APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1984, p. 1047–1053
0099-2240/84/051047-07$02.00/0

Vol. 47, No. 5

# Rapid Enumeration of *Staphylococcus aureus* in Foods by Direct Demonstration of Enterotoxigenic Colonies on Membrane Filters by Enzyme Immunoassay

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Received 14 November 1983/Accepted 24 January 1984

Based on enzyme-linked immunosorbent assay, a convenient method has been devised for the direct demonstration of enterotoxin B production by *Staphylococcus aureus* colonies grown for 24 h on membrane filters. The problem of false-positive reactions due to binding of immunoglobulin G to protein A was turned to advantage by conjugating horseradish peroxidase directly to protein A, which then mediated the labeling of the antitoxin. The test requires 3 h to complete and yields a purple stain at the site of enterotoxin B-producing colonies, thus allowing direct enumeration of confirmed *S. aureus* in foods within 27 h. The method should be applicable to other enterotoxins of *S. aureus*.

As part of a continuing program for the development of hydrophobic grid-membrane filter (HGMF) techniques in food microbiology (8, 17, 19), methods are needed which allow optical discrimination of colonies for manual or automated counting. Since enterotoxigenic *Staphylococcus aureus* accounts for one-third of the food poisoning incidents in Canada (21), the usefulness of HGMF for enumerating these organisms was a prime target for study. A previously published method for enumerating *S. aureus* by using HGMF (2) calls for individual confirmation of presumptive colonies by coagulase tube test, which requires an additional 6 to 24 h to perform (15). A method whereby confirmed *S. aureus* could be counted directly on membrane filters (MF) seemed desirable.

Thermostable nuclease production, one of the screening tests for enterotoxigenic *S. aureus*, can be demonstrated directly on the colony by the toluidine blue-DNA technique of Lachica et al. (12); however, a good correlation does not exist between thermostable nuclease and enterotoxin production (20), and the presence of thermostable nuclease in organisms other than *S. aureus* has been reported (14). We therefore sought to develop a procedure for the direct demonstration of enterotoxin production by *S. aureus* colonies, using the enzyme-like immunosorbent assay (ELISA) (7) as the method of choice both for its colored end product and its enhanced simplicity and safety as compared with solid-phase radioimmunodetection. As the MF itself participates in these reactions (19), a prior study was made of the binding of a staphylococcal enterotoxin and its antiserum to five type of MF. Staphylococcal enterotoxin B (SEB) and its rabbit antiserum (anti-SEB) were used because of their commercial availability in high purity. Results of the ELISA method for the demonstration of SEB production with 9 *S. aureus* strains and 14 nonstaphylococcal organisms, as well as a variety of artificially and naturally contaminated foods, are presented.

## MATERIALS AND METHODS

**Organisms.** *S. aureus* strains and the type of staphylococcal enterotoxin produced were: S6 (A, B), ST 722 (none), ST 243 (B), Cowan I (none), and ATCC 13565 (A), 14458 (B), 19095 (C), 23235 (D), and 27664 (E). Nonstaphylococcal organisms used in the study were: *Bacillus subtilis*, *Proteus rettgeri*, *Proteus mirabilis*, *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Streptococcus faecalis*, and *Streptococcus faecium*. Production of enterotoxins A, B, and C was determined by radioimmunoassay (Health Protection Branch (HPB) method FT-32; production of enterotoxins D and E was determined by the microslide method (HPB method FT-47).

**Materials.** Reagents and media were purchased from the following suppliers: fluorescein isothiocyanate (FITC), protein A, horseradish peroxidase (HRP) type VI, sodium periodate, sodium borohydride, and anti-SEB from Sigma Chemical Co., St. Louis, Mo.; SEB and anti-SEB for binding studies from Makor Chemicals, Ltd., Jerusalem, Israel; nitrocellulose membranes (NCM), 4-chloro-1-naphthol, Tris, gelatin, Tween 20, and goat anti-rabbit HRP were contained in the Immun-Blot assay kit from Bio-Rad Laboratories, Mississauga, Ontario, Canada; brain heart infusion agar, mannitol salt agar (MSA), and Baird-Parker (BP) medium from Difco Laboratories, Detroit, Mich.; and Sephadex G-25, Sephacryl S-200, and CNBr-activated Sepharose 4B from Pharmacia Canada, Ltd., Dorval, Quebec, Canada. Other chemicals were reagent grade. Canned lima beans (Libby's), green beans (York), and frozen chicken were purchased locally.

