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Rapid, Sensitive Assay for Staphylococcal Enterotoxin and a Comparison of Serological Methods

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Reversed passive hemagglutination was used to assay enterotoxin in culture filtrates and in food samples. With cells tanned and then sensitized with antitoxin globulin and preserved with either formaldehyde or pyruvic aldehyde, as little as 0.0007 μg of enterotoxin was detectable. The results of hemagglutination tests compared well with those obtained by quantitative precipitin tests or by immunodiffusion, but hemagglutination was 50 to 100 times more sensitive than the immunodiffusion technique. In addition, results of the hemagglutination test were available within a few hours, and neither elimination of interfering proteins from food extracts nor concentration of the sample, both of which are necessary for immunodiffusion, was required for this procedure.

The purification of staphylococcal enterotoxin has resulted in the recognition of at least four serological types (1, 5, 6) and the development of serological tests for detection and assay. Methods for immunodiffusion in agar, quantitative precipitin assays, hemagglutination (HA)-inhibition, and immunofluorescence have been described for this purpose (8, 9, 11, 15, 17, 21, 23). Measurement of enterotoxin in food samples by immunofluorescence, a micro Ouchterlony technique, and the single diffusion technique of Oudin have been described, as have the procedures necessary to eliminate nonspecific proteins and salts that influence the results (4, 12, 13, 14, 19, 20). HA-inhibition, passive HA, and the quantitative precipitin test have not been used with food samples. It seemed that these procedures would be less influenced by the presence of the nonspecific substances that affect immunodiffusion; thus, the lengthy procedures described for processing food extracts could be eliminated (4, 14). In addition, HA should permit the detection of smaller amounts of enterotoxin than do the other methods without the necessity of concentrating the food extracts.

HA of sheep erythrocytes, to which enterotoxin B was attached by means of bis-diazotized benzidine, has been used in our laboratory for several years to estimate serum antibody titers. Johnson et al. (15) used such cells in an inhibition test to measure enterotoxin in culture filtrates. Cook (7) described a procedure that he termed "reversed passive hemagglutination" for the assay of tetanus toxin. Antitoxin globulin attached directly to erythrocytes permitted direct measurement of toxin by HA rather than by a two-step procedure such as HA-inhibition. Sinitsyn (22) used a similar technique for detecting botulinal toxin. We describe here the use of "reversed passive hemagglutination" for the assay of enterotoxin B in culture filtrates and in food samples to which toxin was added. The report also compares the data obtained by this procedure with those obtained by immunodiffusion and the quantitative precipitin technique.

MATERIALS AND METHODS

Formalin-preserved sheep erythrocytes (SRBC) were obtained from Difco. They were washed once with 0.038 M $NaHSO_3$ in 0.85% saline and then twice with saline. We observed previously (Bacteriol. Proc., 1966, p. 43) that formaldehyde decreases the serological activity of enterotoxin B; therefore, the initial washing with $NaHSO_3$ was included to neutralize free aldehyde. Fresh SRBC, collected in an equal volume of modified Alsever's solution (3), were treated with pyruvic aldehyde as described by Ling (16).

Antienterotoxin globulin was prepared from rabbit antiserum by saturation with $(NH_4)_2SO_4$ to 50%. The precipitate was washed several times with 50% saturated $(NH_4)_2SO_4$, dialyzed against 0.15 M borate-buffered saline (*p*H 8.4) until free of SO_4^{2-} ions, and then freeze-dried and stored at 4 C. Approximately 90% of the antibody was recovered. Rabbits were immunized as described previously (21). Recent data have indicated that initial injections of toxoid in Freund's adjuvant into the foot pad, followed after 3 to 4 weeks by intravenous injections of alum-adsorbed toxoid and the alum-adsorbed toxin, produce high-

titered antitoxin in a shorter time interval than with the original procedure.

