Serological detection of enterotoxin in foods implicated in staphylococcal food poisoning

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SUMMARY

Two methods are described for the extraction of enterotoxin from foods incriminated in incidents of staphylococcal food poisoning. Enterotoxin was detected serologically in 12 of 24 food samples from 20 separate incidents: eight samples contained enterotoxin A, three contained D and one both A and B. The amount of enterotoxin in nine foods, based on 100 % recovery, varied from 0·02 to 0·09 μg./g.

Data are also given on the numbers of *Staphylococcus aureus* isolated from samples of food from 39 food poisoning incidents. Colony counts varied between $7·5 \times 10^5$ and 9×10^9/g. with a median value of 7×10^7/g.

INTRODUCTION

In the past the laboratory investigation of staphylococcal food poisoning has been concerned with the isolation of *Staphylococcus aureus* from suspected foods, from faecal and vomit specimens, and from the hands and nose of suspected food handlers; phage-typing has enabled the strains to be correlated. Since the preparation of purified enterotoxins and specific antisera it has been possible to determine the type of enterotoxin produced by the strains isolated (Casman, Bennett, Dorsey & Issa, 1967; Šimkovičová & Gilbert, 1971). Although the results from such tests provide good evidence that staphylococcal enterotoxin caused an outbreak, the demonstration of enterotoxin in the suspected food itself is even better proof. Such detection is especially important when cooking or other treatment has killed the organisms but left the enterotoxin still active; also, in some foods, e.g. cheese, the organisms may have died during storage.

The demonstration of enterotoxin in food using the monkey feeding test or even the intraperitoneal kitten test is impracticable for routine procedures owing to the cost involved and, furthermore, they are not sufficiently sensitive to detect the amount of enterotoxin ordinarily encountered in foods from outbreaks (Bergdoll, 1969). The kitten test, in particular, has been criticized by various workers (Fulton, 1943; Casman *et al.* 1967; Bergdoll, 1970). However, various workers in

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the U.S.A. (Casman & Bennett, 1965; Hall, Angelotti & Lewis, 1965; Casman, 1967; Zehren & Zehren, 1968) have described *in vitro* tests for detecting enterotoxin in food.

This paper describes our experience with an *in vitro* technique for the detection of staphylococcal enterotoxin in food.

MATERIALS AND METHODS

Foods and cultures

Twenty-four food samples from 20 separate food poisoning incidents in England and Wales, where there was good clinical and bacteriological evidence that staphylococcal food poisoning had occurred, were received during 1969–71. Cultures of *Staph. aureus* from victims were also submitted on most occasions.

The foods used in experiments where measured amounts of enterotoxin were added were ham from a freshly opened can, cooked, peeled prawns and freshly cooked chicken.

Control enterotoxins and antisera

Enterotoxins A, B, C and D, all partially purified, and their specific antisera were supplied by Professor M. S. Bergdoll (A, B and C) and Dr E. P. Casman (D). The enterotoxins and antisera of A, B and C were diluted in 0·02 M phosphate buffer in saline, pH 7·4, containing 0·02 % thiomersal, and those of D were diluted in Difco Brain-Heart Infusion (BHI) broth in saline. Towards the end of the work 10 % of BHI broth was added to the diluents used for enterotoxins A, B and C and their antisera to increase the sensitivity of the gel-diffusion test (Casman, Bennett, Dorsey & Stone, 1969).

Enumeration of Staph. aureus

Counts of *Staph. aureus* were made on phenolphthalein diphosphate agar containing polymyxin (PPAP) (Hobbs, Kendall & Gilbert, 1968) using a modified Miles & Misra (1938) technique with incubation for 48 hr. at 37° C. Selected colonies were tested for coagulase production in 10 % plasma broth.

Information on counts of *Staph. aureus* in foods from a further 19 food poisoning incidents were obtained from Hobbs (1955) and from unpublished data from several Public Health Laboratories. Various selective media including PPAP had been used for counts of *Staph. aureus*.

Production of enterotoxin by cultures of Staph. aureus

Cultures were examined for their ability to produce enterotoxins A, B, C and D. Enterotoxin was produced by means of a sac-culture technique and detected serologically by a slide gel double-diffusion method (Šimkovičová & Gilbert, 1971).

