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Immunofluorescent Detection of Staphylococcal Enterotoxin B

II. Detection in Foods

SUMMARY
Specific staphylococcal enterotoxin B antiserum conjugated with fluorescein isothiocyanate was used successfully for detecting toxin in food smears or food extracts in about 4-5 hr. The fluorescent antibody technique detected minute amounts of enterotoxin—much less than 1 or 0.05 μg/ml, which are the sensitivity limits of single or double gel diffusion tube tests—without involving any extraction procedures.

INTRODUCTION
Staphylococcal enterotoxin B has been demonstrated in culture media by the use of antienterotoxin B serum conjugated with fluorescein isothiocyanate (FITC) (Genigeorgis and Sadler, 1966). Detection of the enterotoxin in smears prepared from fluid culture media requires a concentration of toxin higher than 15 μg/ml. A technique was developed (drop technique), however, which detected enterotoxin around living staphylococcal cells when its concentration was greater than 1 μg/ml as determined by a quantitative single gel diffusion technique (Hall et al., 1963). Impression smears made from colonies growing on solid agar media gave minimum specific immunofluorescence because of diffusion of the toxin into the agar. With the drop technique, purified enterotoxin B in solution was demonstrated and the toxin from the fluid medium was demonstrated in the presence or absence of enterotoxic cells. The possibility of applying the fluorescent antibody technique (FAT) to the detection of enterotoxin B in food smears or food extracts was investigated, and the results are reported here.

MATERIALS AND METHODS
Staphylococcal strains. The strains used in this study were the same strains used to investigate the application of FAT to detect enterotoxin B in culture media (Genigeorgis and Sadler, 1966). These staphylococcal strains produce types A, B, A and B, or no enterotoxin.

Preparation of conjugate. Enterotoxin-B-specific antiserum was conjugated with fluorescein isothiocyanate and fractionated through a diethylaminoethyl cellulose column according to a method described by Corstvet and Sadler (1964).

Preparation and inoculation of foods. The following foods were used: commercially vacuum-packed ham, both with and without sterilizing, a variety of hams prepared in the laboratory, commercially canned ham, raw and boiled chicken breasts, and Cheddar cheese. Discs (14-mm diameter and 2-mm thick) of the different food items were prepared as described by Casman et al. (1963). The discs were placed in sterile Petri dishes, inoculated with staphylococcal strains, covered with another disc, and then incubated for different periods at 24, 30, and 37°C, either aerobically or anaerobically (hydrogen atmosphere), in a high-humidity chamber.

Immunofluorescent demonstration of enterotoxin B. In smears. At the end of the incubation period the two discs of each set were separated, and thin impression smears were prepared on clean coverslips. The coverslips were dried for 30 min at 37°C and then fixed in ethanol for 1 hr at -20°C, dried at the same temperature, and kept there until staining. Frozen sections of the set of the two discs were also prepared with a cryostat and fixed the same way. Six twofold dilutions of the conjugate in phosphate buffer (pH 7.2, 0.02M) ranging from 1:5 to 1:160 were made. Six coverslips prepared from the same sample were each stained with two drops of one of the conjugate dilutions. These coverslips were incubated like those with smears. The washing differed in that the fluid from each coverslip was poured onto wet Millipore filter membranes (0.22-μm pore size and 13-mm diameter), placed on absorbing filter paper, and then washed with 20 drops of buffer. Impression smears were finally made from each membrane on slides (Genigeorgis and Sadler, 1966). Millipore filter membranes have also been used to
catch detached parts of smears made on coverslips or demonstrate enterotoxin in food extracts.

Serological demonstration of enterotoxin B. The same discs used for the preparation of smears or the slurry were also used for the demonstration of enterotoxin B by the single-tube gel diffusion (Hall et al., 1963) and microdiffusion (Crowle, 1958) techniques. The toxin was extracted from the food by first making a 50% slurry in phosphate-buffered saline. The slurry was heated for 25 min at 50°C, left at room temperature for 30 min, and then centrifuged at high speed for 30 min at 30,000 x G. The clear supernatant was further analyzed quantitatively and qualitatively as described (Genigeorgis and Sadler, 1966; Hall et al., 1963) without any concentration and with purified enterotoxin B as a reference.

