14

Staphylococcal intoxications

Merlin S. Bergdoll and Amy C. Lee Wong

1 Historical aspects and contemporary problems

1.1 Historical aspects

Among the toxic substances produced by the staphylococci are the enterotoxins, the causative agents of staphylococcal food poisoning. Ingestion of any of the enterotoxins by humans usually results in intestinal disturbances, involving vomiting and diarrhea within a few hours of the ingestion. The illness can be serious, but usually lasts only a few hours with no sequela.

There is no record of when illnesses similar to staphylococcal food poisoning were first observed, but it is likely that humans have been afflicted with this illness as long as they have been consuming foods in which staphylococci could grow. There are records of illnesses of this type as early as 1830, although the organisms themselves were not recognized until 1878 and 1880 by Koch and Pasteur, respectively. Although Ogston is credited with applying the name ‘Staphylococcus’ to these organisms in 1881 because of the grapelike clusters of cocci he observed in cultures, it was Rosenbach who in 1884 obtained pure cultures of the microorganisms on solid media and accepted the name Staphylococcus.

Dack (1956), in his book Food Poisoning, relates several descriptions of foodborne illnesses similar to staphylococcal food poisoning. A number of food items were

1 deceased
involved – sausage, rabbit pie, ‘pork brawn’, milk, ice cream, and of course cheese, where Vaughan and Sternberg first associated micrococci with the illnesses in 1884. Each of these investigators independently examined cheese that had been implicated in food-poisoning outbreaks in Michigan. Sternberg stated: ‘It seems not improbable that the poisonous principle is a ptomaine developed in the cheese as a result of the vital activity of the above mentioned Micrococcus, or some other microorganisms which had preceded it, and had perhaps been killed by its own poisonous products.’ In 1894 Denys concluded that the illness of a family who had consumed meat from a cow dead of ‘vitullary fever’ was due to the presence of pyogenic staphylococci, and in 1907 Owen recovered staphylococci from dried beef implicated in a foodborne illness characteristic of staphylococcal food poisoning (Dack, 1956).

In 1914, Barber was the first investigator actually to relate staphylococcal food poisoning to a toxic substance produced by the staphylococci (Barber, 1914). He discovered that milk from a mastitic cow caused illness when left unrefrigerated, and showed that the illness was due to growth in the milk of the staphylococci isolated from the mastitis. The significance of this excellent report was not recognized and, as a result, this type of food poisoning was ascribed for the most part to other bacterial agents. For example, an outbreak involving 2000 soldiers in the German army during World War I was attributed to Proteus vulgaris even though cocci were present in large numbers.

In 1929, Dack rediscovered the role of staphylococci in food poisoning with his classical work on two Christmas cakes that were responsible for the illness of 11 people (Dack et al., 1930). These three-layer sponge cakes with thick cream fillings were baked possibly 1 day before delivery and eaten 2 days later. They were presumably refrigerated at the bakery but not after delivery. Dack and his associates showed, with the aid of human volunteers, that the sponge cake substance was responsible for the illness. Staphylococci isolated from this part of the cake produced a substance that caused typical food poisoning symptoms in human volunteers. In essence, this was the beginning of the research on staphylococcal food poisoning.

From 1930 until about 1948 a number of investigators studied this type of food poisoning from various angles, with Dack and Dolman independently carrying out much of the work – particularly during the early part of the period. Beginning around 1948, Casman (of the US Food and Drug Laboratories) and Surgalla and Bergdoll (in the Food Research Institute at the University of Chicago), began intensive studies of this subject. Casman continued his studies until his retirement in 1969. These studies were continued by Bennett, who had worked with Casman before his retirement. Surgalla worked with Bergdoll until 1953, when he left the project; Bergdoll continued the studies at the Food Research Institute until his retirement in 1988. During the middle part of this period, Sugiyama made a number of valuable contributions to the work at the Food Research Institute. Many other investigators have made valuable contributions to the field, although the interest in this area has diminished in the developed countries as other foodborne agents have been identified. However, Bergdoll concentrated his efforts on aiding developing countries in pursuing research in this field, particularly in Brazil where staphylococcal food poisoning is quite common.
1.2 Contemporary problems

Once the etiology of staphylococcal food poisoning had been established, it soon became recognized as a major cause of foodborne illness in the US and in many other countries. In the 1960s and 1970s it was recognized as one of the leading foodborne diseases in the US; however, by the late 1980s very few cases of this type of food poisoning were being reported to the Centers for Disease Control and Prevention (CDC) (Table 14.1; CDC, 1981a, 1981b, 1983a, 1983b; MacDonald and Griffin, 1986; Bean et al., 1990, 1996; Olsen et al., 2000). This was also true in England (Wieneke, 1993). It is suggested that outbreaks involving a few people, such as a family, were still occurring, but because of their relative mildness and short duration, these were not being reported. At the height of this disease in the US a number of large outbreaks were occurring, with only an occasional one reported since that time.

The decrease in the number of outbreaks in developed countries is probably due to greater care taken in preparing and handling foods that are to be served to large groups of individuals. For example, in Japan many outbreaks were due to rice balls, which were made by hand. Today, very few if any outbreaks involve rice balls, primarily because they are now made by machine, which eliminates the major source of contamination – the food handler. However, the situation is different in developing countries, such as Brazil. When Bergdoll started working with the food microbiologists in Brazil in 1976, this type of food poisoning was not being recognized even

### Table 14.1 Staphylococcal food poisoning outbreaks reported in the US, 1978–1997

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<th>Year</th>
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Compiled from Annual Summaries of Foodborne Outbreaks (CDC).

* Number of reported and confirmed bacterial foodborne disease outbreaks and number of bacterial foodborne disease cases.
though there was interest in conducting research in this area. It was not until some 10 years later that the first staphylococcal food-poisoning outbreak was investigated and reported (Noleto and Tibana, 1987). In this case the staphylococci isolated (in large numbers) from the implicated food produced enterotoxin – adequate circumstantial evidence to conclude that the outbreak was due to staphylococcal food poisoning. A second group reported an outbreak in 1988 in which cheese was implicated (Sabioni et al., 1988). Bergdoll had been working with both these groups and directed the PhD research of the leaders of both research groups. Apparently outbreaks were widespread in Brazil, but were not being investigated. For example, many outbreaks were occurring in Belo Horizonte and the state of Minas Gerais, but were not being investigated because no one in the Public Health Laboratory had any experience in this field. Finally Bergdoll was asked to assist in examining the staphylococci isolated from foods implicated in 18 outbreaks for enterotoxin production (do Carmo and Bergdoll, 1990). Bergdoll was able to give the researchers at São Paulo assistance in examining the food, implicated in six outbreaks, for the presence of enterotoxin (Cerqueira-Campos et al., 1993). For the most part complete investigation of the outbreaks was not being performed. In one outbreak that Bergdoll was instrumental in investigating, the food handler was shown to be responsible for contaminating the implicated food (Pereira et al., 1994). As recently as 1996, several hundred individuals became ill with staphylococcal food poisoning after consuming food at a political rally.

In some countries, obtaining information about the importance of staphylococcal food poisoning is impeded because, aside from the fact that other problems may be of more immediate concern, it is difficult to determine how important the staphylococci are in the digestive disturbances common to these countries. Although the type of foods consumed in any given country may affect the incidence of this type of food poisoning, the populations of most countries consume foods that can support the growth of staphylococci and the production of enterotoxin.

## 2 Characteristics of *Staphylococcus aureus*  

### 2.1 Classification of *Staphylococcus*

Taxonomically the staphylococci have been placed in the Family *Micrococcaceae*. Baird-Parker (1963) proposed a system of classification of the micrococi and staphylococci based on certain physiological and biochemical tests. He divided the Family *Micrococcaceae* into Group I (*Staphylococcus* Rosenbach emend. Evans) and Group II (*Micrococcus* Cohn emend. Evans). These groups were then divided into subgroups on the basis of pigment production, coagulase and phosphatase reactions, acetoin production, and formation of acid from glucose (both aerobically and anaerobically) and other sugars. Six subgroups were recognized within the genus *Staphylococcus* and seven within the genus *Micrococcus*. Hajék and Marsálek (1971) further divided the pathogenic staphylococci found in Baird-Parker’s subgroup I into six biotypes with characteristic biochemical and biological properties.
Staphylococcus can be differentiated from the other three members in the family, Micrococcus, Stomatococcus, and Planococcus, on the basis of the guanine plus cytosine (G + C) content of the DNA, cell wall composition, and the ability to grow and ferment glucose anaerobically. Only three species of Staphylococcus (S. aureus, S. epidermidis and S. saprophyticus) were included in the genus in 1974 (Buchanan and Gibbons, 1974). They were differentiated primarily on the basis of the ability to produce coagulase, ferment mannitol (both aerobically and anaerobically) and produce heat-stable endonuclease, and by the cell wall composition (Baird-Parker, 1974).