Extraction of enterotoxin from food

Method 1. The food, usually 50 g. was homogenized in an Atomix beaker and then rehomogenized at half speed with 200 ml. of 0·2 M-NaCl. The slurry was centrifuged at 2000 *g* for 30 min. and the supernatant retained. The sediment was

re-extracted with 100 ml. of 0·2 M-NaCl and centrifuged. The two extracts were pooled and then vigorously shaken with at least two separate one-fourth volumes of chloroform: the chloroform layers were drawn off and discarded. The extract was reduced to a volume of about 20 ml. by dialysis against 30 % (w/w) polyethylene glycol (PEG) Carbowax 20 M, (Union Carbide Co. Ltd., Hythe, Southampton) and centrifuged at 40,000 *g* for 1 hr. at 4° C. The supernatant fluid was concentrated by dialysis against PEG to approximately 0·4 – 1 ml. and examined for the presence of enterotoxin by gel-diffusion.

Method 2 (modified from Casman, 1967). The food, usually 50 g., was homogenized and extracted with 200 ml. of 0·02 M phosphate buffer in saline, pH 7·4. The pH was checked and adjusted to about pH 7·0–7·4. The slurry was centrifuged at 2000 *g* for 30 min. and the supernatant retained. The sediment was re-extracted with 100 ml. of buffer-saline and centrifuged. The two extracts were pooled and after vigorous shaking with chloroform the chloroform layer was discarded and the extract concentrated to dryness in PEG. The residue was taken up in 20 ml. of 0·01 M phosphate buffer, pH 7·4, and shaken again with chloroform. After removing the chloroform layer the extract was diluted with 40 vol. of 0·005 M phosphate buffer, pH 5·7. The pH was checked and if necessary adjusted to pH 5·7. The diluted extract was allowed to percolate at room temperature through a column of 1 g. of Whatman carboxymethylcellulose (CM 32, H. Reeve Angel and Co. Ltd., London, E.C. 4) equilibrated at pH 5·7 in 0·005 M phosphate buffer. After washing the column with the same buffer, any toxin adsorbed was eluted with 100 ml. of 0·2 M phosphate buffer, pH 7·4. The eluate was concentrated to dryness in PEG, re-suspended in 0·2 ml. of 0·02 M phosphate buffer in saline, pH 7·4, containing 10 % BHI broth and 0·02 % thiomersal, and examined for the presence of enterotoxin by gel-diffusion.

Slide gel double-diffusion test for enterotoxin

Slight modifications were made to the method described by Šimkovičová & Gilbert (1971). Two test areas were prepared on an agar-coated slide between three parallel double layers of tape set 2 cm. apart. Each area was filled with 0·2 ml. of an agarose-phosphate buffer-saline mixture and covered with a Perspex matrix with four funnel-shaped wells surrounding a fifth central well. Three of the outer wells were filled with dilutions of food extract or culture filtrate, the fourth outer well with reference enterotoxin and the central well with antiserum of the corresponding type. Slides were incubated in moist chambers at room temperature for 2 days (culture filtrates) or 5 days (food extracts). The staining procedure used to enhance precipitin lines was also modified. Slides were immersed in 0·1 % thiazine red in 1 % glacial acetic acid for 1–2 min (food extracts) or 10 min (culture filtrates). The presence of one or more of the enterotoxins A, B, C and D in a culture filtrate or food extract was verified by the coalescence of its precipitation line with the reference line of the corresponding type.

An optimal precipitin line mid-way between the two reactant wells required 2·5 μg./ml. of enterotoxins A, B or C and dilutions of 1/25 of antiserum A, or 1/75 of antisera B and C: a faint precipitin line was formed with 0·6 μg./ml. of toxin. The

49   HYG 70

reaction for enterotoxin D was optimal with 1/20 dilutions of toxin and antiserum.

When BHI broth was included in the diluent a control line required 1 μg./ml. of enterotoxins A, B, or C and 1/50 dilution of antiserum A, or 1/100 dilutions of antisera B and C: a faint precipitin line was formed with 0·25 μg./ml. of toxin.

