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Strip ELISA for Detection of Staphylococcal Enterotoxins in Culture Supernatants and Foods

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For the easy and rapid detection of staphylococcal enterotoxins (SE) A, B, C, D and E, a sandwich ELISA (enzyme-linked immunosorbent assay) was developed using an activated nylon membrane with covalently bound antibodies as a solid phase. Strips coated with antibodies against one SE type were incubated with culture supernatants of strains of Staphylococcus aureus as well as with the extracts of food previously contaminated with SE. The assay of bound toxin was performed using horseradish peroxidase labelled antibodies which were partly purified by affinity chromatography. After addition of 3,3',5,5'-tetramethylbenzidine, a distinct blue dot developed in the case of positive samples. The detection limit for individual SE is in the range of 0.5 ng ml⁻¹ to 1 ng ml⁻¹. A semi-quantitative evaluation is possible without any special reading equipment. The test can be done within 2 h, sample preparation excluded. While milk needed no extraction, in the case of minced meat or noodles, homogenization with 1.5 or 2 volumes of phosphate-buffered saline followed by centrifugation for 20 min at 10 000 × g and 4°C proved to be adequate. Interferences by constituents of the food extracts were not observed. The results showed good agreement with those of the microtiter plate ELISA and also with those of a commercially available SE ELISA kit.

Keywords: Strip ELISA, staphylococcal enterotoxins

INTRODUCTION

For the detection of staphylococcal enterotoxins (SE), assay systems have been developed in the recent years which show an improved detection limit compared with the previous gel diffusion test and are also less time consuming. They are based on reversed passive hemagglutination (Yamada, 1977), reversed passive latex agglutination (Fujikawa & Igarashi, 1988) and radioimmunoassay (Bergdoll & Reiser, 1980), but most frequently on ELISA (Simon & Terplan, 1977; Freed *et al.*, 1982; Notermans *et al.*, 1983; Fey *et al.*, 1984; Boerlin *et al.*, 1986; Granade *et al.*, 1986). ELISAs with strips or dipsticks as a solid

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phase are easy to handle and do not require any special equipment for the evaluation. The method for the detection of SE reported here is such an ELISA employing strips with an activated nylon membrane as solid phase.

MATERIALS AND METHODS

Purified Enterotoxins and Antisera

The purified toxins and antisera were prepared in this laboratory. The enterotoxins A, B, C₁, D and E were prepared from culture supernatants of *Staphylococcus aureus* strains FRI 722, FRI 243, FRI 137, FRI 1151m and FRI 326 respectively. In the case of SEB and SEC₁, a combination of ion exchange chromatography on CM 52 (Whatman), chromatofocusing and gel filtration on Sephadex G-50 or G-75 sf (Pharmacia) was used (Ende *et al.*, 1983) for isolation. The purification of SEA, SED and SEE was initially based on the same method with some modifications and use of other ion exchanger resins according to different isoelectric points (IPs) (unpublished data). As soon as affinity purified antibodies to the common epitope of SEA and SEE were available (see below), SEA and SEE were isolated in one step from culture supernatants by affinity chromatography on SEE/A-antibody-Sepharose. The purified enterotoxins were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a gradient gel 8–25% (Phastgel, Pharmacia).

Anti-enterotoxin sera were prepared by immunizing sheep with purified enterotoxins (Notermans, 1982). The immunoglobulin G₁ (IgG₁) fractions were obtained by ammonium sulfate precipitation (Hebert *et al.*, 1973) followed by ion exchange chromatography on DEAE-Trisacryl M (IBF) performing a stepwise elution using the following buffers: (i) 25 mmol l⁻¹ TrisHCl + 35 mmol l⁻¹ NaCl, pH 8.8 (starting buffer), (ii) 25 mmol l⁻¹ Tris HCl + 75 mmol l⁻¹ NaCl, pH 8.5 (elution buffer), 0.1 mmol l⁻¹ TrisHCl + 1 mol l⁻¹ NaCl, pH 8.8 (regeneration buffer). Cross-reacting antibodies recognizing the common epitope shared by SEA and SEE, and by SEB and SEC₁, were isolated by affinity chromatography using activated CH-Sepharose 4B (Pharmacia) as a matrix. About 5 mg SE per gram of Sepharose were coupled according to the instructions of the manufacturer. Antisera or IgG₁ fractions against the heterologous SE type were passed through a column with the immunosorbent under binding conditions (0.1 mol l⁻¹ TrisHCl + 0.5 mol l⁻¹ NaCl, pH 8.0). Cross-reacting antibodies were adsorbed and could be eluted, after washing the column with binding buffer, using 0.1 mol l⁻¹ Glycin-HCl + 0.5 mol l⁻¹ NaCl, pH 2.8 for the desorption. In the same manner, specific antibodies against SED were isolated by passing anti-SED IgG₁ through a column of SED-Sepharose.