**MF.** HGMF used in this study were ISO-GRID (QA Laboratories, Toronto, Ontario, Canada) printed with hydrophobic material on polysulfone (Gelman Tuffryn HT-450) or prepared in our laboratory on cellulose-mixed esters (18). MF used in the binding studies are shown in Table 1.

**Fluorescent labeling of toxin and antitoxin.** For the evaluation of their binding properties to MF, SEB and anti-SEB were labeled with FITC (11). Commercial SEB was sufficiently pure to be conjugated directly; anti-SEB was purified by affinity chromatography on SEB coupled to CNBr-activated Sepharose 4B according to the recommended procedure of the manufacturer (Pharmacia Fine Chemicals AB, Uppsala, Sweden) before conjugation to FITC. The

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TABLE 1. MFs used in binding studies

| MF | Lot no. | Polymer composition | Supplier |
|---|---|---|---|
| Sartorius | 061233726<br>060293-0 | Cellulose nitrate[a] | British Drug House, Toronto, Ontario |
| Millipore HAWP | 68K69637 | Cellulose-mixed esters | Millipore, Ltd., Mississauga, Ontario |
| Oxoid Nuflow | 3722 | Cellulose acetate | Oxoid Canada, Inc., Nepean, Ontario |
| Unipore | 81C7A66 | Polycarbonate | Bio-Rad Laboratories, Mississauga, Ontario |
| Tuffryn HT-450 | 3099021 | Polysulfone | Gelman Sciences, Inc., Montreal, Quebec |

[a] Cellulose nitrate and nitrocellulose are the same material; following the usage of the manufacturer, the former term is used for MF, the latter for membranes such as are used in electrophoreses.

immunological activity of both toxin and antitoxin, determined by ELISA microtiter plate test, was unchanged by these manipulations.

**Binding studies.** MF were incubated with FITC-conjugated toxin or antitoxin either in phosphate-buffered saline varying from pH 6 to 11 for 8 h (SEB) or 18 h (anti-SEB) or in phosphate-buffered saline (pH 9) for times varying from 0 to 24 h. For determination of adsorption isotherms, FITC-conjugated toxin or antitoxin in phosphate-buffered saline (pH 9) was incubated with increasing quantities of MF for 6 h (SEB) or 18 h (anti-SEB). For determination of the binding capacities of different MF, FITC-conjugated toxin or antitoxin in phosphate-buffered saline (pH 9) was incubated with MF for 8 h (SEB) or 18 h (anti-SEB). All incubations in a rotary-action incubator at 35°C were done in duplicate in Nalgene bottles in which the headspace air was replaced by nitrogen to control quenching of fluorescence by molecular oxygen. Fluorescent intensity was compared with that of a solution containing no MF in a Perkin-Elmer MPF-3 spectrofluorometer previously standardized with FITC. Preliminary experiments had shown that Nalgene (linear polyethylene) bound neither SEB nor anti-SEB to the container walls.

**Conjugation of HRP and protein A.** HRP was conjugated to protein A (5 mg) by the periodate method of Wilson and Nakane (22) and separated from unreacted material by chromatography on Sephacryl S-200. The pooled fraction of conjugate (HRP-protein A) was stored at 4°C and retained its activity for about 6 weeks.