Recovery of enterotoxin from foods

Measured amounts of enterotoxin were added to separate 50 g. samples of canned ham, cooked prawns and cooked chicken. Method 1 was used to extract enterotoxins A (20 μg.), B (100 μg.) and C (20 μg.) from ham and prawns, and enterotoxins A (15 μg.), B (75 μg.) and C (15 μg.) from samples of chicken. Method 2 was used to extract enterotoxin A from samples of chicken (0·25 and 1 μg.) and prawns (0·25, 1, 5 and 10 μg.).

RESULTS

Recovery of enterotoxin added to foods

The estimated recovery of enterotoxins A, B or C from samples of ham, chicken and prawns, using method 1 for the extraction, varied between 20 and 55 % of the quantity added with a mean value of 33 %. When method 2 was used for extracting enterotoxin A from samples of chicken and prawns the estimated recovery was 20 %. Method 2 was more satisfactory than method 1 for the quantitative estimation in food because the final concentrated extract contained less non-specific components. As a result precipitin lines in gel-diffusion tests were more distinct with less non-specific precipitation.

Detection of enterotoxin in foods implicated in food poisoning

Two methods were used for the extraction of enterotoxin from foods implicated in food poisoning. Enterotoxin was detected in nine of 17 foods using method 1 for the extraction of toxin, and in three of seven foods from the more recent outbreaks using method 2: enterotoxins A, D and A and B together were detected in foods from eight, three and one incidents respectively (Table 1). In each instance *Staph. aureus* was present in large numbers, from 5×10^6 to 2×10^9/g. of food, and the enterotoxin present was the same as that produced by growth of the organisms in sac-cultures: strains isolated from patients also produced the same enterotoxin. In one outbreak, however, strains isolated from the food and from patients produced both enterotoxins C and D, but only enterotoxin D was detected in the food. Subsequent tests have shown that strains from three of the incidents, where enterotoxin A was detected in the food, also produce enterotoxin D. The amount of enterotoxin A or A and B in nine foods, based on 100 % recovery, varied from 0·02 to 0·09 μg./g.

Enterotoxin was not detected in 12 foods. Cultures from eight of these foods and from specimens of patients produced enterotoxins A or C. However, in two incidents where *Staph. aureus* was isolated at concentrations of 2×10^6/g. and from 9×10^6 to 9×10^9/g. (3 foods) none of the cultures produced enterotoxins A, B, C or D.

Table 1. *Detection of enterotoxin in foods implicated in staphylococcal food poisoning in England and Wales* (1969–1971)

Extraction method used (see text)	Incident	No. of persons ill / No. at risk	Place	Food	Count of Staph. aureus/g.	Enterotoxin in food Type	Enterotoxin in food Amount*(μg./g.)
1	1	1/1	Home	Prawns (canned)	40×10^6	A and B	A – 0·03 B – 0·03
	2	30/120	Dog show	Cold chicken	55×10^6	A	0·02
	3	21/350	Mental hospital	Cold chicken	150×10^6	A	0·04
	4	21/82	Convalescent home	Trifle	150×10^6	A	0·05
	5	38/48	Coach outing	Tongue and beef	200×10^6	A	0·04
	6	4/4	Hotel	Ham	250×10^6	A	0·05
	7	35/35	Coach outing	Ham	450×10^6	A	0·08
	8	2/2	Home	Cold chicken	200×10^6	D	†
	9	50/?	Canteen	Ham	1000×10^6	D	†
2	10	4/?	Restaurant	Prawns‡ (frozen)	6×10^6	A	0·02
	11	2/2	Home	Prawns (canned)	5×10^6	A	0·09
	12	7/7	Home	Veal, ham and egg pie	2000×10^6	D	†

* Based on 100% recovery.

† Not calculated because concentration of reference enterotoxin unknown.

‡ After thawing, the prawns were eaten several hours later in the form of a prawn cocktail.

49-2

Table 2. *Counts of* Staphylococcus aureus *in foods implicated in* 39
incidents of staphylococcal food poisoning in England and Wales

	Count of *Staph. aureus*/g.			
	$7 \cdot 5 \times 10^5 - 9 \cdot 9 \times 10^6$	$10^7 - 9 \cdot 9 \times 10^7$	$10^8 - 9 \cdot 9 \times 10^8$	$> 10^9$
No. of incidents	8	15	9	7
% of incidents	21	38	23	18

Counts of Staphylococcus aureus implicated in food poisoning

Table 2 summarizes the plate counts of *Staph. aureus* in foods implicated in 39 incidents where there was good clinical and bacteriological evidence that staphylococcal food poisoning had occurred. Counts varied between $7 \cdot 5 \times 10^5$ and 9×10^9/g. with a median value of 7×10^7/g.