Antibody-Enzyme Conjugate

Horseradish peroxidase (Boehringer, 413170) was conjugated to IgG by the method of Wilson and Nakane (1978). Unreacted horseradish peroxidase was removed by gel filtration on Sephacryl S-200 (Pharmacia). The conjugate was divided up into suitable portions and stored at -20°C until use. For the detection of SEA and SEE the cross-reacting, affinity isolated antibodies were coupled. Affinity purified specific antibodies were also used for the anti-SED conjugate.

Solid-phase Coating

Immunodyne (R) membrane strips (PALL) were dotted with 1–2 µl of an antibody solution of 1 mg ml⁻¹ in phosphate-buffered saline (PBS, 0.116 mol l⁻¹ NaCl + 0.01 mol l⁻¹ Na₂ HPO₄ + 3.2 mmol l⁻¹ KH₂ PO₄, pH 7.2). The dotted strips were dried at 50°C for 15 min. Remaining binding sites were blocked by incubation of the membrane in 1% (w/v)

caseine in PBS over 15 min. The strips were dried again as before and were ready for use. Dry and in the dark they could be stored for months.

Microtiter plates (Nunc, Immuno Plate Maxisorp) were coated by filling 0.1 ml of an antibody-buffer solution of $10 \mu\text{g ml}^{-1}$ in PBS, pH 7.2 to each well—excluding one as a blank—and incubating it for 3 h at 37°C in a humid chamber. After removal of the coating solution, 0.2 ml of $0.015 \text{ mmol l}^{-1}$ bovine serum albumin was added to the wells and incubated for 15 min at room temperature. The microtiter plates were stored at 4°C for several days or at -20°C for months.

Preparation of Culture Supernatants and Food Extracts

Twelve different enterotoxigenic and non-enterotoxigenic strains of *S. aureus* (see Tables 1 and 2) were cultivated in brain-heart-infusion (Oxoid) at 37°C and 60 rpm for 48 h. Supernatants were harvested by centrifugation at $18\,000 \times g$ and 4°C for 20 min and 0.2% NaN_3 added. They were checked for their content of enterotoxin by gel diffusion test (Sommerfeld & Terplan, 1975). Milk, noodles and minced meat were contaminated with defined amounts (12 ng g^{-1} food) of purified enterotoxins before extraction. While milk needed no extraction, in the case of minced meat or noodles the samples were homogenized with 1.5 or 2 volumes of PBS respectively in a Waring Blendor followed by centrifugation for 20 min at $10\,000 \times g$ and 4°C . The supernatant (extraction step I) was adjusted to pH 4.5 with 5 mol l^{-1} HCl and centrifuged as before. The supernatant was neutralized to pH 7.2 with 5 mol l^{-1} NaOH (extraction step II) and once more centrifuged as before in the case of minced meat. Extracts of food to which no enterotoxin was added were used as negative controls. They were also used as a diluent.