Cultures of *Staphylococcus aureus* strain S6, grown for 8 to 24 hr in 2.0% Protein Hydrolysate Powder (Mead Johnson & Co., Evansville, Ind.), were filtered through ultrafine sintered-glass filters to provide sterile filtrates (10). Before immunodiffusion assays, they were dialyzed against 0.02 M phosphate-buffered saline (PBS, *p*H 7.3). Purified enterotoxin B was obtained from E. J. Schantz, Fort Detrick. Frozen cream, custard, meat, and tuna pies were thawed and the pastry portion discarded. The fillings were homogenized in a Waring Blendor with an equal amount of distilled water (v/v), and culture filtrate containing known quantities of enterotoxin B was added. The homogenates were further diluted with equal volumes of 0.02 M PBS (*p*H 6.4) and were heated at 50 C for 15 min. After standing for 45 min at room temperature, they were centrifuged at about 15,000 × *g* for 20 min in a Servall SS-1 centrifuge at 4 C. The clear liquid portion between the sediment and the top fat layer was removed with a syringe having an 18-gauge needle and then was filtered through a single layer of paper towel (Scott Wipers 590, Scott Paper Co., Chester, Pa.) (14). Cheese and milk were prepared essentially as described by Read et al. (19, 20). The culture filtrate containing enterotoxin was added to the homogenized cheese or directly to the milk before separation of the whey. Homogenized food samples were also inoculated with a suspension of either of two strains of *S. aureus* (one a stock culture, S6; the other, A13, a recent isolate obtained from a food-poisoning incident). The suspensions were prepared from the growth on nutrient agar slants incubated at 37 C for 8 hr. For HA tests, serial dilutions of the food extracts were prepared in PBS (*p*H 7.3) containing 1.0% normal rabbit serum (NRS).

SRBC were tanned and sensitized as described by Boyden (2). Antitoxin globulin diluted to contain 50.0 μg of antibody nitrogen per ml was used to sensitize a 2.5% suspension of SRBC or a 1.0% suspension of SRBC for use in microtiter tests.

In ancillary studies not detailed here, we observed that burro antitoxin globulin gave less satisfactory results than did rabbit antitoxin globulin; therefore, the latter was used in these studies. The concentration of globulin required for optimal fixation onto tanned SRBC was determined by titrating known quantities of toxin with SRBC treated with varying concentrations of globulin. The dilution selected, 1:8, was slightly in excess of the minimal concentration required for maximal agglutination with the antiserum globulin used. Bis-diazotized benzidine (BDB), already used to couple enterotoxin to SRBC, was also investigated, but we failed to attain optimal conditions for conjugation of SRBC with globulin using BDB.

In the tube tests, 0.5-ml amounts of serial 1:2 dilutions of the enterotoxin samples were distributed into a series of tubes, and 0.05 ml of SRBC treated with globulin antitoxin was added. After standing at room temperature for 2 hr, the degree of agglutination was determined from the pattern of sedimented cells. For the microtiter test, serial dilutions of the enterotoxin sample were prepared in the wells of plastic dishes with microtiter loops; 0.025 ml of 1.0% SRBC was added to 0.025 ml of dilution, and the tests were read after 2 hr at room temperature. Toxin concentrations in the samples were calculated by multiplying the reciprocal of the greatest dilution of sample that reacted by the smallest amount of purified standard enterotoxin that gave a positive result in the control test. With the antiserum used for these studies, 0.0015 μg of enterotoxin B per ml was generally the smallest amount that caused distinct HA.

The quantitative precipitin tests were performed as described previously (8). After incubation at 37 C for 3 to 4 hr and at 4 C for 4 days, the precipitates were washed and then dissolved in 0.25 N acetic acid; the optical density was determined at $\lambda = 277$ mμ. The concentration of enterotoxin in the sample was determined from a standard curve relating optical density of the dissolved precipitate to the concentration of enterotoxin reacting with the antibody. The antiserum supernatant fluid was tested for excess antigen and excess antibody by double immunodiffusion. In samples with more than 10.0 μg of enterotoxin per test, incubation at 37 C for 6 hr appeared to be sufficient for complete precipitation; thus, assay of culture filtrates could be completed easily within 8 hr.

Immunodiffusion tests were performed by layering the sample on a mixture of equal volumes of 1.0% agar and a suitable concentration of antitoxin. The tests were placed in a 30 C water bath. The distance of the leading margin of the precipitate band from the agar meniscus was measured with a cathetometer three times at approximately 24-hr intervals. The distance of band movement was plotted against the square root of time to obtain the slope *k*. The concentration of toxin was determined from a standard curve relating the value of *k* to the concentration of enterotoxin. The standard curve was prepared from the results obtained with known concentrations of purified enterotoxin B diluted in brain heart infusion broth diluted 1:1 with 0.02 M phosphate buffer in saline, *p*H 7.3 (21).

RESULTS

Table 1 shows a portion of the data obtained when culture filtrates were analyzed for enterotoxin by the procedures studied. In most cases, exceptionally good agreement was obtained with the various techniques. Microtiter results (not shown), obtained after the filtrates were stored at −20 C for about 3 months, corresponded well with the results of the tests performed earlier by the other procedures. In general, titers obtained by the microtiter technique were twofold (one dilution) less than those obtained with the tube test. No cross-reactions were obtained with antigens in culture filtrates formed by either staphylococci that produced type A enterotoxin or nontoxigenic strains.

