Simple Assay for Staphylococcal Enterotoxins A, B, and C: Modification of Enzyme-Linked Immunosorbent Assay

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Received for publication 1 August 1978

The enzyme-linked immunosorbent assay (ELISA) introduced for the detection of staphylococcal enterotoxins by Saunders et al., Simon and Terplan, and ourselves has proved to be a simple, reliable, and sensitive test. A new modification is described that uses polystyrene balls (diameter, 6 mm) coated individually with antibody against one of the toxins A, B, or C. In a single tube, 20 ml of the food extract was incubated with the three balls differently stained, which were then each tested for the uptake of enterotoxin by a competitive ELISA. A concentration of 0.1 ng or less of enterotoxin per ml can be measured, making tedious concentration procedures of the extracts superfluous. Culture supernatants and extracts from foods artificially or naturally contaminated with toxin were successfully examined. Cross-reactions did not occur, and nonspecific interfering substances did not create serious problems.

At present, the diagnosis of staphylococcal food poisoning is mostly done on clinical, epidemiological, and bacteriological evidence, but there is an urgent and widespread need for a simple and economical test. Some diagnostic laboratories use the microslide test in which 10 μ l of the food extract is tested with 10 μ l of antiserum. This technique makes it necessary to concentrate food extracts by a series of lengthy procedures (20, 27).

More sensitive serological methods such as reversed passive hemagglutination (27) need neither the elimination of interfering proteins from food extracts nor concentration of the sample, but are more difficult to handle.

It was therefore logical that the highly sensitive radioimmunological procedures were applied to the detection and quantitation of staphylococcal enterotoxins (4, 11, 12, 14, 15, 18, 19, 21; H. Fey and G. Stiffler-Rosenberg, unpublished data).

However, these methods have many drawbacks (e.g., they require elaborate equipment; autoirradiation occurs and the reagents are, consequently, short-lived; or there is a health hazard involved). Thus, when Engvall and Perlmann (5, 6) described their enzyme-linked immunosorbent assay (ELISA), several authors tested this new serological method for its suitability in the quantitative determination of staphylococcal enterotoxins (22-24, 28). Consequently, we dropped the radioimmunosorbent test in favor of ELISA (7, 9).

ELISA is, in its extreme sensitivity, comparable to radioimmunoassays, but it is simple to

perform, and the reagents can be stored for more than a year.

Based on our experience with the measurement of tetanus antitoxin in humans and horses by using ELISA (10, 29), we prefer the competitive version of the test, which is less likely to be complicated by background problems. Polystyrene tubes are used as adsorbents by the majority of authors (30, 31). We worked with polystyrene balls which made it possible to adsorb the enterotoxin contained in 20 ml of food extract onto the antibody-coated surface of the balls. In this way, we intended to increase the sensitivity of the test compared with methods that use 1 ml or only 10 μ l of the extract.

In Fig. 1, the principle of a competitive ELISA for enterotoxin is outlined in our version as a single-ball test.

In the positive case, enterotoxin bound to the antibody-coated ball, excess toxin was washed away, and enzyme-linked test toxin was added. The latter was competitively prevented from binding to the ball and was washed away; therefore, there was no breakdown of substrate. In the negative case, the labeled toxin was fixed by the immunosorbent, and the chromogenic substrate was enzymatically attacked. The resulting color was measured in a spectrophotometer, and the extinction ratio was inversely proportional to the concentration of the toxin in the food extract.

MATERIALS AND METHODS

Test strains. The strains used in this study were: type A, *Staphylococcus aureus* 722; type B, *S. aureus*

FIG. 1. Single-ball test, competitive ELISA for staphylococcal enterotoxin.

S 6 (producing enterotoxin B and some enterotoxin A) and *S. aureus* B 243. These three strains were obtained from G. Terplan, Munich, West Germany. Type C₁ was *S. aureus* 19095 (ATCC).