ELISA Procedure

1 ml test and standard solutions respectively were placed in polystyrene tubes ($11 \times 100 \text{ mm}$). The strips were dipped in the tubes and incubated under slight agitation on a tumbler for 30 min at room temperature. The strips, about 10 at a time, fixed in a self-made device, were washed twice in washing solution (0.15 mol l^{-1} NaCl + 0.42 mmol l^{-1} Tween 20) in a beaker under slight agitation by a magnetic stirrer. Then strips were placed in tubes containing 1 ml of diluted antibody-enzyme conjugate and again incubated as before. Strips were washed again three times as above and placed in tubes with 1 ml of 3,3',5,5'-tetramethylbenzidine- H_2O_2 substrate solution (Gallati & Pracht, 1985). The substrate reaction was interrupted by removal of the sticks after 15 min. Strips were dried at room temperature and evaluated comparing the dots of test and standard solutions. Also, measurement of the reflectance using a special photometer (Beckman, DU-70) was performed. A similar protocol was used for the microtiter plate assays. 0.1 ml of the sample or standard solution was added to the wells and plates were incubated on a tumbler at room temperature for 30 min. The plates were washed twice with washing solution using an ELISA washer (Dynatech). Then 0.1 ml of dilute antibody-enzyme conjugate was filled into the wells and plates were incubated again as before. After washing of the plates, 0.1 ml of 3,3',5,5'-tetramethylbenzidine- H_2O_2 substrate solution was added to each well and the enzyme reaction was quenched after 15 min by addition of 0.1 ml stopping solution (2 mol l^{-1} H_2SO_4). The absorbance was read at 450 nm by a Microelisa Autoreader (Dynatech).

Serial dilutions of enterotoxin solutions were generated for use as standards. Buffer blanks or negative controls were assayed to account for any non-specific color reaction. When measured by autoreader and photometer the absorbance and reflectance values of the standard dilution series were plotted against the corresponding concentrations of enterotoxins using a logarithmic graduation of the abscissa.

In comparison, samples were also assayed by a commercially available ELISA kit which uses antibody-coated polystyrene balls as solid phase and alkaline phosphatase as enzyme

in the conjugate (Fey & Pfister, 1983). The test which enables the detection of the SE types A, B, C and D was performed according to the instructions of the supplier (Labor Bommeli, Switzerland).

RESULTS

Enterotoxins, Antisera and Antibody-Enzyme Conjugates

The purified enterotoxins showed a single band slightly below 30 000 Dalton in the SDS-PAGE when compared to the calibration kit.

The IgG₁ from the sheep sera emerged in buffer ii from the ion exchange column as a double-headed peak. The antibodies showed cross-reactions with heterologous antigenically related enterotoxins in the gel diffusion test only before absorption on SE-Sepharose. The isolation of SED antibodies by SED-Sepharose yielded an antibody fraction with a 7-fold increased specific activity compared to the original preparation. The isolated antibodies against the common epitope of SEE and SEA showed a higher specific activity against SEE than against SEA in a relation of about 2:1. Antibody-enzyme conjugates could be used in working dilutions of 1:1000 to 1:5000 at a substrate incubation time of 15 min.

Assay of Enterotoxins, Detection Limit

The evaluation of reflectance data from measuring the standard dilution series revealed sigmoid graphs as shown in Figure 1 for SED. The detection limits were 0.1–0.5 ng ml⁻¹ for SE defined as values exceeding the control means by more than twice the standard deviation. When the strips were evaluated by the naked eye, the detection limit as the threshold of perceptibility of the dot against its corresponding background was 0.5–1 ng ml⁻¹ SE (see Figure 2).

Detection of SE in Culture Supernatants

The results of the examinations with the microtiter plate and the strip ELISA agreed and are shown in Table 1. The results obtained with the commercial test kit are presented in Table 2. Presence of SED in supernatants of *S. aureus* strains FRI 13N-2909 and FRI 722 and of SEA in the supernatant of strain FRI 920 was not indicated by this test.

Detection of SE in Food Extracts

Enterotoxins added to food could be detected in all cases and with all test systems. An exact determination of the recovery rate was not performed, but it could be assessed by the dilution series to be above 50%. Those results registered by the naked eye revealed no difference between samples of extraction steps I and II. Interferences by the constituents of the extracts could not be observed.

DISCUSSION

In these experiments, a strip ELISA was performed in comparison to an ELISA based on microtiter plates and a commercially available system using polystyrene balls as a solid phase support.