DISCUSSION

Enterotoxin was detected in 12 foods from 20 incidents of staphylococcal food poisoning. However, the technique used on most occasions (method 1) to extract the toxin was not satisfactory because the final extract contained various soluble constituents of the food which interfered with the gel-diffusion test and was often viscid. Method 2 was more satisfactory in both respects and the final product could be concentrated to a smaller volume. Although method 2 is now used routinely in this laboratory for the extraction of enterotoxin from food a negative result in the gel-diffusion test cannot be interpreted as 'absence of enterotoxin'.

Most food samples submitted from outbreaks weighed *ca.* 50–60 g. However, in one incident where enterotoxin A was detected in the food (Table 1, incident 11) the sample tested weighed only 17 g.

Although human volunteers have been used for the detection of enterotoxin in food and in culture filtrates of *Staph. aureus*, little has been published on the amount of enterotoxin required to cause illness in man. However, such volunteer experiments have now been carried out in the U.S.A. and they indicate that the estimated illness dose (ID50) for adults fed highly purified enterotoxins A, B or C is between $0 \cdot 14$ and $0 \cdot 19$ μg./kg. (Dr D. A. Kautter, personal communication); thus for a man weighing 70 kg. the ID50 is about 10–13 μg. These results are similar to those of Raj & Bergdoll (1969) who reported that a dose of 20–25 μg. of pure enterotoxin B caused typical symptoms of staphylococcal food poisoning in three volunteers. In contrast, Bergdoll (1969, 1970) has reported that 1 μg. of enterotoxin A or less may cause illness in sensitive individuals. By assuming a direct correlation between growth and enterotoxin production and by disregarding any differences in abilities of culture media and certain foods to support the production of enterotoxin, Casman & Bennett (1965) estimated that the concentration of enterotoxin A in food from various outbreaks was from 0·01 to 0·4 μg./g.: thus if 100 g. of food was consumed the dose was from 1 to 4 μg. There is a wide variation in the sensi-

tivity of individuals to staphylococcal enterotoxin (Dack, 1956). Consideration of all the evidence available suggests that 1 μg. or less of enterotoxin may cause illness in sensitive individuals.

In the present work the total amount of enterotoxin A or A and B was estimated to be from 0·02 to 0·09 μg./g. of food. If we assume that 50–100 g. of food was consumed and that no enterotoxin was produced in the time-interval between consumption of the food and testing for toxin in the Food Hygiene Laboratory, then the dosage was from 1 to 9 μg. In all the incidents recorded in Table 1 the victims suffered symptoms typical of staphylococcal food poisoning, and several were admitted to hospital. In one incident (Table 1, no. 2), fourteen persons were admitted five of whom required intravenous fluid therapy.

Recent reports indicate that the biosynthesis of enterotoxin A may be quite distinct from that of enterotoxin B. Enterotoxin A production occurs mainly during the exponential phase of growth (Markus & Silverman, 1970). In contrast, 95 % of enterotoxin B is synthesized and released during the late exponential and early stationary phases of growth (Markus & Silverman, 1969; Morse, Mah & Dobrogosz, 1969). These results may be one of the reasons why enterotoxin B is implicated infrequently in staphylococcal food poisoning.

Except for data by Hobbs (1955) and Casman & Bennett (1965), little information has been published on the numbers of staphylococci present in foods implicated in outbreaks of staphylococcal food poisoning. The results shown in Table 2 indicate that large numbers of staphylococci, usually > 1 million/g., must be present before there is a sufficient concentration of enterotoxin to cause symptoms. During the time-interval between consumption of contaminated food and investigation of the incident, the numbers of staphylococci in the food may have increased or decreased. Nevertheless, we believe that the counts obtained are of importance as well as the phage-typing results and will continue to be so until procedures for the detection of staphylococcal enterotoxin in food are simplified.

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