of 44 kDa, composed of two identical subunits of 22 kDa each and produced only at pH between 5·5 and 6·5.
(4) Staphylococcal cytolysins are α-, β-, γ- and δ-haemolysins in addition to leucocidin. The α-toxin is a hydrophilic molecule present in monomeric form in aqueous solution with a molecular mass of 33–34 kDa and it is isoelectric at pH 8·5–8·6. The β-toxin has a molecular mass of 30 kDa and p*I* of 9·4. The γ-toxin consists of two proteins with molecular masses of 26, and 29 kDa and p*I* values of 9·9 and 9·8, respectively. The δ-toxin has a molecular mass of 103 kDa. The leucocidin consists of a fast (F) migrating component (32 kDa) and a slow (S) component. Both have a p*I* of 9·0.

Table 1 Molecular masses and p*I* values of serologically distinct types of staphylococcal enterotoxins

Type	Molecular mass (kDa)	p*I*
A	27·8	7·0
B	28·3	8·6
C1	34·1	8·6
C2	34·0	7·0
D	–	–
E	29·6	7·0

METHODS OF IDENTIFICATION AND DETERMINATION OF EXOTOXINS

Biological Methods

Laboratory Animals

Both laboratory animals and tissue culture methods are used in identifying exotoxins. Laboratory animals are

used for the detection of the enterotoxin of *S. aureus*, for example by feeding monkeys with the enterotoxin which results in vomiting. For the detection of *Cl. botulinum* toxin mice are injected intraperitoneally (i.p.) with the supernatant of the sample homogenate, both with and without antitoxin. In positive cases mice injected with antitoxin survive while those injected with the sample only die. [*See Bioassays*]

For identification of toxigenic strains of *C. diphtheriae* guinea pigs or rabbits are injected intracutaneously with broth culture, then 5 h later diphtheria antitoxin is injected i.p. or i.v. (intravenously) and 30 min later a control area of the skin is injected intracutaneously with the broth culture. In positive cases necrosis occurs at the first site of injection, while only a pinkish nodule without necrosis appears on the second site.

The ileal loop test is used for the detection of enterotoxins produced by several organisms such as *E. coli* enterotoxins, cholera toxin, diarrhoeic toxin of *B. cereus*, enterotoxin of *A. hydrophila* and shiga toxin. Rabbit or mice ileal loops are injected by culture filtrate and control loops are injected with saline. In positive cases the segments injected with the toxin are swollen compared with the control ones.

Tissue Cultures

Tissue cultures of various cell lines are inoculated with the toxins or sample extract and examined after a certain time for cytopathic effect. Various toxins are tested by this method including shiga toxin, shiga-like toxins and clostridial toxins.

Serological Methods

Several serological methods are used for the detection of exotoxins. They are based on the use of specific antibodies to each of the different toxins. The most commonly used methods are gel diffusion, radioimmunoassay, reverse passive haemagglutination, reverse passive latex agglutination and various versions of enzyme-linked immunosorbent assay (ELISA). [*See Immunoassays, enzyme immunoassays*]

The Elek test used for the detection of diphtheria toxin is a gel diffusion. The tested organism is streaked onto the surface of a medium at right-angles to a paper strip soaked with a defined amount of antitoxin. Positive and negative control strains are also used. In positive cases white lines of precipitation extend out from the line of bacterial growth. A modified Elek test is used for the detection of *Vibrio parahaemolyticus* haemolysin and the LT of *E. coli*.

The gel diffusion test can be done using wells cut in agar plates, as in the case of the optical sensitivity plate test for staphylococcal enterotoxin. Sample or culture filtrate extract, standard enterotoxin and specific antiserum are distributed in the wells. After 24 h incubation the plate is examined for precipitation lines identical to that of the standard toxin. The test can be done on slides, in which case it is called the microslide gel diffusion test.