Assays for enterotoxin added to various food samples are presented in Tables 2 and 3. The data

in Table 2 were obtained with frozen foods that had been thawed but not cooked before homogenization and the addition of enterotoxin; Table 3 shows results obtained with cooked products. It was difficult to clarify the liquid separated from the homogenates of the uncooked tuna or custard pies so that it was not possible to determine the results of the precipitin test of the custard by the procedure used. Again, good agreement was obtained with the different tests. The food homogenates (cheese, chicken, tuna, and banana cream pies) that were inoculated with *S. aureus* contained from 0.31 to 3.1 μg of enterotoxin B per ml produced by the stock culture S6. The recently isolated strain (A13) produced from 0.06 to 0.31 μg of enterotoxin B per ml of food slurry. Because the HA tests are semiquantitative, only a difference greater than fourfold (two tubes) was considered to be significant. No HA reactions were obtained when the samples were incubated with tanned unsensitized cells, and food homogenates did not react with sensitized SRBC in the absence of enterotoxin.

TABLE 1. *Comparison of serological tests for detection of enterotoxin B in staphylococcal culture filtrates*

Filtrate	Enterotoxin B (μg/ml)			
	Immunodiffusion (Oudin)	Hemagglutination[a]		QPT[b]
		HCHO	CH_3COCHO	
WZ14	100.0	99.2	198.0	93.7
WZ19	26.0	19.8	19.8	28.5
WZ27	39.0	19.8	19.8	39.7
Old P2	49.0	19.8	39.7	46.0
WZ21	59.0	39.7	39.7	55.5
Old P3	57.0	39.7	39.7	48.6
P1	<4.0	4.9	4.9	11.0
WZ7	54.0	99.2	99.2	55.9
Old P1	52.0	39.7	39.7	46.0
17-5	40.0	19.8	39.7	36.0

[a] Hemagglutination with sheep red blood cells preserved with formaldehyde (HCHO) or pyruvic aldehyde (CH_3COCHO), tanned, and then sensitized with antitoxin globulin.

[b] QPT = quantitative precipitin test.

TABLE 2. *Comparison of serological tests for determination of enterotoxin in food samples*

Sample[a]	Entertoxin B (μg/ml)		
	Hemagglutination (HCHO-SRBC)[c]	QPT[b]	Immunodiffusion (Oudin)
Tuna pie	3.8	7.1	5.5
Cheddar cheese	7.7	6.8	7.7
Milk	7.7	4.9	4.9
Beef pie	7.7	4.9	5.1
Coconut cream pie	3.8	—[d]	5.5

[a] Frozen pies were thawed but not cooked. All food was homogenized with an equal volume of distilled water and 1 part staphylococcal culture filtrate, containing 31.5 μg of enterotoxin per ml, added to 4 parts of homogenate so that each food sample contained 6.3 μg enterotoxin B per ml.

[b] QPT = quantitative precipitin test.

[c] Sheep red blood cells preserved with formaldehyde.

[d] Acetic acid solution of washed specific precipitate was cloudy, possibly due to starch in the filling.

TABLE 3. *Assay of enterotoxin B in cooked food homogenates*

Food	Enterotoxin added (μg/ml)	Enterotoxin measured (μg/ml)			
		Hemagglutination[a]		QPT[b]	Immunodiffusion
		HCHO	CH_3COCHO		
Cheese	10.0	5.0	10.0	6.9	8.2
	0.1	0.1	0.2		
	0.01	0.01	0.02		
Chicken pie	10.0	10.0	10.0	7.1	6.7
	0.1	0.1	0.2		
	0.01	0.01	0.02		
Banana cream pie	10.0	5.0	10.0	7.4	8.2
	0.1	0.1	0.2		
	0.01	0.01	0.01		
Tuna pie	10.0	5.0	10.0	8.9	5.8
	0.1	0.1	0.2		
	0.01	0.01	0.02		
Control (enterotoxin B)	10.0			6.1	8.8

[a] Hemagglutination tests with sheep red blood cells preserved with formaldehyde (HCHO) or pyruvic aldehyde (CH_3COCHO), tanned, and then sensitized with antitoxin globulin.

[b] QPT = quantitative precipitin tests. The enterotoxin was added to the samples after the frozen foods had been cooked according to the manufacturer's directions.

DISCUSSION

The data presented indicate that reliable, simple serological methods for enterotoxin assay are available. HA is a more sensitive pro-

cedure for detection of small amounts of enterotoxin than are the immunodiffusion procedures described by others (4, 13, 14). The mean end point for about 30 HA tests with purified enterotoxin was 0.0015 μg per ml (or 0.0007 μg, the actual amount in the 0.5-ml volume of the test). Casman and Bennett (4) reported a sensitivity of 0.1 μg for the micro immunodiffusion technique, whereas Hall et al. (13) were able to detect 0.05 μg by the Oakley immunodiffusion procedure (18).