**Labeling of anti-SEB with HRP.** Anti-SEB (1 ml) was gently stirred with HRP-protein A (0.5 ml) for 1 h at 4°C.

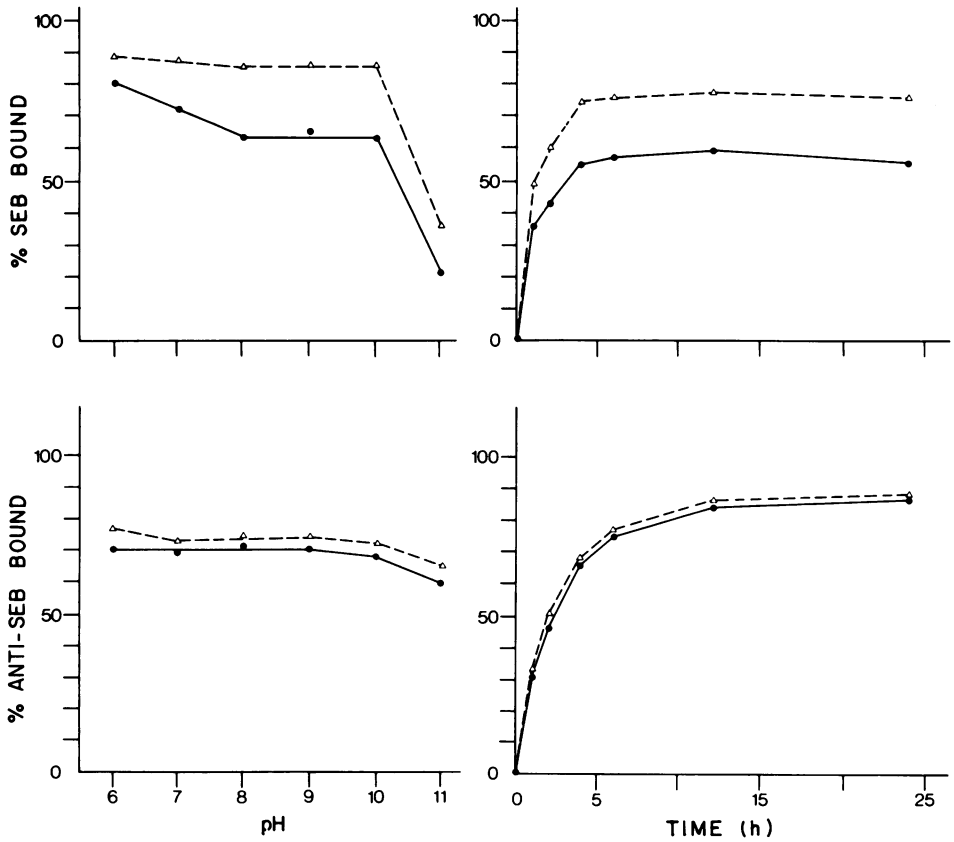


FIG. 1. Variation of SEB and anti-SEB binding to MF with pH and time. Symbols: △-----△, cellulose nitrate; ●——●, cellulose-mixed esters.

Unlabeled protein A (0.1 mg) was added, and stirring was continued for 30 min. The mixture was diluted 1:100 with 20 mM Tris–500 mM NaCl (pH 7.5) (TBS) containing 1% gelatin and was then ready for use (HRP-anti-SEB).

**ELISA method on colonies.** Inoculated HGMF were incubated on brain heart infusion agar (pure cultures) or on BP medium (food samples) for 18 to 24 h at 35°C. NCM previously immersed in TBS and air dried were laid for 30 min onto colonies previously lysed in chloroform vapor ("colony-lifting" or "blotting"; see reference 3). The blots taken from colonies grown on BP medium were bleached by laying the NCM, blot uppermost, on a pad soaked in 30% Javex (vol/vol) for 10 min. NCM were then immersed in 3% gelatin-TBS (blocking solution) for 30 min with gentle agitation and transferred to HRP-anti-SEB for a further 90 min. The membranes were dipped in distilled water, rinsed twice for 10 min each in 0.05% Tween 20-TBS, then immersed in TBS containing 0.05% 4-chloro-1-naphthol, 0.015% $H_2O_2$, and 16% methanol (HRP color development solution; see reference 1) for up to 30 min while purple spots consisting of insoluble product developed. Membranes were then rinsed in distilled water and air dried. Enterotoxin-producing colonies were enumerated by counting purple spots on the NCM and converting this count to most probable number of growth units (16).