Kloos and Schleifer (1975) outlined a simplified scheme for the routine identification of human Staphylococcus species. They divided these into 11 species on the basis of coagulase activity, hemolysis, nitrate reduction, and aerobic acid production from several sugars. Since then the number of species and sub-species had increased to 32 as of 1994 (Holt et al., 1994). This increase included the elevation of two of the S. aureus biotypes to species status, biotype E (from dogs) to S. intermedius and biotype F (from swine) to S. hyicus. An additional coagulase-positive species, S. delphini from dolphins, has been added.

### 2.2 Characteristics of S. aureus

For many years S. aureus was the only recognized species that produced coagulase, with the species further subdivided by biotyping to determine the animal source of the species. Identification of S. aureus was relatively easy, because any organism that produced coagulase was automatically classified as S. aureus. Addition of thermonuclease (TNase) production (Chesbro and Auborn, 1967) to the characteristics of S. aureus aided in the identification because the production of either coagulase or TNase was adequate to classify an organism as S. aureus. Although upgrading of the biotypes to species was helpful in identifying the major source of a particular coagulase-positive species, additional tests were necessary to identify S. aureus. Each biotype was predominately associated with particular animals, with humans being the major source of S. aureus, although S. aureus can be isolated from a wide range of animals. However, it is seldom that the other coagulase-positive species can be isolated from humans. Most of the staphylococcal species recognized to date do not produce coagulase, with the exception of S. aureus, S. delphini, S. hyicus, S. intermedius and S. schleiferi subspecies coagulans. All of the coagulase-positive species produce TNase, with the exception of S. delphini, but only S. aureus ferments mannitol both aerobically and anaerobically, and produces protein A and acetoin (Table 14.2). The fact that not all strains in any one species are positive for all of the characteristics of that species complicates the classification of S. aureus. For example, not all S. aureus strains ferment mannitol or are positive for acetoin (Bennett et al., 1986; Roberson et al., 1992), which could result in their being classified as another coagulase-positive species if additional testing were not performed. Although S. aureus is isolated from many animal species, it is the only coagulase-positive species normally isolated from humans, particularly from the nose and throat. S. intermedius can be transmitted from dogs to the skin, but seldom to the nose or throat (Talan et al., 1989).
S. aureus is the most important species involved in staphylococcal food poisoning, even though S. intermedius strains can produce enterotoxins (De la Fuente et al., 1986; Hirooka et al., 1988). Only one outbreak has been associated with S. intermedius (Khambaty et al., 1994) since the identification of this species. In this outbreak the source of the staphylococci, whether animal or human, was not identified.

2.2 Hosts and reservoirs

The staphylococci are ubiquitous in nature, with humans and animals as the primary reservoirs. They are present in the nasal passages and throat, in the hair, and on the skin of probably 50% or more of healthy individuals. The prevalence is usually higher in individuals associated with hospital environments because many infections and diseases are caused by the staphylococci. These organisms are associated with sore throats and colds, and are found in abundance in postnasal drip following colds. Staphylococci can be isolated from animals, with the bovine being the most important because of the involvement of staphylococci in mastitis. Although animals and humans are the major source, staphylococci also can be found in the air, dust, water, and human and animal wastes.

2.3 Ability to survive and grow in the environment

The staphylococci can survive indefinitely in the nasal passages and throats of humans and animals. From these sources they can be transferred to meat and other foods. Essentially all meats can be contaminated with staphylococci; however, the organisms may persist on raw meats but grow very poorly. In foods that provide a satisfactory medium they can grow to sufficient numbers to produce enterotoxin if the foods are not refrigerated. These organisms can be transferred to equipment; if the equipment is then not adequately cleansed before use, the organisms can be transferred to foods. A common source of contamination of dairy products is cows’ udders, particularly in animals with staphylococcal mastitis. The organisms are destroyed when the milk is pasteurized, but any enterotoxin in the milk will not be inactivated (Read and Bradshaw, 1966; Evenson et al., 1988).
3 The toxins

3.1 Conditions for enterotoxin production

The same factors that affect growth of the organism in general also affect the production of enterotoxin. The methods used for production of the enterotoxins depends to a large extent on the purpose of the production – for example, whether attempting to determine the enterotoxigenicity of a strain or conducting purification studies. In either case it is desirable to produce as much enterotoxin (per milliliter) as possible, but for purification the overall quantity needed is an important factor (Kato et al., 1966).

3.1.1 Enterotoxigenic staphylococci

The staphylococci produce 12 related but serologically distinct enterotoxins – A (SEA) and B (SEB) (Casman et al., 1963), C (SEC) (Bergdoll et al., 1965), D (SED) (Casman et al., 1967), E (SEE) (Bergdoll et al., 1971), G (SEG) (Munson et al., 1998), H (SEH) (Ren et al., 1994; Su and Wong, 1995), I (SEI) (Munson et al., 1998), J (SEJ) (Zhang et al., 1998), K (SEK) (Jarraud et al., 2001; Orwin et al., 2001), L (SEL) and M (SEM) (Jarraud et al., 2001) – and possibly some unidentified ones. There is no enterotoxin F (SEF) because toxic shock syndrome toxin was misidentified as SEF when it was first isolated. Two SEKs were described independently by two different groups at about the same time (Jarraud et al., 2001; Orwin et al., 2001); however they are different proteins based on their deduced amino acid sequences. Enterotoxin C has several members that are very closely related and react with the same antibodies (Reiser et al., 1984; Bohach and Schlievert, 1989; Couch and Betley, 1989). These are labeled C1, C2 and C3, in addition to others that are labeled Cbovine, Cowine, and Ccanine (Edwards et al., 1997). Only an antibody to SEC2 is needed to identify all of the SECs (Reiser et al., 1984).

Enterotoxins A–E and SEH were identified by purification of the protein and their specific reaction to antibodies developed to them. It is important for purification purposes to identify staphylococcal strains that produce a maximum amount of enterotoxin. The SECs and SEB are normally produced in relatively large amounts (over 100 µg/ml), so there is no particular problem in identifying strains for production of these toxins for purification (Robbins et al., 1974). This is not true for the other enterotoxins, as they are produced at only a few micrograms per milliliter, with SED produced at less than 1 µg/ml. To improve the production of SEA, several mutations were generated to increase the amount produced (Friedman and Howard, 1971). This also was done for an SED producer, but still the mutant produces only a few micrograms per milliliter (Chang and Bergdoll, 1979).

3.1.2 Media

The media developed in the Food Research Institute for the production of enterotoxins employed a pancreatic digest of casein from Mead Johnson and Co. (Evansville, Indiana), which had been used by Segalove (1947), supplemented with thiamin, niacin, and glucose (Surgalla et al., 1951; Kato et al., 1966) (Table 14.3). The use of pancreatic digest of casein became standard for the large-scale production of
enterotoxin, and these are still in use today; however, because of the discontinuance of production of Amigen, protein hydrolysate powder (PHP) and N-Z Amine NAK (NAK) (Humko-Sheffield Chemical Co., Lyndhurst, New Jersey), less suitable pancreatic digests of casein are used. One such product is N-Z Amine A (NZA-A) (Humko-Sheffield Chemical Co.), but it is necessary to supplement it with yeast extract (Metzger et al., 1973). The addition of yeast extract has made it unnecessary to add niacin and thiamin to the media. Metzger et al. (1973) obtained excellent production of both SEA and SEB with 4% NZA-A supplemented with 1% yeast extract and 0.2% glucose. Over 100 µg/ml of SEA and over 400 µg/ml of SEB were produced with this medium. Carpenter and Silverman (1974) also obtained good results with 4% NZA-A supplemented with 0.4% yeast extract and 0.1% K₂HPO₄. This product is normally sold in bulk, but can be obtained in small amounts at an increased price. Other pancreatic digests of casein, such as tryptone, available in small quantities (Difco Laboratories, Detroit, MI), have proved useful for enterotoxin production when supplemented with yeast extract (unpublished data). These results show that various enzymatic hydrolysates of casein can be used to produce enterotoxin when they are supplemented, particularly with yeast extract. What is used will be determined to a large extent by the casein hydrolysate available.

Glucose is frequently added to the medium used for enterotoxin production, but in most of the experiments conducted in the Food Research Institute enterotoxin production was not increased when it was present. Metzger et al. (1973) reported a 40% reduction in enterotoxin B production in the fermenter at a constant pH of 7 when glucose was eliminated from the medium.