The radioimmunoassay is based on the addition of a known amount of specific antibody to known amount of sample extract. After a period of reaction a standard amount of radioactive-labelled toxin is added which will react only with unbound antibodies. Staphylococcal cells containing protein A are added to precipitate the toxin–antitoxin complex which is then tested by a scintillation counter. The amount of toxin in the sample extract is inversely proportional to the amount of labelled toxin present. The test requires expensive reagents and equipment in addition to the risk of handling radioisotopes.

The reverse passive latex agglutination test is based on the use of antibody-coated latex particles which will be added to doubling dilutions of the sample extract. The agglutination reaction is read after 24 h incubation.

The reverse passive haemagglutination test is the same as the latex agglutination test but instead of latex particles red blood cells sensitized with specific antitoxin are used.

The ELISA test is used either in the form of a microtitre plate assay or a polystyrene ball assay. The direct, indirect, double antibody sandwich and competitive ELISA techniques can all be used for the detection of toxins. The microtitre plate assay described for the detection of staphylococcal enterotoxin involves the addition of sample extract and control to individual wells. Wells are washed after 2 h and antistaphylococcal enterotoxin-conjugate is added to the wells, left for 1 h, then washed out and the substrate is added, followed by the stop reagent when positive control shows strong reaction. The reaction is read visually or by plate reader. In the case of the polystyrene ball assay, the sample extract is mixed with normal rabbit serum and Tween 20 for 20 min, centrifuged, and the supernatant is added to the antitoxin-coated beads and control beads. After overnight incubation the beads are washed and added to antitoxin-conjugate. After a 6 h wash, the substrate is added and read.

Molecular Biological Techniques

Molecular biological techniques such as DNA probes have recently been introduced in the detection of toxigenic microorganisms. Many toxins are gene-coded and consequently radio- or enzyme-labelled DNA fragments from the toxin genes can be used as probes to detect homologous DNA sequences in the toxigenic strains that may be present in the test sample. The DNA fragment in the sample may be also amplified using the polymerase chain reaction (PCR). [*See Polymerase chain reaction*]

CHEMICAL AND BIOLOGICAL PROPERTIES OF ENDOTOXINS

Endotoxins are complex molecules consisting of lipopolysaccharide. This is an integral part of the cell wall of Gram-negative bacteria and consequently the endotoxins are released only when the integrity of the cell wall is disrupted. The toxicity resides in the lipid A portion of the molecule, which is composed of a β-1,6-linked D-glucosamine.

When endotoxins are released into the body they almost always cause fever because they stimulate host cells to release pyrogens which affect the temperature-controlling centre in the brain. In addition they may cause diarrhoea and reduce rapidly the number of leucocytes and platelets in the blood. In large doses, endotoxins may cause death, primarily through haemorrhagic shock and tissue necrosis.

Methods of Identification and Determination of Endotoxin

Schwarzman Reaction

The injection of endotoxins intradermally into rabbits in doses of few μg induces a mild inflammatory reaction in the skin. The i.v. injection of the same, or another, endotoxin in the same or lower dose 24 h later induces haemorrhagic necrosis at the site of the original injection.

Limulus Assay

The test is based on the induction of clotting of horseshoe crab (*Limulus polyphemus*) amboecyte lysates with traces of lipopolysaccharides. The test can be done in tubes by mixing 0·1 ml test material with 0·1 ml lysate. After 1 h incubation at 37°C the mixture is tested for turbidity by spectrophotometer. The test can be done on microslides or in microtitre plates using serial dilutions of the test material. The test can be automated.