**Determination of method sensitivity.** Standard solutions (2 μl) of SEB were applied to NCM in quantities ranging from 20 ng to 25 pg of toxin. The membrane was treated as above, and the smallest quantity of SEB yielding a visible dot was determined.

## RESULTS

**Binding studies.** MF containing cellulose nitrate (CN), either pure or as mixed esters, adsorbed SEB and anti-SEB strongly (Table 2). Cellulose acetate and polycarbonate adsorbed SEB much less strongly and did not adsorb anti-SEB. Polysulfone showed no affinity for either substance.

With this indication that MFs containing CN would form useful matrices for enterotoxin demonstration, binding conditions of SEB and anti-SEB to such MF were studied. Binding was relatively constant in the range from pH 6 to 10. The amount of bound material increased over a time period of 6 h for SEB and 12 h for anti-SEB and then remained relatively unchanged up to 24 h in both cases (Fig. 1). Adsorption isotherms (Fig. 2) of SEB onto MF containing CN indicated strong binding up to a certain concentration beyond which no further adsorption occurred. The adsorption was essentially irreversible. Polysulfone showed no adsorption. Adsorption of anti-SEB to MF containing CN rose continuously through the concentration range examined and was more reversible. The apparent degree of binding of SEB and anti-SEB was not affected by a 10-fold increase in the label (Table 3).

**ELISA method with pure cultures.** Pure cultures of known enterotoxigenicity were incubated for 24 h on brain heart infusion agar for increased enterotoxin production (10). In early experiments with the Immun-Blot assay kit, SEB-producing colonies could be clearly differentiated from some nonproducers (Fig. 3). However, the double-antibody system using rabbit anti-SEB and goat anti-rabbit HRP also detected those staphylococci which are strong producers of protein A. The problem of nonspecific binding of immunoglobulin G (IgG) to protein A was avoided by conjugating HRP directly to pure protein A, which yielded a product which could be used to label any IgG antibody while saturating protein A-binding sites on the Fc portion of the IgG molecule. The HRP-protein A conjugate showed an

TABLE 2. Binding of SEB and anti-SEB to different MF

| Type of MF | SEB (% bound) | Anti-SEB (% bound) |
|---|---|---|
| Cellulose nitrate | 79 | 89 |
| Cellulose-mixed esters | 62 | 88 |
| Cellulose acetate | 15 | 1 |
| Polycarbonate | 14 | 0 |
| Polysulfone | 1 | 0 |

TABLE 3. Binding of SEB and anti-SEB at different doping levels of the label

| Doping level | SEB[a] (% bound) | | Anti-SEB[a] (% bound) | |
|---|---|---|---|---|
| | CN | CE[b] | CN | CE |
| Low | 76 | 62 | 85 | 84 |
| High (10×) | 77 | 63 | 82 | 79 |

[a] Mean of two experiments.
[b] CE, Cellulose-mixed esters.