Brain heart infusion (BHI) broth was promoted by Casman and Bennett (1963) for enterotoxin production, particularly for small volumes. It is primarily useful in testing staphylococcal strains for enterotoxigenicity because of the larger amount of enterotoxin that can be produced (Table 14.4). There appeared to be a variation in results among sources of the BHI for production of SEB (Reiser and Weiss, 1969), as well as within lots from the same source (Casman and Bennett, 1963). Because this medium is more readily available than casein digests, it is being used on a wider scale than other media for the production of small volumes with high concentrations of

<table>
<thead>
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<th>Table 14.3 Effect of medium on production of SEB*</th>
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<td><strong>Medium</strong></td>
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<td>NZA-A, 2%</td>
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<td>NZA-A, 4%</td>
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<tr>
<td>NZA-A, 4%, 0.1% K₂HPO₄</td>
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<td>PHP, 1% + NZA-A, 1%</td>
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<td>PHP, 3% + NAK, 3%</td>
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*a Incubation at 37 °C for 18 hours on gyrotry shaker (280 rpm), 50 ml in 250 ml Erlenmeyer flasks (Kato et al., 1966).

*b Supplemented with 10 µg/ml niacin and 0.5 µg/ml thiamin and adjusted to pH 6.0.
enterotoxin. Relatively large amounts can be obtained without supplementation with yeast extract; however, the addition of yeast extract does increase enterotoxin production (Bergdoll, unpublished data).

### 3.1.3 Temperature

The temperature range for enterotoxin production varies with the medium, but in general the minimum temperature for production is 10 °C and the maximum is 45 °C. The temperature for optimum production of enterotoxin is 35–40 °C, with 37 °C being the temperature generally used. There are variations with the different enterotoxins and with different media. Scheusner *et al.* (1973) showed that enterotoxins A–D were produced in BHI over a range of temperatures (19–39 °C). All but SEB were produced at 45 °C, but SEB was the only one produced at 13 °C; however, the SED-producing strain did not grow at this temperature. Vandenbosch *et al.* (1973) obtained maximal production of SEB (strain S-6) and SEC (strain FRI-137) in PHP-NAK medium at 40 °C with somewhat lesser amounts at 35 °C and 42 °C. Small amounts of toxin were produced at 15 °C and 45 °C, with none at 10 °C and 50 °C. Thota *et al.* (1973) obtained maximal SEE production at 40 °C. Pereira *et al.* (1982) produced maximum amounts of SEA and SEB in 4 % NAK medium by the sac culture method (Donnelly *et al.*, 1967) at 39.4 °C.

Several investigators have shown that enterotoxin production can occur in certain foods at temperatures as low as 10 °C (Genigeorgis *et al.*, 1969; Tatini, 1973) and as high as 40 °C (Fung, 1972). Normally growth is much slower at the lower temperatures, and since enterotoxin production is related to growth, a much longer period at these temperatures would be required before enterotoxin might be detectable.

### 3.1.4 pH values

Most strains of staphylococci will grow at pH values between 4.5 and 9.3, with the optimum being 7.0–7.5; however, the conditions for enterotoxin production are more restricted than for growth. For example, enterotoxin production is limited to pH 5.15–9.0 (Scheusner *et al.*, 1973), without any attempt to control the pH during fermentation. With the use of a fermenter, Metzger *et al.* (1973) produced the greatest

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**Table 14.4  Enterotoxin production methods (µg/ml)**

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<tr>
<th>Toxin</th>
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<sup>a</sup> 1, 3% PHP + 3% NAK medium; 2, BHI(1X); 3, BHI(2X) (Robbins *et al.*, 1974).

<sup>b</sup> The higher values were obtained with new Spectra/Por 1 molecular porous dialysis membrane with a molecular weight cut-off of 6000–8000; the lower values were with 1-year-old membrane.
amount of SEB when the pH was controlled at pH 7.0 (580 µg/ml), with much less enterotoxin produced at pH values of 6.0 (268 µg/ml) and 8.0 (32 µg/ml), after 10 hours of incubation using a 4% N-Z Amine A medium containing 1% yeast extract and 0.2% glucose. Carpenter and Silverman (1974) also found that the greatest amount of SEB was produced in a fermenter when the pH was held constant at 7.0. Metzger et al. (1973) obtained more SEB production when the pH was controlled than when it was not controlled (starting pH, 6.5) (580 µg/ml vs 440 µg/ml), although Carpenter and Silverman (1974) obtained no more SEB at a constant pH of 7.0 than when the pH was not controlled. Jarvis et al. (1973) obtained higher levels of SEA when the pH was controlled at 6.5 than under any other conditions tested.

Most experiments have been done by adjusting the medium to a specific pH value with no attempt to control the pH during incubation. Experiments at the Food Research Institute in which the starting pH was adjusted to 5.0–8.0 at 0.5 pH-unit intervals resulted in the maximum amount of enterotoxin being produced at initial pH values of 6.0 and 6.5. It has become normal practice to adjust the pH of the medium to 6.5 before fermentation. It would appear that controlling the pH is not generally necessary to obtain high enterotoxin production.

3.1.5 Methods of production

Several different methods have been used for the production of enterotoxin, with the choice usually depending on the purpose of production, the equipment available, and the ease of operation. Semisolid media (Casman and Bennett, 1963) and solid media (membrane-over-agar) (Hallander, 1965; Robbins et al., 1974) in Petri dishes are used for the production of small volumes of concentrated enterotoxin to identify enterotoxigenic strains (Table 14.4). More enterotoxin can be produced by these methods than when shake flasks are employed – up to 10 times as much for SEA, SED and SEE in the membrane-over-agar method. The increase for SEB and SEC is less than for the other enterotoxins (up to two-fold), but is of less concern because strains producing these enterotoxins normally produce much larger quantities than is the case with SEA, SED and SEE. Another method that is used in testing strains for enterotoxin production is the sac-culture technique of Donnelly et al. (1967) (Table 14.4). In this method, medium is placed in a dialysis tube that is positioned in the bottom of an Erlenmeyer flask to which is added about 20 ml of inoculum. Incubation is carried out for 24 hours, by shaking at a moderate speed, in order for the inoculum to make good contact with the sac. This method yields the largest amounts of SEB and SEC of any of the methods, and equivalent amounts of SEA, SED and SEE to those obtained with the membrane-over-agar method (Robbins et al., 1974). Other investigators have found this method superior to alternative methods for obtaining concentrated solutions of enterotoxin (Simkovicova and Gilbert, 1971; Untermann, 1972). The membrane-over-agar method was used in the Food Research Institute because of the relative simplicity of the method and adequate production of all enterotoxins for classification of strains as enterotoxigenic. One problem with the membrane-over-agar method is that not all types of membranes, such as cellophane used by Hallander (1965), give satisfactory results. The material found to be most satisfactory is Spectra/Por 1® molecularporous dialysis membrane.
with a molecular weight cut-off of 6000–8000 (Spectrum, Houston, TX, USA) (Robbins et al., 1974), which should be obtained fresh and stored in the refrigerator.

The production of enterotoxin for purification is normally carried out using much larger volumes of medium than can be obtained with the methods described above. Normally, large-scale production of enterotoxin is accomplished in Erlenmeyer or similar-type flasks with some method of shaking, or in fermenters. For the former method, flasks of different sizes and shapes have been used as well as different methods of shaking – such as reciprocal and gyrotory – with satisfactory results (Table 14.3) (Kato et al., 1966). The method used in the Food Research Institute to produce relatively large quantities of enterotoxin was the incubation of 400–600 ml of inoculated media in 2-liter Erlenmeyer flasks for 18–24 h at 37 °C on a gyrotory shaker at 280 rpm (Kato et al., 1966). This method can also be used to produce small volumes of more concentrated toxin by incubating 15 ml of medium in 250-ml Erlenmeyer flasks. The sac-culture method has been used for the production of higher concentrations of enterotoxin for purification, particularly for SED, by using larger sacs in larger Erlenmeyer flasks.

Production of enterotoxin in volumes greater than 10–15 l is best accomplished by deep culture aeration. Fermenters of various sizes, 0.5–2000 l, have been used for the production of enterotoxin. Metzger et al. (1973) reported high production of SEB (up to 600 µg/ml) in a 50-l volume of medium in a fermenter (Fermentation Design, Bethlehem, Pennsylvania) and up to 150 µg/ml of SEA (personal communication). Carpenter and Silverman (1974) produced equivalent amounts of SEB in 0.5-l of medium in a 1-l fermenter. Jarvis et al. (1973) were able to produce more SEA in 2 l of medium in a fermenter than in shake flasks, but somewhat less SEB and much less SEC in the fermenter. The aeration rate appeared to affect SEC production: the higher the rate, the less enterotoxin was produced. Carpenter and Silverman (1974) found that more SEB was produced at 10–50 % dissolved oxygen (DO) levels than at zero or 100 % levels. In the larger fermenters, such as that used by Metzger et al. (1973), it was impossible to raise the DO level above zero during active fermentation, but this appeared to have little effect on toxin production.