The sensitivity of HA is a disadvantage when relatively high concentrations of enterotoxin are present, e.g., in culture filtrates. The long series of dilutions necessary to obtain an end point introduces considerable error. Furthermore, in calculating the concentration, the inordinately large value of the dilution factor can also introduce considerable error. On the other hand, the sensitivity and simplicity of the procedure is of extreme value for estimating small concentrations of toxin, such as might be found in food samples obtained from a food-poisoning event. When materials containing higher concentrations of enterotoxin (e.g., culture filtrates) are to be assayed, greater accuracy can be obtained by preparing the initial dilutions in volumetric flasks with analytical techniques and precautions.

No significant differences were observed in tests performed with SRBC preserved with the two aldehydes. Formaldehyde seems to be the reagent of choice because cells treated with this substance appeared to store better at 4 C than did cells treated with pyruvic aldehyde.

Occasional HA tests gave results that were in disagreement with those obtained by the other procedures. Examination of the data indicated that the cause of this disagreement was the variation of the end point for the standard toxin from the mean of 0.0015 μg/ml. With some titrations, the smallest amount of purified toxin that caused agglutination was only 0.1 of this value. However, the results obtained with the unknown samples were in agreement with those obtained by immunodiffusion or by precipitation when, for the calculation of the toxin in unknown samples, the mean value for sensitivity of the globulin (0.0015 μg) was used rather than the actual value obtained with the standard enterotoxin sample tested simultaneously with the unknowns. Furthermore, if the smallest amount of toxin in the unknown that produced HA was calculated on the basis of toxin concentration as determined by other procedures (e.g., immunodiffusion), it was close to the value, 0.0015 μg/ml, which was the end point for the antitoxin globulin used in these experiments. The explanation for this disagreement can probably be traced to deterioration of the standard toxin preparations. Stock toxin solutions containing 1.0 μg/ml were dispensed in small volumes and stored at −20 C. Occasional vials seemed to give aberrant results. Precautionary steps have been introduced to avoid this. Standard toxins are now stored in more concentrated solutions (25 or 50 μg/ml), and the actual concentration is confirmed by immunodiffusion or precipitin tests. The results of any HA tests with the standard solution that are significantly different from the mean value of 0.0015 μg/ml are regarded as unreliable and the titration is repeated with a fresh standard solution.

The agreement obtained with the three tests indicates that all of them are reliable procedures. The choice of test to be used for investigative purposes is, in part, dependent upon the speed and accuracy required. When high concentrations of enterotoxin are to be assayed, quantitative precipitin assay appears to be the procedure of choice. Accurate assays have been obtained within an 8-hr day when a serum containing sufficient antibody for rapid precipitation was used. In this case, the samples were kept at 37 C for 4 to 6 hr before the precipitates were washed, dissolved, and assayed. The standard curve was prepared under identical conditions. Immunodiffusion by the Oudin technique has also been useful for samples containing from 5 to 200 μg of toxin per ml. However, longer incubation is necessary and, in the case of such samples as food extracts, elimination of interfering salts and proteins is necessary. For low concentrations, "reversed passive hemagglutination" is the method of choice. A screening technique for preliminary assay could be devised by sensitizing the red blood cells with antitoxin globulin prepared against the various serological types of enterotoxin as these become available.

Preliminary studies on the storage of tanned, formaldehyde- or pyruvic aldehyde-preserved SRBC sensitized with antitoxin indicated that SRBC preserved with formaldehyde were satisfactory for at least 4 months; the pyruvic aldehyde-preserved cells became unsatisfactory in about 1 month. The cell suspensions were stored at 4 or −20 C and were tested at intervals. A few studies indicated that tanned, sensitized formaldehyde-treated cells were also satisfactory when freeze-dried and reconstituted as described by Cook (7).

Formaldehyde-treated SRBC sensitized with antitoxin were tested against several lots of purified enterotoxin and a number of staphylococcal culture filtrates. The end points of these titrations (0.001 to 0.0002 μg) were not significantly differ-

ent. They do indicate, however, the variations inherent in a test based on serial dilutions.

"Reversed passive hemagglutination" is a sensitive procedure for the detection of staphylococcal enterotoxin B. With the reagents and procedures currently in use, 0.0015 μg/ml or the absolute amount of 0.0007 μg of toxin can be detected. This method is more rapid than the immunodiffusion procedures currently in use, and, because of its sensitivity, does not require prior concentration of the sample. Other proteins and constituents present in filtrates or food extracts do not appear to interfere. However, the sample should be near neutrality in reaction and, if necessary, should be dialyzed to remove excess salts.

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