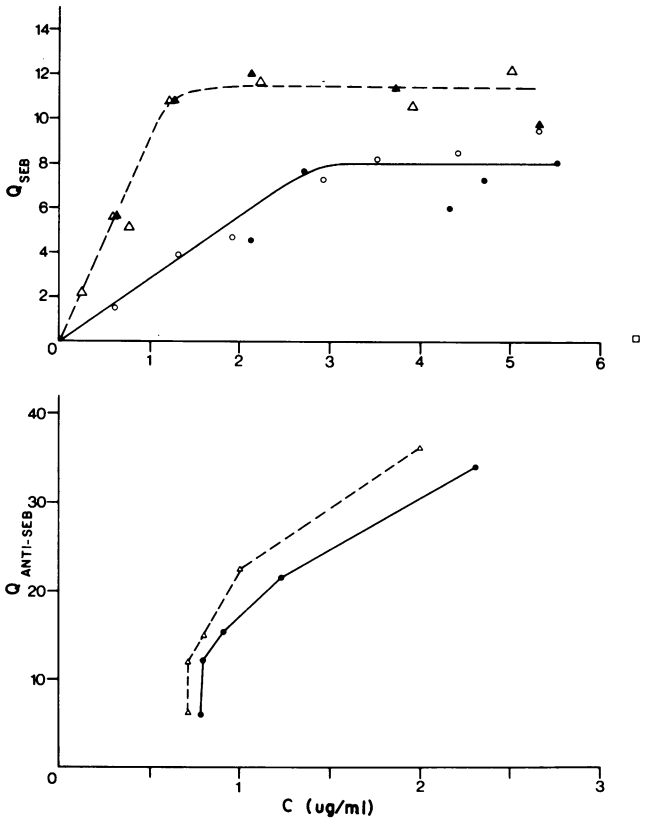


FIG. 2. Adsorption isotherms. Q equals the weight of SEB or anti-SEB adsorbed to MF at the final concentration (C) per unit mass of filter. Symbols: △, ▲, cellulose nitrate; ○, ●, cellulose-mixed esters; □, polysulfone. Solid and open symbols represent duplicate experiments.

$E_{403}/E_{280}$ ratio of 0.5, indicating a superior product with 1 to 2 mol of HRP per mol of protein A (22). Saturation of protein A-binding sites was ensured by adding additional unlabeled protein A during the preparation of HRP-anti-SEB.

By using this system to demonstrate enterotoxigenic colonies, improved specificity was obtained with nine *S. aureus* strains. SEB producers were consistently positive; Cowan I strain, a nonenterotoxigenic strain, and ATCC strains pro-