### 3.1.6 Characteristics of the enterotoxins

#### 3.1.6.1 Physicochemical characteristics

The enterotoxins are simple proteins that are hygroscopic and easily soluble in water and salt solutions, and have relatively low molecular weights of 25 000–29 000 Da. They are basic proteins with isoelectric points (pI) of 7.0–8.6, with the exception of SEG and SEH, which have pIs of 5.6 and 5.7 respectively. Different pIs for the same enterotoxin may be observed, which is due to the loss of amide groups (Chang and Dickie, 1971). The maximum absorbance of the SEs is 277 nm; the absorbance is higher than for normal proteins, primarily because of the high content of tyrosine.

One characteristic of the enterotoxins is the presence of two cysteine residues near the center of the molecule that are joined by a disulfide bond, forming what is referred to as the cystine loop (Bergdoll et al., 1974). The exceptions are SEI, one of the SEKs (described by Orwin et al.), and SEM, which lack a second cysteine...
normally present in the center of the enterotoxin molecule. The sequence and number of residues in the loop are not the same for the different enterotoxins. The loops for SEA and SEE are identical, and those for the different SECs are identical (Figure 14.1). The significance of the loops is not known. It is assumed that they stabilize the molecular structure, although the cystine molecule can be reduced and the -SH’s substituted so the –S–S–bond cannot be reestablished without neutralizing the emetic reaction (Dalidowicz et al., 1966). Two of the enterotoxins, SEB (Spero et al., 1973) and SEC₁ (Spero et al., 1976), can be nicked by trypsin in the cystine loop, again without neutralizing the emetic reaction. The compactness of the enterotoxin molecule is demonstrated by the fact that the SEB and SEC molecules can be split into two fragments by mild tryptic treatment without the two parts separating or the loss of the emetic reaction (Spero et al., 1973, 1976). Directly downstream from

Figure 14.1  Amino acid sequence alignment in the cystine loop region. Residues conserved in all the SEs with the cystine loop are shaded. Residues conserved in the majority of them are in bold.
the cystine loop a number of amino acid residues are conserved among all the enterotoxins (Figure 14.1). It has been suggested that this region is involved in the emetic activity of the enterotoxin molecule (Bergdoll, 1989).

There is significant nucleotide and amino acid sequence homology among the enterotoxins. The SECs are the most similar (96–98% amino acid identity), with only a three or four residue difference between them (Couch and Betley, 1989; Hovde et al., 1990); antibodies to each of the SECs react with all SECs (Reiser et al., 1984). SEB is most similar to the SECs; some antibodies produced against SEB cross-react with the SECs (Lee et al., 1980). SEA and SEE have a high degree of homology

![Figure 14.1—Cont’d](image-url)
(82 %) (Betley and Mekalanos, 1988; Couch et al., 1988); they have common antigenic sites in addition to specific sites (Lee et al., 1978), and are also closely related to SED and SEJ (Zhang et al., 1998). SEG shares 39 % and 38 % deduced amino acid sequence identity with SEB and SEC, respectively (Munson et al., 1998).

**Biological characteristics**

The enterotoxins are immunosuppressive (Smith and Johnson, 1975) and mitogenic (Peavy et al., 1970), and can stimulate production of interferon (Archer et al., 1979), interleukin-1 and -2 (Johnson and Magazine, 1988), and tumor necrosis factor.
A. Other activities were observed when the toxins were injected intravenously into rhesus monkeys (Beisel, 1972).

All the enterotoxins have been shown to have superantigenic activity (Dinges et al., 2000). The enterotoxins have been labeled superantigens because they can activate as many as 10% of the mouse’s T-cell repertoire, whereas conventional antigens stimulate less than 1% of all T cells (Hoffman, 1990). Although they are considered to be T-cell mitogens, the amount required for stimulation is several magnitudes lower than those of conventional T-cell mitogens, such as phytohemagglutinin and concanavalin A (White et al., 1989). They require antigen-presenting cells bearing major histocompatibility complex (MHC) class II molecules to stimulate T cells, but do not require preprocessing to peptides as conventional antigens do (Marrack and Kappler, 1990). The cystine loop must be intact for T-cell activation, as nicking the loop by mild trypsinic action negates SEB’s activity (Grossman et al., 1990). Also, the superantigens attach to the outer face of the MHC molecule rather than in the groove as the conventional antigens do (Marrack and Kappler, 1990). The T-cell receptor (TCR) is a heterodimer composed of α and β chains, which include variable portions Vα, Jα, Vβ, Dβ and Jβ. The T lymphocytes stimulated by the staphylococcal toxins are those that contain particular Vβ elements, regardless of other cell surface proteins. Thus, they can stimulate both CD4+ and CD8+ cells (Misfeldt, 1990). Stimulation of T cells can be blocked by antibodies to MHC class II molecules and antibodies to the appropriate Vβ element.

### 3.1.7 Stability of the enterotoxins

The staphylococcal enterotoxins are more stable in many respects than most proteins. In the active state, they are resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin and papain. Although pepsin can digest the enterotoxins at pH values of 2.0 and below (Bergdoll, 1970), this acidic level does not exist in the stomach under normal conditions, particularly in the presence of food. This makes it possible for the enterotoxins to pass through the stomach to the intestinal tract, where they stimulate the emetic and diarrheal actions.

The enterotoxins are not easily inactivated by heat. Initial studies revealed that their emetic activity was not completely destroyed after solutions of crude enterotoxin were

![Figure 14.1 — Cont’d](P588365-Ch14.qxd 24/10/05 7:03 PM Page 537)
boiled for 30 minutes and fed to human volunteers (Jordan et al., 1931) or injected intravenously into monkeys (Davison et al., 1938). Ordinarily the effect of heat on the enterotoxins is not of much concern, because the majority of staphylococcal food-poisoning outbreaks are due to human contamination of foods that are not heat treated after their preparation. If the foods were heated within an hour after preparation, any staphylococci would be destroyed before sufficient growth took place to produce enterotoxin.

Milk in the US has not been involved in food poisoning because it is kept cold until it can be pasteurized. The one food-poisoning outbreak that did occur from milk happened because the milk was inadvertently held at a warm temperature for several hours because of a problem with the pasteurizer (Evenson et al., 1988). Soo et al. (1974) reported that little loss of SEA and SED occurred in milk, skim milk or cream during pasteurization — that is, 72 °C for 15 seconds. Read and Bradshaw (1966) concluded that SEB in milk was affected very little by pasteurization. They also concluded that spray-drying processes used for milk would not inactivate the enterotoxin; spray-dried milk has been involved in several staphylococcal food-poisoning outbreaks. Cheese has been involved in food poisoning, but this was before pasteurizing milk was the norm.

It is unusual for commercially processed foods to be implicated in staphylococcal food poisoning, but a number of cases occurred in England from corned beef canned in Argentina, Brazil and Malta (Anonymous, 1979a, 1979b, 1979c). However, this was not due to the failure of the processing procedures, but to leaky seams or improperly sealed cans that allowed staphylococci to enter the cans during the cooling process. Sufficient growth of the staphylococci in the can occurred to produce enterotoxin before the corned beef was consumed. Several outbreaks in the US from mushrooms canned in China resulted in their importation being stopped (Anonymous, 1989). It was never satisfactorily determined how the mushrooms were contaminated, except that apparently some cans leaked. Although it was reported by the Food and Drug Administration laboratories that enterotoxin was present in mushrooms from several Chinese canneries (Anonymous, 1990), other scientists were unable to detect enterotoxin in the Chinese mushrooms they examined. How the mushrooms could have become contaminated before they were processed, or if they actually were, was never determined. One study indicated that staphylococci could grow during transportation in sealed plastic bags (Hardt-English et al., 1990); however, this was not confirmed (Brunner and Wong, 1992).

