Comparative Evaluation of Different Enzyme-Linked Immunosorbent Assay Systems for the Detection of Staphylococcal Enterotoxins A, B, C, and D

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We compared four versions of the enzyme-linked immunosorbent assay for their suitability for detecting staphylococcal enterotoxins. The sandwich with labeled antibody proved to be the best. We used it with a sorbent consisting of antibody-coated polystyrene spheres reacted with 20 ml of food extract. The sensitivity of the test was 0.1 ng of enterotoxin per ml, which is far below clinical relevance. The succinimidyl-pyridyl-dithio-propionate enzyme coupling method of Pharmacia was superior to the two-step glutaraldehyde technique. Interfering protein A was eliminated by the simple addition of normal rabbit serum to the extracts. A diagnostic kit is now available.

There is an urgent need for a reliable and sensitive method for diagnosing staphylococcal food poisoning. This type of intoxication is very frequent; 45% of all food-borne disease outbreaks in the United States in 1971 were due to staphylococcal food poisoning (4). Enterotoxins A and D occurred most commonly (6, 31). However, relatively few laboratories are capable of diagnosing the causative toxin(s) in food extracts or culture supernatants. The microslide precipitation test is used if any test is, but the antisera for it are expensive and not readily available.

It is known that less than 1 μg of enterotoxin per 100 g of food is sufficient for inducing clinical symptoms (31). Reiser et al. (31) even emphasize that one should be able to detect 125 to 250 ng of staphylococcal enterotoxin per 100 g of food, and Freed et al. (18) state that the minimum amount of enterotoxin required for the development of food poisoning is considered to be 100 ng.

The radioimmunosorbent assays (5, 9, 21, 26, 30, 32, 33) fulfill these requirements but have many well-known disadvantages. The enzyme-linked immunosorbent assay (ELISA) is equally sensitive and is gaining increasing credit in this field. Saunders and Clinard (35) and Saunders and Bartlett (34) were the first to use the ELISA for the detection of staphylococcal enterotoxin A (SEA). In 1977, Simon and Terplan (36) described a competitive system for staphylococcal enterotoxin B (SEB) with a sensitivity of 1 to 0.1 ng/ml, and they were followed in the same year by Fey and Stiffler-Rosenberg (15). In 1978, Stiffler-Rosenberg and Fey (37) replaced this assay with a triple sphere test with polystyrene spheres. This test was positive with 0.1 ng of SEB, or staphylococcal enterotoxin C (SEC). Thereafter, several authors applied the ELISA to the detection of staphylococcal enterotoxins (3, 12, 16–18, 22–25, 27–29). After 7 years of experience in detecting staphylococcal enterotoxin by ELISA, we decided to test four different modifications of this test for their sensitivity and applicability with the intention of choosing the best one for a publicly available diagnostic kit. We also compared two conjugation methods of rabbit anti-immunoglobulin G (IgG) with phosphatase by using glutaraldehyde and succinimidyl-pyridyl-dithio-propionate (SPDP).

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MATERIALS AND METHODS

Reference strains, toxins, and antisera were obtained from M. Bergdoll (Food Research Institute, Madison, Wis.), to whom we are very much indebted. The strains used were as follows: SEA, *Staphylococcus aureus* 722; SEB, *S. aureus* 243; SEC₁, *S. aureus* 1 ATCC 19095; staphylococcal enterotoxin D (SED), *S. aureus* 1151m; non-enterotoxinogenic strain, *S. aureus* FRI184.

Our toxins were purified by a combination of cationic exchange with CM-cellulose and chromatofocusing (SEA, SEB, and SEC) or by isoelectric focusing in the flat-bed gel (C. Müller, Phil. Nat. Lic. thesis, University of Bern, Bern, Switzerland, 1981) as described by Fey et al. (12, 16). These methods will be published in detail in the near future. The enterotoxins were coupled with phosphatase by the method of Stiffler-Rosenberg and Fey (37).

For the immunization of rabbits we followed a schedule given to us by G. Terplan, Munich (37). Rabbit IgG was isolated with protein A (SPA)-Sepharose (20).

IgG-phosphatase conjugates were prepared by the two-step glutaralydehyde coupling method of Avrameas et al. (2) and the heterobifunctional agent SPDP (Pharmacia, Uppsala, Sweden), which was used according to the instructions of the manufacturer. Phosphatase (alkaline type VII-s no. P-5521) was purchased from Sigma Chemical Co., St. Louis, Mo.