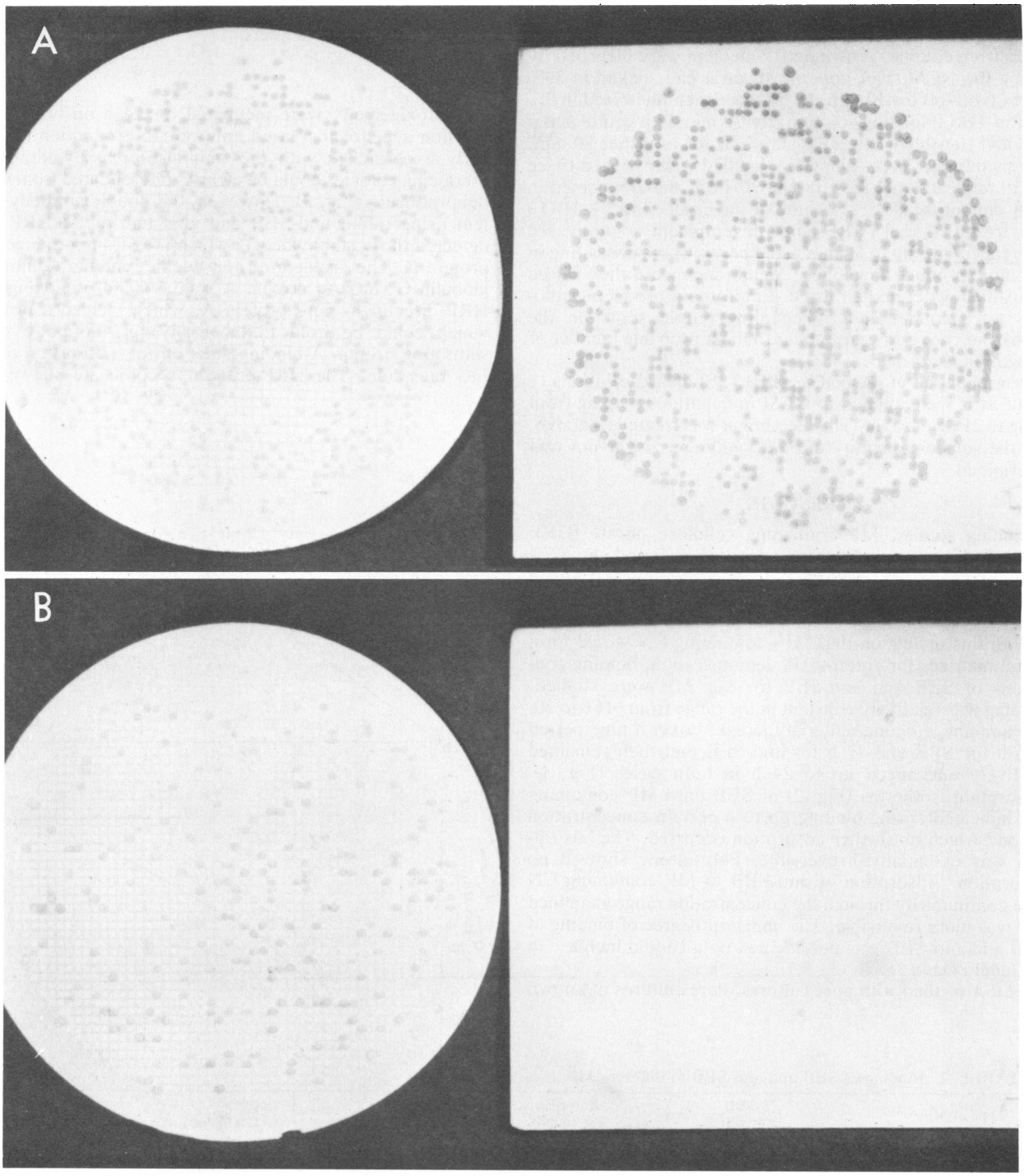


FIG. 3. *S. aureus* colonies grown on HGMF (circles) incubated on brain heart infusion agar; and ELISA for SEB production on NCM (squares). (A) SEB producer (ST 243); (B) SEA producer (ATCC 13565).

ducing SEA, SED, and SEE were consistently negative. There was occasional cross-reactivity with an SEC producer. Fourteen nonstaphylococcal organisms showed no reaction, whereas *S. aureus* S6 on the same blot was positive (results not shown). Absence of endogenous peroxidase and catalase activity under experimental conditions was demonstrated by placing blots not exposed to HRP-anti-SEB in the HRP color development solution. No purple color appeared.

**ELISA method in foods.** HGMF were inoculated with 1 ml of a stomached decimal dilution of foods either spiked with *S. aureus* S6 (lima or green beans) or naturally contaminated (frozen chicken) and incubated on BP medium or MSA for 24 to 48 h at 35°C. ELISA tests performed on colonies proved positive for growth on BP medium and negative for that on MSA. Even after 5 days of incubation, colonies grown on MSA showed only a very faint reaction. The purple color of the dots from enterotoxin-producing colonies grown on BP medium was obscured by the black color of the blots due to reduced tellurite. However, blots bleached on Javex-soaked pads before the blocking step yielded an obvious positive reaction (Fig. 4). The method is sensitive at a level of 500 pg (Fig. 5). By using lima beans spiked with *S. aureus* S6 at a level of about 8,000 organisms per gram, the recovery of demonstrated enterotoxigenic organisms was 101%.

## DISCUSSION

Confirmation of the presence of pathogenic bacteria directly on the colony, either on agar plate or MF, has been reported previously for hemolytic streptococcus and enterotoxigenic *E. coli* (4, 6). To the advantages claimed by these workers—simplicity, speed, and retention of enumeration during resuscitation—can be added those of improved numerical operating range and enhanced potential for automated counting if used with HGMF (17).

Of the polymers examined in the binding studies, CN has the best characteristics for demonstrating enterotoxin production since it binds enterotoxin directly. Early efforts were made to develop an ELISA technique directly on colonies grown on MF which contained CN, but this proved difficult due to variable false-positive reactions probably caused by the great complexity of materials built up within the MF at the site of a bacterial colony during its growth. We then found that the blot or colony-lifting step markedly improved the specificity of the method. As NCM binds SEB directly, the need to precoat with anti-SEB or Fab fragments (3, 9) was eliminated; however, non-specific anti-SEB binding sites must be blocked, for example with gelatin. The use of a chromogen yielding an insoluble product (1) also improved the reaction sensitivity.