Controls. To check for nonspecific immunofluorescence, smears from uninoculated food were first checked for autofluorescence and then stained the same way as smears from inoculated foods. A second control was based on the demonstration of enterotoxin produced in the food, by single or double gel diffusion in tubes or by the double microdiffusion tests (Hall et al., 1965). As a third control, the same foods were inoculated with nontype-B enterotoxin-producing strains and then compared with the foods inoculated with type B strains. Finally, positive smears were treated first with antienterotoxic antiserum and then the conjugate and checked for positive staining.

All preparations were examined by Zeiss fluorescent microscope with an Osram HBO 200-W maximum-pressure mercury-vapor arc. The UG5 filter was used as an ultraviolet excitation filter, and the combination 0/41 as a barrier filter.

RESULTS AND DISCUSSION

Demonstration of enterotoxin B. Enterotoxin B was demonstrated in well-fixed thin food smears by the FAT around the bacterial cells producing it. The microscopic appearance of the smears positive for enterotoxin was the same as that described for smears prepared from broth cultures (Genigeorgis and Sadler, 1966). There were halos of amorphous precipitates around the toxin-producing cells, sometimes in the form of clouds covering them completely or precipitates only in the interstitial spaces or completely separated from the cells (Figs. 1, 2). Depending on the dilution of the conjugate used, there was a variation in the morphology of the fluorescent precipitates or clouds. The problem of optimum proportions of the reagents which was found in the studies with broth cultures was not so critical here. Smears prepared from the same sample but stained with different dilutions of the conjugate showed a wide range of positive reactions. Smears from meats with more than 120 µg toxin/ml were stained positively with dilutions of the conjugate ranging from 1:5 to 1:80 and sometimes even to 1:160. Smears from broth cultures with the same toxin concentration were positive only with conjugate diluted 1:5. The results obtained with food smears seem to simplify the method of staining by eliminating the use of many conjugate dilutions to get optimum proportion with the antigen. The wider range of positive reactions would appear to be due to the different amounts of toxin present around the individual cells or colonies. Sugiyama et al. (1960) demonstrated a variation in the

Fig. 1. Positive Reaction. Growth and enterotoxin B production by strain S-6 in ham after 48 hr of incubation at 37°C. Impression smear fixed in cold ethanol and stained with 1:20 diluted conjugated antienterotoxin-B. 2500x.

Fig. 2. Positive Reaction. Growth and enterotoxin B production by strain S-6 in chicken meat after 48 hr of incubation at 37°C. Impression smear fixed with gentle heat and stained with 1:40 diluted conjugated antienterotoxin-B. 2500x.
amount of toxin produced by different cells. Good positive reactions can, therefore, usually be obtained since some of the bacterial colonies will have toxin in optimum proportions with the conjugated antibody.

With food smears as with broth smears, washing decreased the brilliancy of the fluorescent precipitates. This effect depended greatly on the thickness of the smears and the amount of enterotoxin present. The precipitates were denser in the presence of more enterotoxin, so the effect of washing was minimal. The zone of optimum staining was narrower for smears prepared from foods with small toxin concentration than for smears prepared from foods with higher toxin concentration. Positive reactions were better when there was an additional 4 hr of incubation of coverslips at 4°C.

Frozen section gave the same results as smears. Cross sections of the set of two inoculated discs demonstrated the growth of cells and presence of enterotoxin between the two layers, and a decrease in fluorescent precipitates as the distance increased from the interface.