Media. For the production of enterotoxin A and B, we used 4% protein hydrolysate powder (Mead Johnson International, Evansville, Indiana). The pH for enterotoxin A was 6.6 (13, 25, 26), and that for enterotoxin B was 6.8 (G. Terplan, personal communication). For enterotoxin C₁, the same medium was supplemented with thiamine at 0.5 mg/liter and niacin at 10 mg/liter, and the pH was adjusted to 7.6 (2).

Portions (300 ml each) of the medium were poured into 1-liter Erlenmeyer flasks with indentations in the sides, and the culture was incubated for 48 h at 37°C on a shaker at 95 rpm.

Purification of the toxins: enterotoxin A. Enterotoxin A was purified by the method of Schantz et al. (26), using cation exchange on Amberlite CG-50 (Serva Heidelberg) and carboxymethylcellulose CM 52 (Whatman, W. & R. Balston Ltd., Maidstone, Kent, England, Bender & Hobein, CH-Zurich, Switzerland) followed by gel filtration on Sephadex G 75 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Ion exchange on hydroxylapatite was omitted.

Enterotoxin B. Enterotoxin B was purified by the method of Schantz et al. (25) as modified by Terplan who used only one purification step with Amberlite CG 50 (personal communication). A final gel filtration on Sephadex G 75 was performed.

Enterotoxin C₁. For the purification of enterotoxin C₁, the method of Borja and Bergdoll (3) was used, omitting the lyophilization of the culture supernatant. The protein measurement was done by the Lowry method (16).

Antisera. Antisera against the enterotoxins were prepared in rabbits by using a procedure described by Terplan (personal communication). The enterotoxin was incubated for 24 h at 37°C with 0.2% formaldehyde in phosphate-buffered saline (PBS; 0.01 M; pH 7.4), then dialyzed against PBS for 24 h. Shortly before the

injection, the necessary amount of the Formalin-treated toxoid was taken up in 0.2 ml of PBS. A 0.2-ml amount of 2% Al(OH)₃ was added (Alugel S; Serva, Heidelberg), and the mixture was left at 4°C for 1 to 2 h. It was then emulsified with 0.6 ml of incomplete Freund adjuvant. At intervals of 8 to 10 days, the rabbits were injected with 5, 10, 25, 50, 100, 250, and 500 µg of the toxoid, followed by three 1,000-µg injections, and then bled 10 to 12 days after the last injection. The evaluation of the antisera was done by Ouchterlony double diffusion and immunoelectrophoresis in 1.0% agarose in 0.02 M barbital buffer (pH 8.6). The preparations were stained with Coomassie brilliant blue (1).

The toxin concentration during the purification process was checked by the single radial immunodiffusion test of Mancini (8). The antisera were mixed with the agarose at dilutions ranging from of 1:400 to 1:500 or occasionally 1:1,000.

Reference toxins EB 3 and antibody were kindly supplied by G. Terplan, and toxins A, B S6, and C were a gift from M. S. Bergdoll, Madison, Wis.

Labeling of enterotoxin. For all tests which are read spectrophotometrically, we prefer enzyme alkaline phosphatase with 4-nitrophenylphosphate as substrate. This method is very easy to perform, and less antigen is needed for the coupling than with the peroxidase method. For visual reading, the peroxidase system with H₂O₂/ABTS [2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate)] diammonium salt (Boehringer Mannheim Corp., New York) as substrate is better.

Coupling with phosphatase. Coupling with phosphatase was done by the method of Engvall and Perlmann (6). Alkaline phosphatase, grade VII, was purchased from Sigma Chemical Co., St. Louis, Mo. For the substrate solution, 4-nitrophenylphosphate disodium salt (hexahydrate; Merck, Darmstadt) was dissolved in a concentration of 1 mg/ml in 0.05 M sodium carbonate bicarbonate (pH 9.8) and 1 mM MgCl₂.

Coupling with peroxidase. Coupling with peroxidase was performed by the method of Nakane and

Kawaoui (17); horseradish peroxidase (type VI, RZ 3.2, no. P-8375) was obtained from Sigma.