The problem remained of reaction between IgG antibody and protein A in the cell walls of strong producers. However, as suggested by Dubois-Dalcq et al. (5), an HRP-protein A conjugate can be used in the immunolabeling of antibodies by occupying the protein A-binding sites on the Fc portion of IgG. Thus, a problem may be turned to advantage, providing a single reagent useful for a wide range of immunolabeling studies. This colony-lifting enzyme-linked antibody reaction (CLEAR) technique should, with appropriate antibody, be capable of displaying toxins or other antigens of interest in the identification and enumeration of microorganisms. Preliminary work in our laboratory has indicated its potential in the demonstration of the presence of *Salmonella* spp. (J. M. Farber, personal communication).

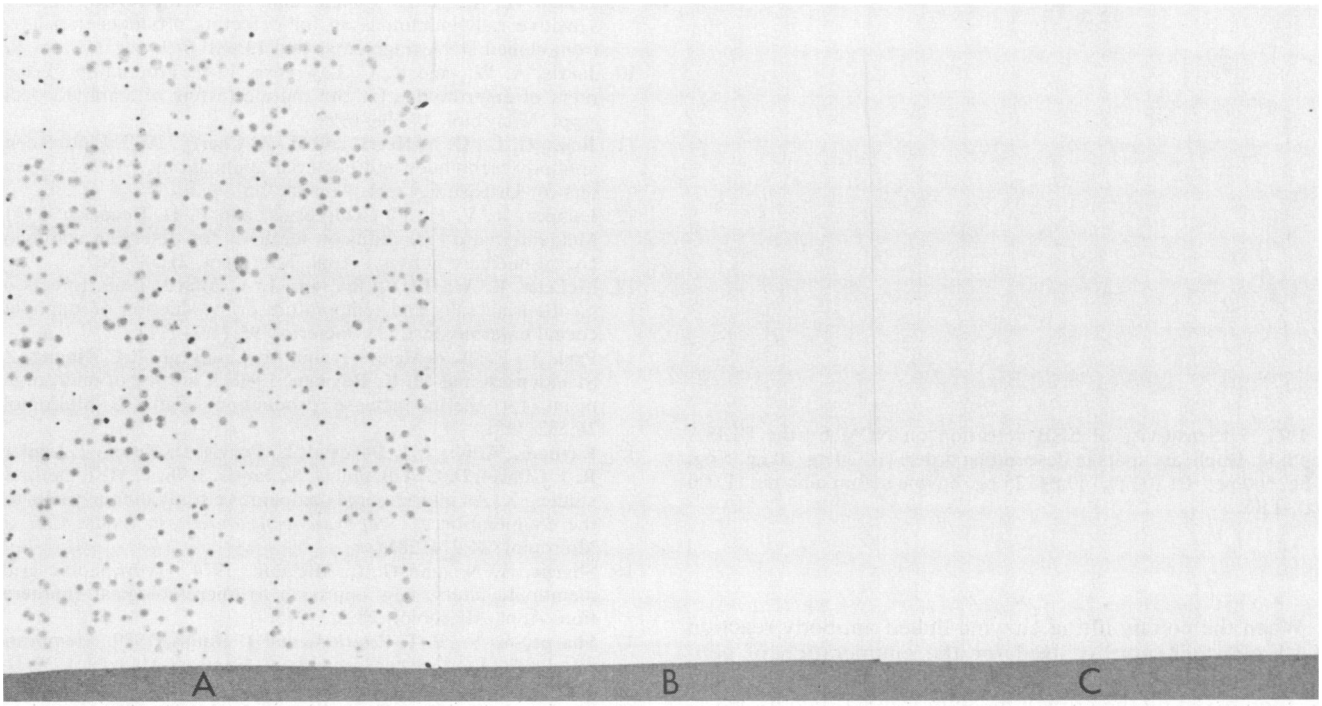


FIG. 4. Detection by ELISA of SEB production in foods. Blots are from colonies of *S. aureus* on ISO-GRID membranes inoculated with artificially contaminated green beans and incubated on BP medium at 35°C for 24 h. *S. aureus* strains are S6 (A) Cowan I (B) and ATCC 13565 (C).