Sheep antibodies were used as test reagents to prevent interferences by staphylococcal protein A in the test. Because for sheep IgG₂, an affinity to protein A has been reported (Kessler, 1976), IgG₁ was isolated by ion exchange chromatography. To optimize the specificity of the test system the known cross-reactivities between antibody fractions and heterologous enterotoxins (Bergdoll *et al.*, 1973) were largely removed by affinity chro-

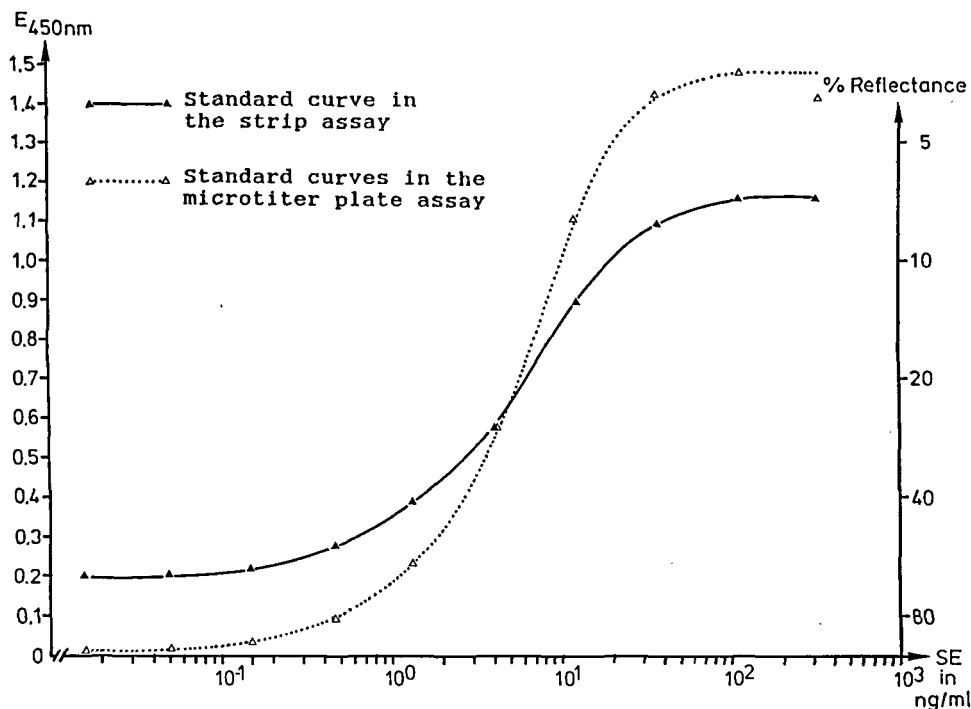


FIG. 1. Standard curves for the detection of SED by a sandwich ELISA comparative for strip and microtiter plate assay.

matography on SE-Sepharose (Lee *et al.*, 1978; Terayama *et al.*, 1976). Affinity-isolated antibody preparations proved to be highly active and were suitable for enzyme conjugation. In previous investigations, it had become evident that for the strip ELISA, antibody-enzyme conjugates were desirable which could be highly diluted, because in this way the background staining of the membrane could be lowered. In the strip ELISA the enzyme substrate reaction was not stopped by acid addition, because this led to a color change of the dot from blue to pale violet and the dot became less distinct. The evaluation of the reflectance data from standard dilution series showed that the substrate reaction yielded comparable standard curves in both systems. While the detectability in both systems is approximately the same, the strip ELISA shows a slightly lower sensitivity.

The examination of culture supernatants had to show whether the strip method is equal to the microtiter plate assay in its specificity and absence of non-specific reactions. The homologous SE produced by different *S. aureus* strains were indicated by all test systems. The results did not correspond in each case. The commercial system did not indicate the presence of SED in supernatants of *S. aureus* strains FRI 13N-2909 and FRI 722. With the strip and the microtiter plate assay, weakly positive results for the presence of SED were obtained which could not be discriminated according to the underlying criteria (see above). The evaluation instructions for the commercial test kit suggested discrimination on a more generous scale. This had also to be considered regarding the indication of SEA in the supernatant of *S. aureus* strain FRI 920 in our own test systems. The presence of SEA was indicated by all systems in the supernatant of *S. aureus* FRI 326 which is a more potent producer of SEE than FRI 920. While this result had been expected for the commercial test due to the known antigenic relationship, the weakly positive result of our own test systems was slightly disturbing. Probably a pronounced but asymmetric relationship between the antigenic properties of SEA and SEE is responsible for the failure in absorbing the SEA