The conjugates were evaluated in two ways. (i) Polystyrene tubes (Petraplastic, Chur, Switzerland; 12 by 54 mm) were coated with 1 ml of enterotoxin (2 μg/ml; 0.1 M PO₄, pH 8) overnight at room temperature. After three washings with NaCl–Tween 20 (0.05%), dilutions of the conjugate were reacted for 6 h at room temperature. (ii) Tubes were coated with antibody IgG (2 μg/ml; 0.1 M carbonate-bicarbonate buffer, pH 9.6) for 6 h at room temperature. After washing, 100 ng of enterotoxin in 1 ml of phosphate-buffered saline–Tween was added and left at room temperature overnight. Again, dilutions of the conjugates were reacted for 6 h.

The substrate was *p*-nitrophenyl phosphate (Sigma; 1 mg/ml; 0.1 M sodium carbonate-bicarbonate buffer plus 1 mM MgCl₂, pH 9.8). The reaction was stopped with 0.1 ml

TABLE 1. Comparative conjugation of antibody IgG[a]

Reagent used for coupling with SPDP	Mol wt ($\times 10^3$)	Protein (mg)	SPDP excess		Reduction
			Fold	μmol	
IgG	160	4	3.5	0.0875	50 mmol/liter
Phosphatase	100	2.5	25	0.625	of DTT[b]

[a] The two-step glutaraldehyde system and the Pharmacia SPDP procedure were used. IgG and phosphatase were used in equimolar amounts (0.025 μmol). It was not possible to use more than a 3.5-fold excess of SPDP for rabbit IgG because of precipitation.

[b] DTT, Dithiothreitol.

of 2 M NaOH. Measuring was done at 403 nm with a VITATRON photometer linked to Texas Instruments programmable calculator TI 59 as described by Fey and Gottstein (14). The Sigma capsules of p-nitrophenyl phosphate proved to be superior to the bottled powder of E. Merck AG (Darmstadt, Federal Republic of Germany) in giving a stable, colorless substrate without any yellowish tint.

The conjugates were used without fractioning by gel filtration. They were stored with 50% glycerol at −20°C. The strength of the conjugate was calculated as (micrograms of antibody IgG used)/(volume of the conjugate × working dilution) and was expressed as micrograms of IgG per ml of working dilution, assuming no loss of IgG. The working dilution was the dilution of the conjugate yielding an optical density at 405 nm (OD_{405}) of 0.8 to 1.0 in 60 min. The antigens used were crude culture supernatants. With the reference toxins of Bergdoll and with our antisera, we measured the toxin content of the supernatants by a Mancini test in which we first optimized the antiserum dilution by checkerboard titration. The supernatants were mixed with 5 to 10% normal rabbit serum for complexing SPA, centrifuged 20 min at 10,000 rpm, and thereafter used as laboratory standards. They were stored at −20°C.

As sorbents we used (i) polystyrene plastic tubes; (ii) polystyrene spheres 6 mm in diameter (Precision Plastic Ball Inc., Chicago, Ill.); (iii) polystyrene microtiter plates (Microelisa M 129A; Dynatech, Kloten, Switzerland); (iv) Protapol disks, which are made of isothiocyanate-substituted Teflon (ICI, Melbourne, Australia) as used by Catt et al. (7, 8); and (v) nitrocellulose membrane filters (type HA; pore size, 0.45 μm; Millipore, Kloten, Switzerland).

SPA was either produced by the method of Hjelm et al. (20) or purchased from Pharmacia. For conjugation with phosphatase, we developed a two-step dialysis glutaralde-

hyde method which we afterwards found published already by Engvall (10).

For the evaluation of positive results we used a statistical discrimination system as described earlier (11, 14, 16), Briefly, the negative control sample, which was either phosphate-buffered saline or culture supernatant of the non-enterotoxigenic strain FRI184, was measured five times. Sample values which exceeded the mean of the negative control by three standard deviations or more were considered positive (99% confidence limit).

RESULTS

Our rabbit antisera proved to be specific as checked by Ouchterlony immunodiffusion. No cross-reactions with other enterotoxins were observed. In earlier immunization procedures, we occasionally had minor reactions with culture supernatants of non-enterotoxigenic strains of S. aureus, but these could easily be absorbed by affinity chromatography on Sepharose 4B, to which we coupled a culture supernatant of S. aureus FRI184, which was depleted of SPA by absorption with normal rabbit serum. It is even possible to add liquid SPA-free culture supernatant concentrated 10-fold to the antiserum to absorb unwanted antibodies. Therefore, it may not be necessary to aim at a very high degree of purity in enterotoxin production since antibodies against contaminants can easily be removed. From immunofluorescence tests it is known that nonspecific factors can either be absorbed with tissue or rendered inactive by dilution. Unlike the microslide test, ELISA uses conjugates at such high working dilutions that nonspecific activities have no diagnostic consequences.