For the gel filtration at the end of the coupling procedure, Ultrogel AcA 44 (LKB-Produkter AB, S-161 25 Bromma, Sweden) was used instead of Sephadex G 200, because columns with AcA 44 are easier to run and allow a higher flow rate for the same separation efficiency. As a substrate, we used H_2O_2 /ABTS, as did Saunders and Bartlett (23), but in a slightly modified form (5 mg of ABTS per 50 ml of 0.1 M $\text{NaH}_2\text{PO}_4\text{-HCl}$, pH 4.0, and 10 μl of 30% H_2O_2).

The fractions eluted from gel filtration were tested for the presence of enterotoxin in an Ouchterlony test against anti-enterotoxin. The specificity of the conjugate was demonstrated by an ELISA, and polystyrene tubes were coated with anti-enterotoxin (see below). The tubes were filled with 1 ml of 0.01 M PBS (pH 7.4), to which 10 μl of each fraction of the eluate was added. The specific uptake of the conjugate was then shown by the peroxidase reaction. Positively reacting fractions were pooled, and portions of the conjugate were stored at -20°C . Addition of sodium azide was avoided.

Coating of polystyrene. Polystyrene balls (diameter, 6 mm) were purchased from Precision Plastic Ball Co., Chicago, Ill. For the coating of the balls, the antisera were precipitated with 1/3 saturated ammonium sulfate, and the sediment was dissolved and reprecipitated twice. The dialyzed globulins with a protein content of 10 mg/ml were diluted to a concentration of 0.5 to 2 $\mu\text{g}/\text{ml}$ in 0.1 M NaHCO_3 (pH 9.6). The polystyrene tubes were incubated with 1 ml of this solution for 3 h at 37°C or at room temperature overnight.

A 10-ml amount of the globulin solution was used for 20 polystyrene balls, which were gently agitated overnight at room temperature. After coating, the polystyrene adsorbents were washed twice with 0.9% NaCl containing 0.05% Tween 20 (Tween-NaCl). The tubes were then completely filled, and the balls were covered, respectively, with 0.01 M PBS (pH 7.4) containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20, and 0.02% sodium azide (BSA buffer) and left at room temperature for 1 h.

Extraction of foods. Different foods (milk, minced meat, and cheese), naturally or artificially contaminated with enterotoxins, were extracted according to the first part of the procedure described by Reiser et al. (20); 100 g of the food was mixed with 1 to 2 volumes of distilled water and homogenized in a blender. The pH was adjusted to 4.5 with 2 N HCl, and the homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was brought to pH 7.5 with 2 N NaOH, stirred with 10% chloroform for 5 min, and centrifuged again as described above. The supernatant was readjusted to pH 4.5, centrifuged, and neutralized. Tween 20 was added to the extracts at a final concentration of 0.25% (23).

Triple-ball test. In the triple-ball test, our modification of the competitive ELISA for the enterotoxins A, B, and C (Fig. 2), polystyrene balls, marked with a pen that used waterproof, instant-dry colored ink, were coated with enterotoxin antibody anti-A, -B and -C, respectively, as described above. Three different balls were incubated with 20 ml of the extract at room

temperature and gently agitated overnight. After two washings with Tween-NaCl, the balls were put individually into a polystyrene tube which had been precoated with BSA buffer and washed with Tween-NaCl to saturate the protein binding sites. The balls were incubated for 4 h at 37°C with 0.5 ml of each of the homologous enterotoxin-phosphatase conjugates (Ent-A-PH, Ent-B-PH, and Ent-C-PH), in appropriate dilutions (e.g., 1:1,000). The three tubes were washed twice with Tween-NaCl, and 0.5 ml of the substrate nitrophenylphosphate was pipetted into the tubes, which were left on the bench for 30 min or until the negative control tube showed an optical density at 403 nm of 0.8 to 1.0. Then the enzymatic reaction was stopped by the addition of 50 μl of 2 N NaOH, and the tubes were read photometrically at 403 nm. A negative control without enterotoxin and three positive controls containing 1 ng of enterotoxin A, B, and C, respectively, per ml (i.e., 20 ng/20 ml) were included every day in the test.