SAMPLING AND SAMPLE HANDLING

It is very important to collect representative samples. Liquid material should be well-agitated before sampling, and dry material should be well mixed. If this is not easy to accomplish several samples should be taken at random from different places within a large container and from various containers of the same lot or shipment. Samples must be collected in clean, leak-proof containers. If it is intended to isolate toxigenic bacteria from the sample it should be collected under sterile condition in a sterile container and transported as quickly as possible under refrigerated conditions to the laboratory. The size and number of samples to be collected depend on the sampling plan and the type of toxin to be tested for. Samples should be accompanied with a sampling report containing all information such as date, place and time of sampling, nature of food or feed, name of manufacturer, importer, seller, etc., the number and size of samples, the reason for sampling, suggested tests, etc. [***See Sampling, overview***]

After collection samples are generally homogenized and the toxin is subjected to extraction and perhaps purification. The extract is then concentrated so as to be able to detect small amounts of toxins. If the sample is to be tested by biological methods the sample extract should be subjected to sterilization by filtration to get rid of any contaminants that may adversely affect the results. If the toxigenic bacteria is to be isolated the sample homogenate may be serially diluted or may be enriched in selective and specific enrichment media.

SELECTED EXAMPLES

Reverse Passive Latex Agglutination (RPLA)

This test is used for the detection of enterotoxins of *S. aureus, E. coli, B. cereus, V. cholerae, Cl. perfringens,* etc.

Principle

Latex particles are sensitized with antiserum taken from rabbits immunized with purified toxin. Such latex particles will agglutinate in the presence of the homologous toxin.

Procedure

(a) Make serial dilutions of the food extract or culture filtrate to be tested in 2 rows of V-well microtitre plate using 25 μl amounts.
(b) Add to each well of the first row 25 μl of the sensitized latex and in the second row the same amounts of nonsensitized latex (control).
(c) Mix, cover the plate and leave at room temperature for 20–24 h.
(d) Examine each well for agglutination. The control row should be negative. Results of positive agglutination are classified as +, ++, +++ and ++++ according to the degree of agglutination.

ELISA

This method is now increasingly used in the detection of various toxins using poly- or monoclonal antibodies. There are several techniques of ELISA. The most sensitive and specific ones commonly used are the sandwich and the competitive ELISA. The sandwich method is described here.

Procedure

(a) Use microtitre plates precoated with the respective

poly- or monoclonal antibodies specific for the toxin to be detected.
(b) Wash with buffer.
(c) Add test solution (food extract or culture filtrate) in serial dilution and controls to control wells.
(d) Incubate for 2 h at 37°C, then empty wells and wash 3–4 times.
(e) Add antitoxin conjugate, incubate for 1 h at 20–25°C, then empty wells and wash 3–4 times.
(f) Add substrate, incubate at 20–25°C until positive control shows strong reaction, then add stop reagent.
(g) Mix and read.

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MICROBIOLOGICAL METHODS

Overview
Vitamin and antibiotic analysis

Overview

There are many different types of microbiological analysis available to the modern microbiologist. These are based on a wide variety of detection principles and range from direct to indirect, manual to automated, fast to slow and total to specific. The choice of method will be largely affected by the specific application and the user's requirements in terms of determinant and sensitivity. In general the methods can be used for a wide range of applications, e.g. food, pharmaceutical, water, medical etc. Microbiological methods can be used to enumerate 'total numbers' of bacteria, yeasts and moulds in test materials, 'indicator organisms' of faecal or other contamination and pathogens which may cause food poisoning or even death. In addition, there are methods for identifying bacteria, yeasts and moulds isolated or enumerated by these techniques. A list of many of the pathogens, indicator and spoilage organisms of interest to the microbiologist are included in **Table 1**.

TYPES OF METHODS

Methods for estimating microbial numbers can be divided into two groups, direct and indirect. Direct methods count cells directly, either by the ability of viable cells to grow and form colonies, or microscopically. Indirect methods measure either a chemical constituent, enzyme, metabolite or changes produced by organisms during growth. This measurement is converted into numbers by reference to a calibration graph. The 'true' numbers of organisms for the calibration graph are usually assessed by direct methods. Generally, direct methods are more sensitive and accurate than indirect methods, and cultural methods take longer to give a result than microscopic or indirect methods.

DIRECT METHODS

The plate colony count isolates bacteria, yeasts or moulds in a quantitative manner. A range of dilutions, usually 10-fold, of the sample is prepared in a sterile diluent, and 1 ml of each dilution is mixed with melted agar cooled