We now examined two methods of phosphatase conjugation. We have always preferred phosphatase to peroxidase for the same reasons as those expressed by Avrameas et al. (2) and Engvall (10), who hold that phosphatase gives the most efficient conjugates and the most sensitive colorimetric enzyme assays and is best for accuracy and reproducibility (Tables 1 and 2).

In Table 2 it is demonstrated that the SPDP conjugates were superior to those obtained with glutaraldehyde. The quality of a conjugate is expressed in micrograms of IgG per ml of working dilution. Engvall (10) reported 0.5 μg of SPA per ml, assuming a 40% loss of the protein. In our calculations, we incorrectly assumed no loss of IgG or SPA. Had we assumed a loss, the factor would be even lower. The glutaraldehyde products were highly satisfactory, but the SPDP conjugates were still superior. In particular, the SPA-

TABLE 2. Comparison of two conjugation procedures for anti-enterotoxin IgG and SPA

Procedure	Conjugate	IgG or SPA used (mg)	Phosphatase used (mg)	Conjugate (ml)	Working dilution	IgG or SPA (μg/ml of working dilution)
Glutaraldehyde	Anti-SEA	1.25	2.5	2	1:2,500	0.25
	Anti-SEB	1.25	2.5	3	1:900	0.46
	Anti-SEC	1.25	2.5	3	1:5,000	0.08
	Anti-SED	ND[a]	ND	ND	ND	ND
	SPA	2.5	2.5	10	1:1,000	0.25
SPDP	Anti-SEA	2.2	1.4	5	1:3,000	0.15
	Anti-SEB	2.2	1.4	5	1:2,000	0.22
	Anti-SEC	2.2	1.4	5	1:8,000	0.06
	Anti-SED	8.5	5.0	8	1:5,000	0.21
	SPA	1.0	2.5	5	1:9,000	0.02

[a] ND, Not done.

SPDP conjugate can be used at a 10-fold higher dilution than the glutaraldehyde conjugate. Consequently, we always used this method to our entire satisfaction.

Different sorbents. The most conventional sorbent is polystyrene as tubes or microtiter plates. In our triple sphere test we used polystyrene spheres because they are capable of collecting antigen from a relatively large volume (for instance, 20 ml of food extract) and yield highly reproducible results. In addition, we tested nitrocellulose membranes (Millipore) and Protapol disks (ICI). Nitrocellulose membranes, which are now widely used for blotting procedures (38), bind protein very firmly, and we had encouraging results, but the handling of filter membranes was not practical. Later, Hawkes et al. (19) had the important idea of using concentrated antigen or antibody in the form of tiny dots. Using a peroxidase conjugate and 4-chloronaphthol as chromogen, they obtained easily readable violet dots as a positive reaction. The dot test seems to be useful for the examination of culture supernatants but needs further elaboration.

Protapol disks also bound protein very efficiently, but it was difficult to saturate free isothiocyanate binding sites by the addition of lysine or glycine or by hydrolysis. Consequently, we abandoned this method too and did all our work with polystyrene, mostly in tubes or with spheres.

Different test systems. (i) Competitive ELISA with polystyrene spheres. Polystyrene spheres (37) were coated in bulk overnight at room temperature with 0.5 ml ammonium sulfate-precipitated antibody globulin per sphere (2 to 4 µg/ml; 0.1 M carbonate buffer, pH 9.6). After washing, 1 or 20 ml of the enterotoxin was added and incubated overnight. After washing, the spheres were reacted for 6 h with phosphatase-labeled enterotoxin. In the absence of unlabeled enterotoxin, the label gave an extinction value at 405 nm of 0.8 to 1.0 in 60 min.

(ii) Sandwich ELISA with labeled antibody. Tubes were coated with 1 ml of antibody as described above and were incubated with a 1-ml sample containing toxin. The binding of enterotoxin was detected with 1 ml of a second antibody coupled to phosphatase. In the region of antigen excess, the labeled antibody yielded an OD_{405} of 0.8 to 1.0 in 60 min.

(iii) Inhibition test with labeled antibody. The toxin-containing sample was incubated with enzyme-labeled antibody, and after incubation the complexes were transferred to the toxin-coated tubes, previously washed. If positive, the labeled antibody was prevented from binding to the sorbent.