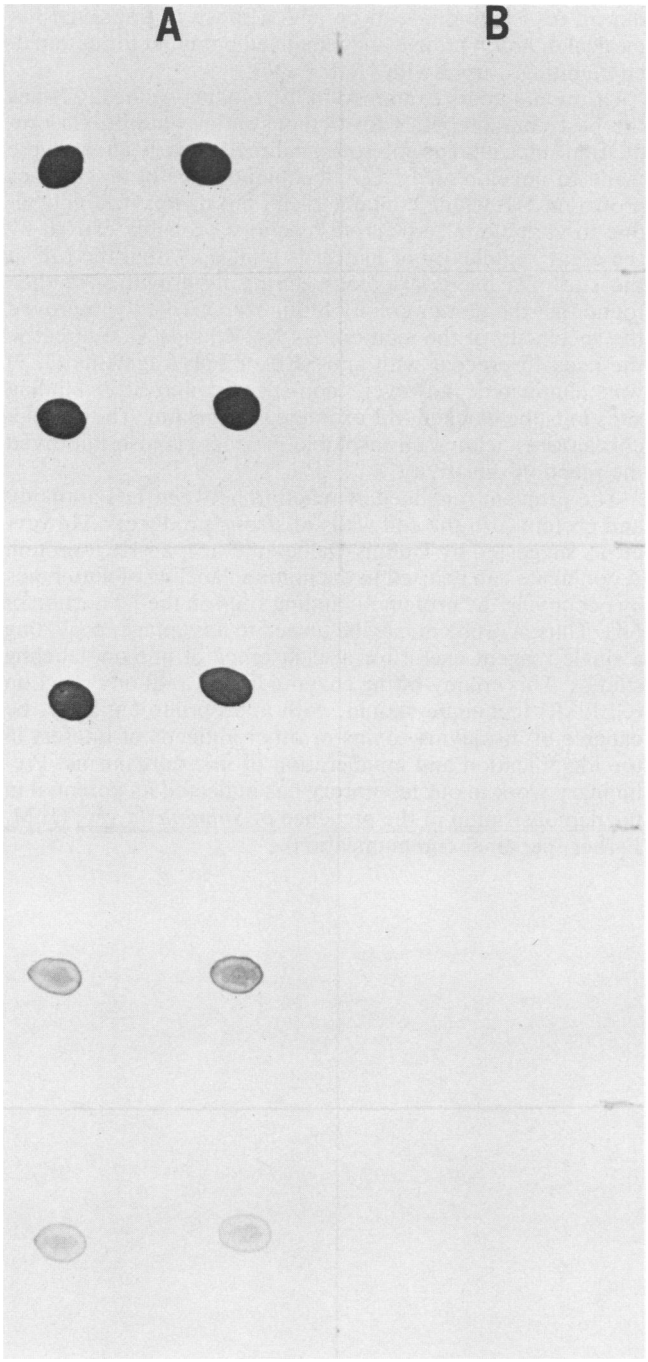


FIG. 5. Sensitivity of SEB detection on NCM by the ELISA method. Duplicate spots in descending order: (A) 20 ng, 10 ng, 5 ng, 1 ng, 500 pg; (B) 100 pg, 50 pg, 25 pg, bovine serum albumin (1,000 ng), TBS.

When the colony-lifting enzyme-linked antibody reaction (CLEAR) technique is used for the enumeration of confirmed *S. aureus* in foods, enterotoxin production must not be suppressed by the growth medium. Media such as MSA, which use high salt as a selective agent, are to be avoided, as staphylococcal enterotoxin production is suppressed by >3% NaCl (13). In contrast, enterotoxin is expressed during growth on BP medium, widely used for selective growth of *S. aureus* in foods, and a positive result is obtained. Since this study was begun, monoclonal antibodies to SEB from seven mouse myeloma lines have been prepared by M. A. Gidney (National Research Council, Ottawa, Canada). Work will now be directed towards development of a standardized antibody reagent capable of detecting all of the known staphylococcal enterotoxins.

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