Quantitation of the toxin with the single-ball test. Quantitation of the toxin with the single-ball test (Fig. 1) was carried out for measuring a given enterotoxin if the triple-ball test revealed a positive reaction in one or more tubes.

A standard toxin which had been measured with a standard antiserum by the Mancini test was used at the following four concentrations: 5, 2.5, 1.25, or 0.6 ng/ml. In a duplicate test, 1 ml of each of these dilutions was incubated with one ball coated with the respective antibody in a polystyrene tube pretreated with BSA buffer and Tween-NaCl. The test then proceeded as described above. With the four extinction values obtained, a regression curve was drawn. Three concentrations of the unknown toxin were tested in duplicate in the same way; i.e., it was tested undiluted and in the dilutions 1:10 and 1:100. The means of the double extinction values were calculated, and the value which fits the linear part of the standard curve was used for the calculation of the enterotoxin concentration of the unknown sample. Thus, we propose a two-step procedure. The extract is first qualitatively examined for the presence and the type of enterotoxin by using the triple-ball test. In the positive case, the toxin(s) is (are) then quantitated by the single-ball test.

RESULTS

As indicated above, the antigens used for the immunization of rabbits were intentionally not highly purified. For enterotoxins A and B, the eluates from Amberlite CG 50 and from Amberlite rechromatographed on CM-cellulose were used. For enterotoxin C₁, chromatography on CM-cellulose was the only purification step. In addition, the crude culture supernatant of the A and B strains was used as an immunogen. The rabbits produced antisera with a major line against contaminants from the culture medium.

In a microslide test, this would be troublesome, but it could be demonstrated in the ELISA that, due to the high dilutions of the antisera, neither cross-reactions with other en-

terotoxins nor any considerable nonspecific interference by contaminants occurred (see Tables 1, 2, and 3).

Application of the single-ball test. By increasing the volume of the antigen extract used in the test from 1 to 20 ml, we hoped to enhance its sensitivity. We supposed that the antibodies insolubilized on the polystyrene balls would collect more enterotoxin molecules from 20 ml of extract than from 1 ml. For the same reason, we incubated the reaction mixture overnight instead of for a few hours. Balls were coated with anti-enterotoxin A and B at a 1:5,000 dilution and with anti-enterotoxin C at a 1:7,500 dilution. The enterotoxins were diluted to concentrations of 1, 0.1, and 0.05 ng/ml, respectively. The different balls were then incubated overnight with 1 and 20 ml, respectively, of the three homologous enterotoxin dilutions. PBS (0.01 M; pH

7.4), instead of enterotoxin, was included as a negative control.

It can be seen in Table 1 that, with 1 ml of extract, 1 ng of all three enterotoxins per ml could be measured. The toxins in two lower concentrations did not significantly inhibit the uptake of the respective enterotoxin conjugates. The limits of detection were lowered 10 (to 20) times when 20-ml amounts of extract were used; 0.1 ng of the enterotoxins A and B per ml was clearly detected (> -2 standard deviations), and the values of the lower concentrations (0.05 ng/ml) were at the limit of significance. Enterotoxin C at 0.05 ng/ml was considered to be positive at an extinction value close to the margin of error. By repeating these experiments several times, we detected 0.1 ng of enterotoxin A per ml, 0.06 ng of enterotoxin B per ml, and 0.06 ng of enterotoxin C per ml.

FIG. 2. Competitive triple-ball test for staphylococcal enterotoxin. Symbols: $\bigcirc$, $\bullet$, $\odot$, polystyrene balls coated with antibody anti-enterotoxin A, B, or C respectively. PH, Phosphatase; A, enterotoxin A; A-PH, phosphatase-labeled enterotoxin A; NPP, p-nitrophenylphosphate (substrate); NP, nitrophenol.