(iv) Inhibition test with unlabeled antibody. The inhibition test with unlabeled antibody was performed with SPA-phosphatase conjugate as a universal label (Fig. 1). The labeled antibody was replaced by crude antibody. Its presence was detected by SPA-phosphatase conjugate (Fig. 1).

In the very beginning of our experiments, it became evident that SPA, which is abundant in culture supernatants
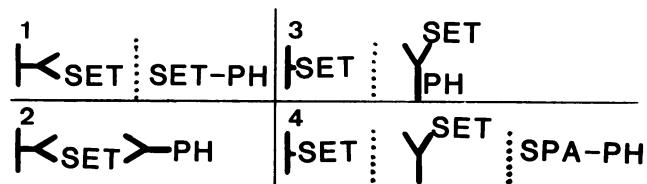
TABLE 3. Lowest detection limits with four versions of ELISA

ELISA[a]	Enterotoxin	No. of tests with lowest detection limit of (ng/ml):		
		0.1	1	10
1a	SEA	5		
	SEB	10		
	SEC	5		
1b	SEA	5		
	SEB	10		
	SEC	5		
2	SEA	11	1	
	SEB	18	10	
	SEC	5	6	1
	SED	10		
3	SEA	7	2	
	SEB	12	18	2
	SEC	2	5	2
4	SEA	5	1	
	SEB	2	4	2
	SEC	5	1	

[a] ELISAs: 1a, competitive ELISA with polystyrene balls, 1 ml of enterotoxin; 1b, same test but with 20 ml of enterotoxin; 2, sandwich ELISA with labeled second antibody, 1 ml of enterotoxin in tubes; 3, inhibition test with labeled antibody, 1 ml of enterotoxin; 4, inhibition test with labeled SPA, 1 ml of enterotoxin. Tests for SED were performed only by test 2. For diagrams of test procedures, see Fig. 1.

(13) but apparently not abundant in food extracts (18), interferes seriously with tests 2, 3, and 4 but not with test 1. Fey and co-workers, who attempted to use phosphatase-labeled SPA as a universal reagent for all enterotoxins, discussed these problems (12, 16) and tried to solve them by using F(ab')₂. The results were unsatisfactory. Koper et al. (23) and Notermans et al. (28) met the same difficulties and overcame them by using sheep antibody, which in their hands was not susceptible to SPA interference, for coating.

Evaluation of the four test systems. After numerous experiments for optimizing the different parameters, we performed the four different tests repeatedly and checked the frequency with which we diagnosed 0.1, 1, or eventually 10 ng of enterotoxin per ml. The source of enterotoxin was crude culture supernatant; the negative control was supernatant from strain FRI184.

From Table 3, it is evident that test 1b, the competitive ELISA with a 20-ml enterotoxin-containing sample, is highly sensitive and regularly detects 0.1 ng of enterotoxin per ml. Its main drawback became evident when we started to produce SED with its extremely low yield of some 0.25 µg of enterotoxin per ml. It became clear that the necessity of labeling antigen is a serious disadvantage of this test. When we later used a 20-ml sample for the sandwich ELISA (test 2), we also regularly obtained a detection limit of 0.1 ng/ml. We therefore decided to drop our original system in favor of the sandwich ELISA. G. Terplan (personal communication) reported a similar experience.

Variation of sample volumes. In an earlier publication (37), we showed that the sensitivity of ELISA systems can be increased with a simple manipulation. It is only necessary to use a multiple volume of antibody coat, sample, and conjugate and then to add 1 volume only of substrate and rotate

FIG. 1. Diagrams of the four versions of the ELISA. Abbreviations: SET, Staphylococcal enterotoxin; PH, phosphatase conjugate.

TABLE 4. Comparison of extinction values at two different reaction volumes in two tests

Test method	SEB concn (ng/ml)	OD_{405}[a]		OD_{405} I/ OD_{405} II
		I	II	
Tubes	1.0	0.712 ± 0.06	0.173 ± 0.02	4.1
	0.1	0.125 ± 0.05	0.034 ± 0.01	3.7
Spheres	1.0	1.09 ± 0.07	0.13 ± 0.01	8.4
	0.1	0.14 ± 0.04	0.04 ± 0.003	3.5

[a] Test conditions for tubes: I, 3-ml coat, toxin and conjugate, 1 ml of substrate; II, 1-ml coat, toxin and conjugate, 1 ml of substrate. Test conditions for spheres: I, 20 ml of toxin, 1 ml of conjugate, 1 ml of substrate; II, 1 ml of toxin, 1 ml of conjugate, 1 ml of substrate.

the substrate-filled tubes during incubation. Results of a single experiment (of many) of this kind with tubes instead of spheres are shown in Table 4.