Although studies were done to show that heat did inactivate enterotoxins in buffer and different types of foods, this was done before sensitive techniques for detection of the enterotoxins were available (Denny et al., 1966, 1971; Humber et al., 1975). The major problem in determining the effect of heat on the enterotoxins is the relationship between the laboratory methods for detection of the enterotoxins and a suitable biological method. Normally if the enterotoxins are not detectable by methods employing specific antibodies they will be considered biologically inactive. The problem is with the biological methods, because the only reliable one is the oral administration of enterotoxin to monkeys. Although it compares to the ingestion of enterotoxin by humans, humans are several times more sensitive. The other biological method
frequently used is the intravenous injection of cats or kittens (Casman and Bennett, 1963; Denny and Bohrer, 1963; Hammon, 1941), but intravenous injection does not compare to oral administration. It is necessary to treat the sample with trypsin or pancreatin to destroy any interfering substances that can produce reactions similar to those of enterotoxin. The use of this method to test the biological activity of heated enterotoxin is inconclusive, especially if the heated sample is not treated with a proteolytic enzyme, as apparently was the case in the results reported by Bennett and Berry (1987). The heated enterotoxin would probably be destroyed in the stomach by pepsin, as was the case in the studies by Schwabe et al. (1990) when the heated enterotoxin was administered to monkeys. The fact that SEA heated at 121 °C lost its mitogenicity more rapidly than its serological activity indicated that biological activity may be lost before serological activity (Stelma et al., 1980). The studies of Bennett and Berry (1987) and Schwabe et al. (1990) showed that enterotoxins were inactivated, as determined by ELISA methods, by heating procedures normally used in commercial processing of foods.

Attention has been given to the possible renaturation of heated enterotoxin by treatment with urea. It is known that urea can partially unfold the enterotoxins, with refolding occurring when the urea is removed (Avena and Bergdoll, 1967); however, heat denaturation of proteins is more complicated than a mere unfolding of the molecule, and it has not been shown that treatment with urea can restore the molecule to its original structure. Only relatively small amounts of enterotoxin were detectable after urea extraction of heat-treated enterotoxin (Brunner and Wong, 1992; Bennett, 1994), whereas Akhtar et al. (1996) were unable to show any increase in recovery after urea treatment of heated enterotoxin.

All the information available indicates that enterotoxin in food is not easily inactivated by heat, and that the larger the amount present, the more heat is required to reduce the quantity to below detection levels. However, in general, the higher the temperature the more rapidly the enterotoxin is denatured, with the times and temperatures used in normal processing of canned food sufficient to destroy the quantity of enterotoxin usually present in foods involved in food-poisoning outbreaks (< 0.5–10 µg/100 g food). However, the processing procedures should not be depended upon to eliminate any hazards that may arise from mishandling the food at any stage during the preparation of the food for canning. No matter what the circumstances, there is no justification for allowing organisms to grow in foods with the possible production of deleterious substances, even though no one may be affected.

The effect of gamma irradiation on SEB and SEA has been reported. A dose of 50 kGy (cobalt-60 source) was required to reduce the concentration of SEB in 0.04-M Veronal buffer (pH 7.2) from 31 µg/ml to less than 0.7 µg/ml (Read and Bradshaw, 1967). In milk, a dose of 200 kGy was needed to reduce the concentration from 30 µg/ml to less than 0.5 µg/ml. These authors concluded that irradiation processes used for pasteurization or sterilization of foods would not inactivate the enterotoxin.

A dose of 8 kGy was insufficient to inactivate all of 111.1 ng/ml SEA (27–34% remained) in lean minced-beef slurries, although SEA was denatured in gelatin phosphate buffer (Rose et al., 1988; Modi et al., 1990). The more concentrated the beef slurries, the less SEA was denatured.
4 Nature of the intoxication in man and animals

4.1 Symptoms

The symptoms of staphylococcal food poisoning are quite characteristic for this illness in comparison to other foodborne diseases, with one possible exception. In 1974–1975 in England, symptoms similar to staphylococcal food poisoning were noted for outbreaks due to *Bacillus cereus* in fried rice. The onset of symptoms is quite rapid, usually 1–6 hours after the ingestion of food containing enterotoxin, with the average time in a large outbreak being 2–3 hours. Occasionally, symptoms will occur in less than 1 hour or later than 6 hours. The development of symptoms is determined by the amount of toxin consumed and the sensitivity of the individuals involved. The amount of toxin consumed in any given outbreak varies with the amount of food eaten and the distribution of the toxin within the food, which can vary considerably.

The most common symptoms are nausea, vomiting, retching, abdominal cramping and diarrhea. Vomiting is the symptom most frequently observed. In one typical outbreak involving a relatively large number of people, of 48 interviewed, 22 reported nausea, 33 cramps, 37 vomiting, and 34 diarrhea. Of these, 33 had both vomiting and diarrhea, 9 vomited without having diarrhea, and 6 had diarrhea without vomiting (Dennison, 1936). Only five had either nausea or cramps or both symptoms, but without vomiting or diarrhea. Three became ill in less than 2 hours, 39 in 2–5 hours, and 6 after 5 hours, with 1 in 7 hours and 1 in 8 hours.

In severe cases, headache, muscular cramping and marked prostration may occur. In the latter instances, fever may develop or the temperature may drop slightly. Normally there is no change in blood pressure, but in one severe case a dramatic drop in blood pressure was noted – from 120/80 to 60/40. In most cases recovery is rapid, occurring in a few hours to 1 day; the more severe the symptoms, the longer the recovery period. The mortality rate is extremely low but occasionally deaths do occur, usually involving the elderly or very young.

The variation in severity of staphylococcal food poisoning symptoms experienced by individuals suggests development of resistance to previous exposure to the enterotoxin, but there is no evidence to support this. One unreported attempt to check individuals involved in an outbreak for antibodies was unsuccessful.

4.2 Emetic dose

The amount of SE required to produce food poisoning in humans is difficult to determine. Reliable results from the examination of food implicated in food-poisoning outbreaks are difficult to obtain because normally the enterotoxin is not uniformly distributed in the food and it is impossible to know how much food any one individual consumed. A food-poisoning outbreak among school children following the consumption of chocolate milk provided the opportunity to obtain an estimate of the amount of enterotoxin required to cause illness. The quantity of milk consumed by a majority of the children who became ill was half a pint (~240 ml). The analysis of
12 half-pint cartons produced an average of 144 ng SEA/half-pint (94–184 ng SEA/half-pint). Although the SEA was not distributed uniformly in the milk, the minimum amount of SEA required to result in illness in sensitive individuals was not more than 184 ng (Evenson et al., 1988) (Table 14.5).

The emetic dose for rhesus monkeys is somewhat variable for the different SEs; SEA appears to be the most potent. Monkeys were the animals used at the Food Research Institute as they most closely resemble the human in their response to the SEs. The least amount of SEA that was observed to produce an emetic reaction when given intragastrically was 5 µg/3 kg monkey, with SEB, SEC and SEE requiring 10 µg and SED requiring 20 µg. Much smaller amounts were needed to produce an emetic action when the SE was given intravenously; 20 ng/kg monkey for SEA and SEC₃ (Bergdoll, 1989). The minimum amounts required for the other SEs were not determined.

4.3 Diagnosis

Any foodborne illness with the symptoms outlined here, particularly if it involves more than one person, is suspected of being staphylococcal food poisoning. A list of foods consumed at the previous meal or meals is needed to aid in the diagnosis, as there are certain foods that support the growth of staphylococci and are frequently involved in this type of illness. Any suspected food should be examined for the presence of staphylococci; if large numbers are present, it can be concluded with some degree of certainty that the illness is staphylococcal food poisoning. Additional information that can remove any doubt is whether the staphylococci are enterotoxigenic and/or whether enterotoxin can be detected in the suspected food. Although the latter is definite proof of the cause, often an insufficient quantity of food (10 g can be used, but larger amounts are better) is available for examination. The presence of enterotoxigenic staphylococci in the food is reasonable assurance that these organisms were the cause of the illness.

4.4 Incidence

The true incidence of staphylococcal food poisoning in the US, as well as in other countries, is unknown, because this illness is not a reportable disease in most countries.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ng per ml</th>
<th>ng per 1/2 pint (~240 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.40</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>0.73</td>
<td>172</td>
</tr>
<tr>
<td>3</td>
<td>0.48</td>
<td>113</td>
</tr>
<tr>
<td>4</td>
<td>0.63</td>
<td>149</td>
</tr>
<tr>
<td>5</td>
<td>0.78</td>
<td>184</td>
</tr>
<tr>
<td>6</td>
<td>0.65</td>
<td>153</td>
</tr>
<tr>
<td>Average</td>
<td>0.61</td>
<td>144</td>
</tr>
</tbody>
</table>

From Evenson et al. (1988).
The numbers of confirmed outbreaks reported in the US to the CDC from 1977 to 1997 are given in Table 14.1. Many additional outbreaks of food poisoning reported were probably due to staphylococcal food poisoning but not classified as such because insufficient information was given – such as the staphylococcal count. Over 40 outbreaks were reported in some years, with staphylococcal food poisoning being the leading bacterial foodborne disease. After 1982 no more than 14 confirmed outbreaks were reported per year, and less than 10 have been confirmed for several years.

Todd (1996) reviewed the worldwide surveillance of foodborne diseases between 1985 and 1989, looking at 17 countries. The highest incidence of staphylococcal foodborne disease per 107 population was 58.5 in Cuba, 9–15 in Hungary, Finland, Japan and Israel, and 0.4 in the United States. The largest number of outbreaks was 128 per year in Japan, compared to only 9.4 per year in the US. Denmark and the Netherlands reported no outbreaks.