TABLE 1. Comparison of the sensitivity of the single-ball test, using 1 or 20 ml of enterotoxin dilutions

Concn of homologous enterotoxins A, B, and C (ng/ml)	Extinction value at 403 nm for the following antibody-coated balls: ^a		
	Anti-A	Anti-B	Anti-C
1-ml extract			
1	<u>0.66</u>	<u>0.58</u>	<u>0.7</u>
0.1	<u>0.75</u>	<u>0.72</u>	<u>0.85</u>
0.05	<u>0.77</u>	<u>0.71</u>	<u>0.82</u>
PBS control $\bar{x}$	0.79 ± 0.025	0.75 ± 0.05	0.86 ± 0.025
$\bar{x} - 2s$	<u>0.74^b</u>	<u>0.65^b</u>	<u>0.81^b</u>
20-ml extract			
1	<u>0.33</u>	<u>0.29</u>	<u>0.47</u>
0.1	<u>0.65</u>	<u>0.58</u>	<u>0.71</u>
0.05	<u>0.72</u>	<u>0.66</u>	<u>0.75</u>
PBS control $\bar{x}$	0.78 ± 0.03	0.69 ± 0.015	0.88 ± 0.05
$\bar{x} - 2s$	<u>0.72</u>	<u>0.66</u>	<u>0.78</u>

^a For extract samples, each value indicates the mean of three determinations; for the controls, each value indicates the mean $\pm$ standard deviation based upon five measurements. Underlined values represent measurable inhibition (unbroken line) and the limits of measurable inhibition (broken line).

^b An inhibition of the label's uptake of $\geq 2s$ (standard deviation) was considered positive.

With the single-ball test, 42 coagulase-positive *Staphylococcus* strains from human material cultivated in 4% protein hydrolysate powder for 48 h at 37°C were examined for enterotoxin production. Four strains were positive for enterotoxin B, one was positive for enterotoxin A, and four strains were positive for enterotoxins A and B. Repeated experiments gave the same results.

A number of different foods were contaminated with enterotoxin B and were afterwards extracted as described above to prove the validity of our tests in practice.

To 100-g samples of milk and yogurt, 1, 0.5, and 0.1 µg each of enterotoxin B were added. Even the products to which 0.1 µg of toxin per 100 g had been added gave positive results; i.e., 1 ng of toxin per ml of extract was detected without considering the losses of toxin during the extraction procedure.

Mayonnaise was contaminated with 2×10^4 enterotoxin B-producing staphylococci per g and incubated for 36 h at room temperature. The extracts were shown to contain 3 to 6 ng of enterotoxin B per ml.

In 4 of 14 diagnostic samples of cheese (mostly soft cheese from goat's milk), enterotoxin A was detected, and in one sample toxins A and B were detected.

Application of the triple-ball test. When the three balls coated with antibody anti-A, anti-B, and anti-C, respectively, were incubated with 20 ml of enterotoxin dilutions containing 1 ng of one or two enterotoxins per ml, no cross-inhibition of the uptake of the enterotoxin-phosphatase conjugate was observed (Table 2). The anti-A and anti-B balls were treated with antibody diluted 1:5,000, and anti-C antibody was diluted 1:10,000. For the negative control, 20 ml of PBS was incubated with each of 3 anti-A, anti-B, or anti-C balls, respectively. The extinction value of the negative controls (mean of three determinations) which resulted from the uptake of the homologous enterotoxin-phosphatase conjugate was considered as 100%. The extinction ratio of the test balls (mean of two measurements) was also given as a percentage of the extinction value of the respective control.

From Table 2 it is evident that the triple-ball test is specific; i.e., a given enterotoxin blocks only the ball coated with the homologous antibody from binding the respective conjugate and not the other balls. The specificity of the interactions has been confirmed by including standard toxins from M. S. Bergdoll (concentrated crude culture supernatants) in the experiment.

The test was further controlled by a blind experiment. A total of 15 cultures of the test strains of staphylococci were mixed so that they contained either no enterotoxin or a single toxin

in the original crude concentration or combinations of the three toxins. All 15 samples were diagnosed correctly with the qualitative triple-ball assay.