In tubes, theoretically, the factor OD_{405} I/OD_{405} II would be 3. In fact, factors of 3.7 to 4.1 can be found. The same observation has been made with microtiter plates. When we used 250 μl of reagents versus 50 μl of substrate and vibrated the plates, the extinction values increased two- to four-fold.

SPA in culture supernatant and food extract. Since our system depends on rabbit antibodies, it is necessary to eliminate SPA. Originally we added 10% porcine IgG insolubilized with glutaraldehyde (1). But whereas porcine IgG absorbed all SPA which could react with human IgG, some SPA remained which reacted with rabbit IgG. Therefore, we changed to rabbit IgG and simplified the procedure even more by simply adding 2.5 to 10% normal rabbit serum to the sample. This absorbs the SPA of even highly producing staphylococcal strains (13). We did not eliminate the SPA-IgG complexes by centrifugation, and we left the sample slightly turbid.

Principle of the final test with polystyrene spheres. After these comparative tests, we finally decided to apply the sandwich version of ELISA with polystyrene spheres and 20-ml samples. The sandwich version has been used before by Berdal et al., who used microtiter plates (3); by Freed et al., who compared microtiter plates and polystyrene spheres (18); and by Notermans et al., who used tubes (29). We examined its practical applicability with three different foods artificially contaminated with SEA, SEB, SEC, and SED.

The test was performed as follows. Spheres with color codes were coated with 0.5 ml of antibody IgG per sphere (2 μg/ml; 0.1 M sodium carbonate buffer, pH 9.6) and incubated for 6 h at room temperature. Four spheres coated with normal rabbit IgG served as a negative control. After three washings with NaCl-Tween, 20 ml of food extract was added, and the bottle was slightly agitated overnight at room temperature. After three washings, the spheres were distributed in individual prewashed tubes and reacted for 6 h at room temperature with 1 ml of homologous conjugate. After another washing procedure, 1 ml of p-nitrophenyl phosphate substrate was added. Its color was spectrophotometrically measured (405 nm) after 60 min and was evaluated as described above (see also 16a).

Artificial contamination of food with SEA, SEB, SEC, and SED. We chose mincemeat, yogurt, and rice salad with mayonnaise as representative foods. One hundred grams of each was homogenized with 100 ml of phosphate-buffered saline. To this suspension we added 1,000, 100, and 10 ng of SEA, SEB, SEC, or SED. The slurry was then extracted as described (37), and 20 ml of each sample was examined by

the test mentioned above. We invariably found the sample containing 10 ng of staphylococcal enterotoxin to be positive with all four enterotoxins, i.e., the detection limit was ≤0.1 ng/ml.

DISCUSSION

It is possible to measure 0.1 to 1.0 ng of enterotoxin per ml by the competitive or sandwich version of ELISA. After we decided in favor of the sandwich ELISA, passing over our earlier competitive test, we wanted to characterize these two procedures.

Competitive method. The advantages include higher specificity because of lower nonspecific uptake. In addition, it is not sensitive to SPA, which interferes by binding to antibody Fc in tests. The disadvantages are that enterotoxins, especially SED, are hard to produce and coating antibody and label concentrations are critical.

Sandwich method. The advantages are that it is somewhat more sensitive than the competitive ELISA; relatively small amounts of enterotoxin antigens, which must not be entirely pure, are sufficient for the production of a large amount of specific antibody; the titration of reagents is not critical since they are used in excess; and the system is suitable for monoclonal antibodies. The disadvantages are that antibody IgG cannot be purified by immunosorption for the conjugation owing to lack of antigen; antibody reagents have a higher tendency to nonspecific stickiness owing to aggregate formation; and SPA absorption of food extracts or culture supernatants is necessary. An alternative is the use of sheep antibody (28).

The sandwich method described above is useful for the examination of both 20 ml of food extracts and 1 ml of culture supernatants (G. Burkhard, D.V.M. dissertation, University of Bern, 1980), as proved by the successful recovery of enterotoxin from artificially contaminated food at the level of 0.1 to 1 ng/ml. If necessary, the system can be made quantitative by a parallel running of a standard curve as described by Fey (11). On the other hand, a semiquantitative model is possible by replacing the negative control by a standard containing, say, 1 ng of enterotoxin per ml. The final diagnosis would then be ≥1 ng of enterotoxin per ml detected.

The final test kit has been described (16a). This diagnostic kit is now available.

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