In a later report of food poisoning in Korea and Japan (1971–1990; Lee et al. 1996), 14.9 % of outbreaks in Korea were due to staphylococcal food poisoning and 24.6 % of outbreaks in Japan were due to staphylococcal food poisoning. In Taiwan, 169 of 555 (30.5 %) foodborne disease outbreaks from 1986 to 1995 were due to staphylococcal food poisoning (Pan et al. 1997). The average number of outbreaks from 1988 to 1993 was 21.5 per year, with only 13 and 12 outbreaks reported in 1994 and 1995, respectively.

### 4.5 Prevention and control

The staphylococci are ubiquitous organisms that cannot be eliminated from our environment. At least 30–50 % of individuals carry these organisms in their nasal passages or throats, or on their hands. Any time a food is exposed to human handling, there is the possibility that the food will be contaminated with staphylococci. Not all of these may be enterotoxin producers, but 30–50 % may well be. Heating of the food after handling will normally assure against food poisoning unless the food has been held unrefrigerated for several hours before the heating; if enterotoxin has formed in the food, the heating might not be sufficient to destroy it. In many cases foods are not processed further after handling, and unless proper care is taken the organisms may grow and produce enterotoxin. The course of action recommended is to keep susceptible foods refrigerated at all times except when being prepared and while being served. Refrigeration should be carried out in such a manner as to facilitate quick cooling of the entire food mass. Most food-poisoning outbreaks could be prevented if this simple precaution were taken. To illustrate its importance, in one food-poisoning outbreak, cream-filled coffee cake that was kept unrefrigerated by one outlet caused a number of illnesses, while the same cake kept refrigerated by another outlet resulted in no illnesses. If it is impossible to guarantee that a susceptible food will be kept refrigerated, special care should be taken in its preparation to avoid contamination if at all possible. Bryan (1968, 1976a) has reviewed the subject and listed helpful hints for those responsible for handling foods.
5 Prevalence of *S. aureus* in foods

Most meat is contaminated with staphylococci, but normally this is not of concern because the organisms do not usually multiply rapidly on raw meat and they are destroyed in the cooking process. Staphylococci that are present on the meat before processing are rarely involved in food-poisoning outbreaks. In the case of ham involvement in food poisoning, the causative organism is a result of post-processing contamination. One exception is fermented sausage – particularly sausages prepared without sufficient heating to destroy the staphylococci. It has been demonstrated by laboratory experiments that sufficient growth of the staphylococci can occur and produce enterotoxin before the fermenting organisms produce enough acid to inhibit the staphylococcal growth (Bergdoll, unreported results). In fact, outbreaks from this type of fermented sausage (Genoa) did occur, due primarily to inadequate fermentation conditions. The contamination of raw meats is probably from multiple sources, including human handlers. Examination of the fermented sausage revealed the presence of several different strains of staphylococci.

Several surveys have been conducted on different types of foods in the marketplace in different countries. Normally foods were found to be contaminated with staphylococci, particularly if they had been handled by humans. However, staphylococci would not grow sufficiently on many of the foods to produce enterotoxin, and cooking before consumption would destroy the organisms. In a majority of staphylococcal food-poisoning outbreaks, the foods were contaminated during their preparation for eating and mishandled after the preparation. One example is the cream-filled coffee cake mentioned above.

6 Foods most often associated with Staphylococcal food poisoning

Any food that provides a good medium for the growth of staphylococci may be involved in this type of foodborne illness. In the US, pork – particularly baked ham – is the food most frequently involved in outbreaks; poultry, salads (meat, potato, etc.) and cream-filled bakery goods are responsible for many of the remaining outbreaks. The frequency of the involvement of baked ham may be for a number of reasons. It is a common food item for picnics, some of which involve large numbers of people. In the latter cases, particularly, it is very difficult to keep the ham properly refrigerated until the food is consumed. Refrigeration in shallow pans is necessary to prevent the growth of any staphylococci that may be present. The warmer summertime temperatures complicate the situation. Another factor is that some people believe that cooked food is safe and do not realize the necessity for adequate refrigeration. Cream-filled bakery goods can pose a particular problem in the summer if they are not stored under adequate refrigeration from the time of preparation until they are consumed. This is much less of a problem in the US now than it was a few years ago (Bryan, 1976b).
The foods involved in other countries vary with the diet as well as the local conditions. In Japan, rice balls are a common item taken on picnics and outings, and once were the major item involved in staphylococcal food poisoning. Rice is an excellent growth medium for staphylococci. The balls used to be prepared by hand and were not usually refrigerated, but now they are made by machine to avoid human handling. In some of the European countries, such as Poland and the former Czechoslovakia, ice cream made by small producers is a cause of this type of foodborne illness. In Brazil, the two foods involved most frequently are cream-filled cake and a white cheese frequently produced on the farm or in small establishments.

7 Principles of detection of \textit{S. aureus}

No specific test may be useful in every case to isolate the staphylococci from the wide variety of foods in which they are found. As a result, attempts have been made to find a combination of selective and enrichment media that will support the growth of the staphylococci and at the same time suppress the growth of other microflora present that tend to overgrow the staphylococci. A three-tube isolation procedure using trypticase soy broth with 10% sodium chloride and 1% sodium pyruvate was accepted as the official method for recovery of the largest numbers of coagulase-positive staphylococci from the widest variety of foods (Lancette and Lanier, 1987); however, thermally stressed cells of \textit{S. aureus} are unable to grow in the medium. As a result, food samples likely to contain a small population of injured cells were incubated in double-strength trypticase soy broth before the addition of 13% NaCl and spread-plating on Baird-Parker agar (Lancette and Tatini, 1992). For detecting small numbers of \textit{S. aureus} in raw food ingredients and non-processed foods expected to contain large numbers of competing organisms, incubation is in trypticase soy broth containing 10% NaCl and 1% sodium pyruvate before transferring to Baird-Parker agar plates. For detecting relatively large numbers of staphylococci, the food extract is plated directly on Baird-Parker agar.

Typical colonies of \textit{S. aureus} on Baird-Parker agar are circular, smooth, convex, moist, ~1.5 mm in diameter on uncrowded plates, gray-black to jet-black, smooth with entire margins and off-white edges, and may show an opaque zone with a clear halo extending beyond it. Normally those colonies that appear to be \textit{S. aureus} will be counted, and one or more of each type tested for coagulase and TNase production. However, the upgrading of biotypes E and F to \textit{S. intermedius} and \textit{S. hyicus} complicates the species classification because all three species can be coagulase- and TNase-positive. If at least one test is positive and the food being examined is from a food-poisoning outbreak, the staphylococci are probably \textit{S. aureus} from human contamination. An additional positive anaerobic mannitol fermentation test will confirm \textit{S. aureus}. The number of colonies on the triplicate plates represented by the \textit{S. aureus} positive colonies is multiplied by the dilution factor, and the result reported as the number of \textit{S. aureus} per gram of food.

Agglutination kits employing the clumping factor, protein A, and specific antigens of \textit{S. aureus} are available for identification of \textit{S. aureus} strains. However, these kits
are designed primarily for use in the clinical field where large numbers of staphylococci are being examined and where large numbers of coagulase-negative species are also encountered (Personne et al., 1997; Wilkerson et al., 1997). The clumping factor test is not satisfactory because *S. intermedius* and some coagulase-negative species can be positive for this factor.

An alternative method has been proposed by Roberson et al. (1992) in which P agar supplemented with acriflavin and the β-galactosidase test are used. Of the coagulase-positive species, only *S. aureus* will grow on the supplemented P agar and is negative with the β-galactosidase test. *S. intermedius* does not grow on the modified P agar and is 100% positive with the β-galactosidase test, whereas *S. hyicus* is negative by both tests. This method is useful if the staphylococci being tested are from sources other than clinical.

Another method that has been proposed to identify *S. aureus* from non-clinical sources employs an immunoenzymatic assay using a monoclonal antibody prepared against endo-β-acetyl-glucosaminidase – an enzyme produced by all isolates of this species. Comparison of this method with six kits available for identification of *S. aureus* has shown it to be specific for *S. aureus*, whereas the kits were positive for *S. intermedius*, *S. schleiferi*, and *S. lugdunensis* (Guardati et al., 1993).