Finally, three samples of cheese were examined diagnostically. The extinction ratios (sample/negative control) are shown in Table 3.

Cheese no. 251 contained enterotoxin A, and it is noteworthy that no cross-inhibition occurred with the anti-B and anti-C balls. Therefore, the triple-ball test has a "built-in" control system; i.e., if at least one ball shows an extinc-

TABLE 2. *Specificity of the triple-ball test: competitive inhibition of the uptake of the enterotoxin-enzyme conjugate by the homologous enterotoxins*

Enterotoxin ^a	Extinction ratio with the following antibody-coated balls: ^b		
	Anti-A	Anti-B	Anti-C
Toxins (this study)			
A	<u>33%</u>	96%	97%
B	97%	<u>48%</u>	92%
C	93%	91%	<u>37%</u>
Combinations			
A + B	<u>32%</u>	<u>45%</u>	89%
A + C	<u>26%</u>	87%	<u>30%</u>
B + C	81%	<u>40%</u>	<u>34%</u>
A + B + C	<u>30%</u>	<u>47%</u>	<u>43%</u>
Bergdoll toxins			
A	<u>30%</u>	92%	95%
B	103%	<u>49%</u>	95%
C	101%	97%	<u>45%</u>
Controls	100%	100%	100%

^a Culture supernatants at 1 ng/ml.

^b Values indicate ratio of sample to negative controls. Underlined values indicate measurable inhibition.

TABLE 3. *Practical detection of enterotoxin in cheese extracts*

No. of cheese extracts	Extinction ratio (%) with the following antibody-coated balls: ^a		
	Anti-A	Anti-B	Anti-C
1,232	100	98	103
1,239	105	98	103
251	<u>27</u>	90	100
Controls	100	100	100

^a Values indicate ratio of sample to negative controls. Underlined value represents measurable inhibition.

tion value comparable to that of the negative control, the absence of nonspecific inhibiting/interfering substances in the extract is proved. However, it is evident that some cheese extracts may cause considerable background problems. We therefore discussed the possible use of a negative cheese extract as a negative control instead of PBS. But in normal diagnostic work this would be impractical.

DISCUSSION

In our experience, the production of potent antisera against the enterotoxins A, B, and C is not difficult. When the competitive ELISA is used, the ammonium sulfate-precipitated globulins prepared from these antisera can be diluted to a concentration of 0.5 to 2.0 $\mu\text{g}/\text{ml}$ of protein. The labeled enterotoxin is also diluted considerably (1:500 to 1:1,000), which renders the method highly economical. It was possible to detect 0.1 ng or less of enterotoxin per ml. A 1- μg amount of toxin causes clinical symptoms in sensitive persons. Therefore, the method which we describe measures a concentration of enterotoxin which is 10 to 100 times lower than that provoking clinical symptoms.

At this level of sensitivity, we should now focus on the practicability of the technique for routine diagnosis. We do not have much experience in the control of foods for staphylococcal enterotoxins, but our experiments with artificially and naturally toxin-contaminated foods showed that our triple-ball test is obviously suited to this purpose.

With some foods, especially certain cheese products, we occasionally had troubles with non-specific interfering substances. But this can be overcome by titration of the extract and/or by employing an extract of normal food as a negative control. We think that the ELISA with this or another modification now offers the possibility of a reliable and economical diagnosis of staphylococcal food poisoning.

The triple-ball test makes use of three labeled enterotoxin reagents. We are now proceeding to replace these by a universal label, i.e., *Staphylococcus* protein A coupled with peroxidase, which is used in a sandwich technique. Preliminary experiments have been very promising.

ACKNOWLEDGMENTS

The kind help offered in the starting period by G. Terplan and E. Simon, Munich, is highly appreciated. We are indebted to M. S. Bergdoll for his valuable gift of reference antisera and antitoxins.

This research was supported by a grant from Fraunhofer-Gesellschaft, Munich, InSan I-0673-V-043.

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