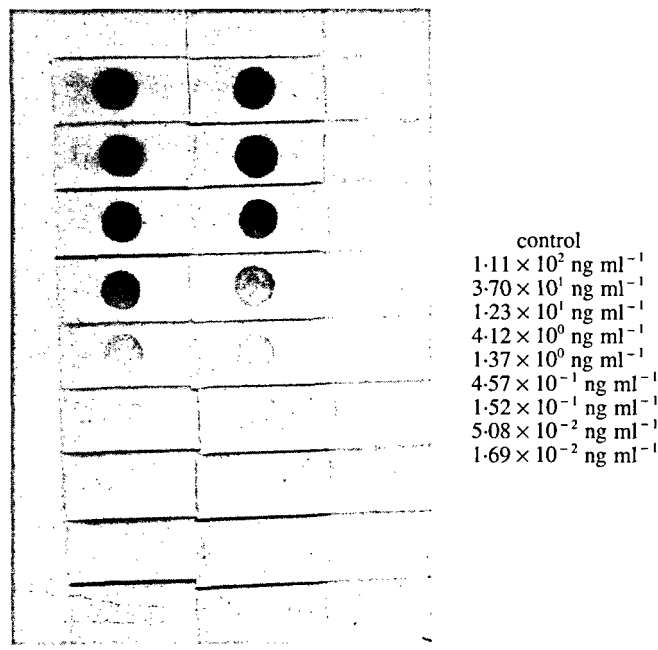


FIG. 2. Strips from the standard curve given in Figure 1, except the one of concentration $3.33 \times 10^2 \text{ ng ml}^{-1}$.

antibodies completely against SEE. The results showed that absorption had been successful in the other direction. *S. aureus* strain FRI 184 which should be non-enterotoxigenic seemed to produce SEA and SEC₁ as indicated clearly by all test systems. No unspecific reactions were observed with supernatant of *S. aureus* strain 20372, a producer of *S. aureus* protein A in any test system, as was expected.

Experiments not shown here demonstrated that strips stored at room temperature and protected against light and humidity were stable for at least 3 months.

A convenient finding was the fact that the strip method was compatible with a shortened extraction procedure in the examination of food. Interferences by constituents of the food

TABLE 1. Positive reactions of culture supernatants of enterotoxigenic and non-enterotoxigenic *S. aureus* strains with homologous (●) and heterologous (○) antisera in the microtiter plate and the strip ELISA

<i>S. aureus</i>		Reaction with				
strain no.	SE type	Anti-SEA	Anti-SEB	Anti-SEC ₁	Anti-SED	Anti-SEE
13N-2909	(A)	●			○	
722	(A)	●			○	
901	(B)		●			
243	(B)		●			
137	(C ₁)			●		
361	(C ₂)			●	○	
1230	(C ₃)			●		
1151m	(D)				●	
326	(E)	○				●
920	(E)	○				●
184	—	○		○		
20372	(Prot. A)					

TABLE 2. Positive reactions of culture supernatants of enterotoxigenic and non-enterotoxigenic *S. aureus* strains with homologous (●) and heterologous (○) antisera in a polystyrene ball-based commercially available ELISA

<i>S. aureus</i>		Anti-SEA	Anti-SEB	Reaction with	
strain no.	SE type			Anti-SEC ₁	Anti-SED
13N-2909	(A)	●			
722	(A)	●			
901	(B)		●		
243	(B)		●		
137	(C ₁)			●	
361	(C ₂)			●	○
1230	(C ₃)			●	
1151m	(D)				●
326	(E)	○			
920	(E)				
182	—	○		○	
20-372	(Prot. A)				

extracts did not occur. Homogenization and centrifugation at 4°C may be performed easily in most laboratories. The strip test itself also requires minimal equipment as far as it is performed as a semi-quantitative test. Further advantages of the strip ELISA may be seen in easy and rapid performance, reliability and specificity adequate to microtiter plate assays, economic expense of reagents, and stability of the immunosorbents at room temperature. Especially in the case of varying and low quantities of samples the use of a corresponding number of strips is preferable to microtiter plates. The strip ELISA for the detection of SE presented in this report met the requirements of a modern, rapid and easy-to-handle test system.

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