### 8 Principles of detection of the enterotoxins

#### 8.1 Introduction

It was not possible to develop specific methods for the detection of the enterotoxins before Bergdoll et al. (1959a) identified and purified the first enterotoxin. Until that time, the only means of detecting the presence of the enterotoxins was by the use of animals that gave emetic reactions to the toxin, either intragastrically or intravenously. Fortunately, at the time Surgalla and Bergdoll began their research to identify the enterotoxin, immunological methods were being developed for the specific detection of individual proteins. These investigators were able to show that specific antibodies could be produced to the enterotoxin when the emetic reaction in monkeys was neutralized by antisera produced against the crude toxin (Bergdoll et al., 1959b; Surgalla et al., 1954). Subsequently, all laboratory methods for the enterotoxins have been based on the use of specific antibodies to each of the enterotoxins for their detection, because it is almost impossible to detect individual proteins by chemical methods.

#### 8.2 Biological methods

Before the first enterotoxin was purified, many types of animals (such as pigs, dogs, cats and kittens, and monkeys) were tested in the search for an inexpensive specific test method. All of these animals, with the exception of monkeys, were relatively insensitive to the enterotoxins, unless the toxin was injected intraperitoneally or intravenously. Emesis is the most readily observable reaction to
enterotoxin; hence animals without a vomiting mechanism, such as rodents, were of little value as test subjects.

The feeding of young monkeys (Surgalla et al., 1953) – preferably rhesus although cynomologus monkeys have been used successfully – provides the most reliable bioassay for enterotoxins because, of the biologically active substances produced by the staphylococci, only enterotoxins cause emesis when administered by the oral route. Assays are performed by administering solutions of the enterotoxins (up to 50 ml) to monkeys (2–3 kg) by catheter. The animals are observed for 5 hours for emesis. A response in at least two animals is accepted as a positive reaction. Other investigators have used the production of diarrhea as well as emesis as a criterion for positive reactions (Schantz et al., 1965), as this occurs in humans suffering from staphylococcal food poisoning about as frequently as emesis. The sensitivity of the monkey-feeding test was increased by feeding 20-fold concentrates of bacterial culture supernatants. This made it possible to show that strains once thought to be non-enterotoxigenic did produce enterotoxin. The cost of monkeys, the expense of their upkeep and the difficulty in getting approval for the use of animals in research has limited their use for routine testing. One additional drawback is that the animals become resistant to the enterotoxin after several feedings.

The intravenous injection of cats and kittens (Hammon, 1941) also proved useful for the detection of the enterotoxins. When materials other than the purified enterotoxins are injected intravenously, it is necessary to inactivate any interfering substances by treatment with trypsin (Denny and Bohrer, 1963) or pancreatin (Casman and Bennett, 1963). Cats are not as reliable as monkeys because they are subject to non-specific reactions. More recent studies have suggested that the Asian house shrew (Suncus murinus) could be used as an animal model. Hu et al. (1999) showed that the 50 % emetic dose of SEA by oral and intraperitoneal administration was 32 µg and 3 µg per kg body weight, respectively.

Because antibodies are specific for each enterotoxin, it is necessary to continue the use of animal testing until each new enterotoxin has been purified and antibodies produced against it. Animal testing is also necessary for assessing the effect of various treatments, such as heat, on the enterotoxins.

### 8.3 Immunological methods

The most specific and sensitive tests for the enterotoxins are based on their reactions with specific antibodies. The first tests developed were based on the reaction of the enterotoxin with the specific antibodies in gels to give a precipitin reaction. These were the only laboratory methods available until radioimmunoassay (RIA) was applied, and later the enzyme-linked immunosorbent assay (ELISA) and the reversed passive latex agglutination (RPLA) method were developed. The gel-diffusion methods have been used primarily for the detection of enterotoxin production by staphylococcal strains, although the RPLA method is used for testing strains for low production of enterotoxin. The RIA method was used for testing for enterotoxin in foods until the ELISA and RPLA were available.
8.4 Detection of enterotoxigenic strains

8.4.1 Gel-diffusion methods

Many types of gel reactions have been used in the detection of the enterotoxins, the most common ones being some form of either the Ouchterlony gel plate or the microslide (Ouchterlony, 1949, 1953; Crowle, 1958). A modification of the Ouchterlony gel-plate test used in the Food Research Institute for detection of enterotoxin-producing staphylococcal strains, and recommended to others, is the optimum sensitivity plate (OSP) method (Robbins et al., 1974). It is easy to use and, in conjunction with production of the enterotoxins by the membrane-over-agar method (Robbins et al., 1974) or the sac-culture method (Donnelly et al., 1967), is adequate in sensitivity to detect most enterotoxigenic staphylococci. The normal sensitivity is 0.5 µg/ml, but this can be increased to 0.1 µg/ml by a five-fold concentration of the staphylococcal culture supernatant fluids.

The microslide method is the most sensitive of the gel-diffusion methods (0.05–0.1 µg/ml), but care is needed in preparing the slides (Casman et al., 1969); even so, the results are often difficult to interpret. For example, Gibbs et al. (1978) reported that a high percentage of staphylococcal strains isolated from poultry produced SEA as determined by the microslide method. These strains were examined in the Bergdoll laboratory and found to be negative for SEA. Careful examination of the microslides produced in the Gibbs et al. laboratory showed a line of impurity that was incorrectly observed to form a line of identity with the SEA control precipitin line. Many things can go wrong with this method, and experience is very important in using it successfully (Casman et al., 1969). A sensitivity of 30 ng/ml has been reported for this method, but this is exceptional as most operators are unable to achieve a sensitivity of greater than 100 ng/ml.

Although monoclonal antibodies have been developed for the enterotoxins (Thompson et al., 1985), they cannot be used in gels because their reactions with the enterotoxins do not result in the formation of precipitates, even when a mixture of monoclonals is used.

8.4.2 The RPLA method

Through the application of the RPLA method to the detection of enterotoxin production by staphylococci it was possible to detect low-producing strains (approximately 10–20 ng/ml) that were not detectable by the OSP gel-diffusion method (Igarashi et al., 1986; Bergdoll, 1990; Table 14.6). Some staphylococcal strains that were implicated in food poisoning by the monkey feeding test but negative by OSP were positive by ELISA (Table 14.7; Kokan and Bergdoll, 1987). The production of 10–20 ng of enterotoxin/ml is probably of significance, because only 100–200 ng of enterotoxin A was shown to be necessary to produce food poisoning (Evenson et al., 1988), with the amount present in the vehicle (2 % chocolate milk) being 0.40–0.78 ng/ml (Table 14.5). The OSP method can be used as a preliminary method for the testing of strains, as only a small percentage of strains are low enterotoxin producers.
Since DNA sequence information is available for all the described SEs, DNA probes (Notermans et al., 1988; Ewald et al., 1990; Neill et al., 1990; Jaulhac et al., 1992) and PCR (Johnson et al., 1991; Wilson et al., 1991, 1994; Tsen and Chen, 1992; McLauchlin et al., 2000) are commonly employed for detection of the SE genes. Several reports have described the development of multiplex PCR for the detection of multiple SE genes (Becker et al., 1998; Mehrotra et al., 2000; Martin et al., 2003). Sharma et al. (2000) were able to detect five enterotoxin genes, sea–see, in a single PCR reaction in 3–4 hours. PCR is highly sensitive and specific, and allows the detection of enterotoxigenic staphylococci in a relatively short time with little sample preparation. One advantage of the method is that dead enterotoxigenic cells can also be detected, which is important in the analysis of heated foods.

### Table 14.6 Staphylococcal strain testing by RPLA method

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enterotoxins detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>188</td>
<td>SEA, SED</td>
</tr>
<tr>
<td>228</td>
<td>SEA, SED</td>
</tr>
<tr>
<td>311</td>
<td>SEA</td>
</tr>
<tr>
<td>365</td>
<td>SEC</td>
</tr>
<tr>
<td>452</td>
<td>SEA</td>
</tr>
<tr>
<td>581</td>
<td>SEC</td>
</tr>
<tr>
<td>609</td>
<td>SEA, SED</td>
</tr>
<tr>
<td>754</td>
<td>SEA, SEB</td>
</tr>
<tr>
<td>802</td>
<td>SEB</td>
</tr>
<tr>
<td>887</td>
<td>SEA</td>
</tr>
<tr>
<td>896</td>
<td>SEA, SEB</td>
</tr>
<tr>
<td>965</td>
<td>SEA, SED</td>
</tr>
</tbody>
</table>


* a Strains were received from Dr James K. Todd, The Children’s Hospital, Denver, CO.

### Table 14.7 S. aureus strains negative for enterotoxin by OSP

<table>
<thead>
<tr>
<th>Source</th>
<th>Monkey positive</th>
<th>ELISA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food poisoning</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>Foods</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Fish (raw)</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Nares (human)</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Nares (horse)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

* OSP, optimum sensitivity plate (Kokan and Bergdoll, 1987).

8.4.3 Polymerase chain reaction (PCR) and DNA hybridization
8.5 Detection in foods

The detection of enterotoxin in foods requires methods that are sensitive to less than 1 ng/g of food. The quantity of enterotoxin present in foods involved in food-poisoning outbreaks may vary from less than 1 ng/g to greater than 50 ng/g. Although little difficulty is usually encountered in detecting the enterotoxin in foods involved in food-poisoning outbreaks, outbreaks do occur in which the amount of enterotoxin is less than 1 ng/g – such as the case of the 2% chocolate milk. In such instances, the enterotoxin can be detected only by the most sensitive methods. Another situation in which it is essential to use a very sensitive method is in determining the safety of a food for consumption, where it is necessary to use the most sensitive methods available in order to show that no enterotoxin is present.

8.5.1 The ELISA method

ELISA methods were applied to the detection of the enterotoxins in foods soon after they were originally developed for the detection of other proteins. The most common type of ELISA is the sandwich method, in which the antibody is reacted with the unknown sample before the antibody-enterotoxin complex is treated with the enzyme-antibody conjugate (Saunders and Bartlett, 1977). This format is preferred because the amount of enzyme, and thus the color developed from the enzyme-substrate reaction, is directly proportional to the amount of enterotoxin present in the sample. This eliminates the need for highly purified enterotoxins, as crude or only partially purified enterotoxin is needed for preparation of a standard curve.

The majority of users of ELISA methods employ microtiter plates or strips to which the antibodies are attached. The large number of wells in a microtiter plate provide for analyzing several samples at one time. A plate reader is useful for recording the results, which adds to the expense of the method, although in many cases the test can be read visually. One commercial kit that uses microtiter strips is the RIDASCREEN®, developed in Germany by Kraatz-Wadsack et al. (1991). The polyclonal antibodies were prepared in sheep in the Institute of Milk Hygiene, University of Munich, Germany. A collaborative study conducted in Canada indicated that the specificity, sensitivity, repeatability and reproducibility met food safety criteria (Park et al., 1996a).

An alternate procedure has been developed; this is the use of polystyrene balls to which the antibodies are attached (Stiffler-Rosenberg and Fey, 1978; Freed et al., 1982; Fey and Pfister, 1983). The ball method is more cumbersome because each ball must be handled separately. The main advantage is that a relatively large volume of the unknown sample can be used, thus increasing the amount of enterotoxin adsorbed per sample. This makes possible the use of 1-ml volumes of substrate so that the color developed can be read in a simple colorimeter, an instrument that most laboratories have available. The sensitivities of the ELISA methods are between 0.5 and 1.0 ng/g of food. One kit that employs the ELISA ball method was developed in Switzerland by Fey and Pfister (1983) and produced by Diagnostische Laboratorien, Bern, Switzerland. Those who used it have found it to be a very good method for detecting enterotoxin in foods (Wieneke and Gilbert, 1987). Although the test could
be completed in a single day, the recommendation was that the antibody-coated balls be shaken with 20 ml of food extract overnight to obtain the highest sensitivity. The method could be used quantitatively, although this is not necessary in checking foods for the presence of enterotoxins. This method was found to be superior when compared to other methods (Wieneke and Gilbert, 1987; Wienecke, 1991). Unfortunately this kit is no longer available.

A third method is based on a fluorimetric reaction with a sensitivity of less than 1 ng/ml (Armstrong et al., 1993). This method is produced and marketed by bioMérieux Vitek, Inc. (Hazelwood, Missouri) as VIDAS® (Vitek ImmunoDiagnostic Assay System) S.E.T. An automated detection system is used with this assay.

A dip-stick method was developed by IGEN, Inc., with monoclonal antibodies developed at the Food Research Institute, and licensed to Transia, Transia-Difchamb, Lyon, France. The antibodies to each of the enterotoxins were adsorbed onto nitrocellulose paper attached to wells in plastic sticks. There was some problem with the SEC antibodies, as only one monoclonal antibody for SEC was available and the second antibody was a polyclonal antibody. One report indicated that false-positive reactions were obtained for SEC in some food samples (Wieneke, 1991). This method was very easy to apply, as only one test was needed to check for all of the enterotoxins and it could be done in 1 day. It did not prove to be as sensitive as the other methods (Wieneke, 1991).

Many investigators have developed ELISA methods for the detection of the staphylococcal enterotoxins with slight differences in procedures. One difference involved the use of biotinylated antibodies and avidin-alkaline phosphatase instead of coupling the enzyme directly to the antibodies (Hahn et al., 1986; Edwin, 1989). The biotinylated antibodies apparently were more stable than the enzyme antibodies. A sensitivity of 0.1 ng/ml was possible.

### 8.5.2 The RPLA method

An RPLA kit also is available commercially for use in the detection of enterotoxins in foods. It is produced by Denka Seiken Co. Ltd., Niigata, Japan. The method is adequately sensitive for the detection of enterotoxin in most foods that are implicated in food-poisoning outbreaks (Igarashi et al., 1985); however, it may be inadequate for detection of the small amounts of enterotoxin that are sometimes present. This was indicated when Wieneke and Gilbert (1987) compared the RPLA method to ELISA methods for the detection of enterotoxin in foods. The RPLA method was adequate in all but two tests; in one case the sensitivity was inadequate, and in the other case the food extract gave a non-specific agglutination.

### 8.5.3 Screening methods

In some instances it may not be necessary to determine the type of enterotoxin if enterotoxin is present; for example, in examining the marketability of suspect foods. The food would not be marketable if any enterotoxin were present. For this purpose, including all of the enterotoxins in one test saves time. However, this would not save time in examining foods implicated in food-poisoning outbreaks, as identification of the type of enterotoxin is valuable in tracing the source of the contamination.
Two kits that include all of the enterotoxins in one test are available commercially. One kit utilizes small tubes coated with monoclonal antibodies to all of the enterotoxins (Transia, Transia-Diffchamb, Lyon, France). The method is sensitive to 0.2 ng/ml of extract; a bright blue color is developed with this concentration of enterotoxins. Although this is a screening test, if the enterotoxins were present at these low levels it would be difficult to analyze for individual enterotoxins because the other methods are less sensitive. If more than one enterotoxin were present, it would be necessary to concentrate the extract. An international collaborative study using this method has been reported in which acceptable results were obtained by the collaborators and were validated by the French Normalization Agency for identification of staphylococcal enterotoxins in foods and culture fluids (Lapeyre et al., 1996).

The second kit, TECRA®, is produced by Bioenterprises Pty Ltd., Roseville, New South Wales, Australia. It utilizes microtiter plates and has a sensitivity of at least 1 ng of SEA/g of ham (Park et al., 1996b). Although the sensitivity is adequate for most foods involved in food-poisoning outbreaks, it is doubtful that the SEA in the chocolate milk outbreak would have been detectable. This is critical, particularly in the case of testing suspect foods whose marketability is contingent on the absence of enterotoxin. Two collaborative studies have been conducted utilizing the TECRA kit, one by Bennett and McClure. (1994) in the US for the purpose of establishing it as an official method, and one by Park et al. (1996b) in Canada. Unfortunately, the minimum amount of enterotoxin included in any of the foods was 4 ng/g of food in the Bennett and McClure study and 1 ng/g of food in the Park et al. study. Although all collaborators were successful in detecting these minimum amounts, they were not tested for detecting 0.5 ng/g – the minimum amount that should be detectable by any acceptable method, as was pointed out by Bergdoll (1994). Despite this shortcoming, the Bennett and McClure collaborative study was accepted by the Official Methods Committee as an official method for detection of enterotoxin in foods.

9 Summary

The staphylococci, isolated in the 1800s as specific organisms, were identified as the cause of human infections that included food poisoning. It was not until the 1900s that the cause of food poisoning was identified as a toxin produced by the staphylococci growing in foods. This toxin was given the name enterotoxin because of its effect on the intestinal tract. The staphylococci produce several enterotoxins (SEA, B, C, D, E, G, H, I, J, K, L, M) that are related proteins, with varying degrees of sequence homology. Although the staphylococci produce other toxic substances that may be involved in human infections, the emphasis of this chapter is on the enterotoxins and their involvement in food poisoning. Staphylococcal food poisoning occurs worldwide, but has declined in the developed countries, such as the US and England. Many cases may not be reported because the illness is relatively mild and short-lived, with no sequelae. The enterotoxins are low molecular weight, single-chain proteins, with a cystine loop in the center of the molecule. They are identified by specific antibodies, which are the basis of the detection methods. The gel-diffusion
methods in which a precipitin reaction occurs between the enterotoxin and its specific antibody are used for detecting enterotoxin production by staphylococcal strains. The majority of the methods used for detection of enterotoxins in foods are some form of the enzyme-linked immunosorbent assay (ELISA), with a sensitivity of less than 1 ng/g of food.

Bibliography