Sensitivity of the tests. The demonstration of enterotoxin B in food smears by the FAT appeared to be more sensitive than the presently applied method of extraction and gel diffusion. A series of sterile ham discs were inoculated with the same number of cells of strain S-6 (producing both A and B enterotoxins) and checked for toxin production at 3-hr intervals by the FA and gel diffusion (single and double) techniques. Food samples were demonstrated positive for enterotoxin by the FAT much earlier than the time when 1 μg toxin/ml had been produced, the amount needed for its detection by gel diffusion (Genigeorgis, 1965). The FA test showed fluorescent precipitates and clouds around the cells which became denser as the concentration of toxin increased. The FAT showed masses of cells covered with precipitates while the food extract was still negative for enterotoxin by gel diffusion, although enterotoxin could be demonstrated by the latter technique if the food extract was first concentrated 5 times or more. Strongly FA-positive smears were obtained when the extracts were weakly positive by gel diffusion technique. It is considered that the toxin in the extract is the average amount of toxin per unit of volume of the food buffer mixture, and this amount should be many many times smaller than the amount of toxin around the toxic cells per unit volume of food in that area. This may also explain the difference in the sensitivity of FAT when applied to smears prepared from broth cultures or solid foods.

Demonstration in impression smears or drops from toxic food slurries. Smears prepared from slurries (50% in phosphate-buffered saline) were positive for fluorescent precipitates when the concentration of enterotoxin was greater than 5 μg/ml. With smaller concentrations, results were variable. The sensitivity was increased when less than 1:1 buffer was used to prepare the slurry. It appears that, during the preparation of the slurry, the foci of the toxin concentration are destroyed, and so the toxin becomes evenly distributed. It is obvious that if the concentration of toxin around the cells was already small, it would become smaller, and with the added effect of washing, the smear could be negative or be only weakly positive even though the food contained enterotoxin.

Smears made from centrifuged slurry were FA negative when the supernatant liquid showed small amounts of toxin by gel diffusion. Possibly, homogenization and centrifugation completely removed toxin that was only loosely associated with the cells. This seems to be substantiated by the fact that the cell walls were intensely stained, showing little or no toxin, and the appearance was the same as a nontoxic strain stained as a result of the naturally occurring antibodies in the conjugate reacting with surface antigens (Genigeorgis and Sadler, 1966).

The results were more consistent when the drop technique was used. Since the amount of slurry used is more than can be used to make a smear, more toxin was available to react with the conjugate in an unfixed fluid medium. This aided in the formation of precipitates which were caught later by the filter membrane.

Demonstration in food extracts. Clear food extract from which the fat was removed
by cooling at 4°C was tested for the presence of enterotoxin by the drop technique. Large precipitates were formed with low dilutions of the conjugate. Positive results were more consistent than those obtained with undiluted supernatants from broth cultures. These broths, with over 120 µg toxin/ml, were negative or weakly positive by the drop FAT; however, they proved to be positive when they were diluted 1:3 or 1:5 with buffer, and an increased range of dilutions giving positive reactions was obtained (Genigeorgis and Sadler, 1966). This may be explained by both an excess of antigen and by the effect of constituents in the broth itself. Food extracts gave better reactions by far with the conjugate than did broth cultures. Smaller amounts of toxin were also detected, possibly because there were less soluble solids to affect the antigen-antibody reaction.

**Staining of controls.** Foods tested for enterotoxin following inoculation with enterotoxin-B-producing strains, S-6 and 243, were also tested for toxin after inoculation with non-enterotoxin-B-producing strains. The inoculations were heavy to assure good growth. Sterile foods (cheese, ham, and chicken meat) which were inoculated with strains that do not produce type B enterotoxin were negative for precipitates or clouds around the bacterial cells by the FAT. Depending on the strain, the cells demonstrated varying classical fluorescence, and the microscopic appearance was the same as that of smears made from broth cultures. Ham, vacuum-packed commercially, showed a variety of rods, cocci, and yeast in addition to the cells of the inoculum. Some of the yeasts were stained intensely green, while the rods and other cocci were blue-violet or gray with an amorphous, thin, uniform cloud of fluorescence which was easily distinguished from specific fluorescent precipitates or clouds around enterotoxin-B-producing cells.

Smears were made from uninoculated nonsterile foods and treated the same way as smears made from foods inoculated with strains S-6 and 243. No typical fluorescent precipitates or clouds were demonstrable. A nonspecific fluorescence was obvious in muscle fibers. Natural flora composed of rods and cocci did not show any typical precipitates or clouds around the bacterial cells. With sterile food there was little nonspecific staining, if any, and when present it was weak.

Smears made from foods which had been found to contain large amounts of enterotoxin B by the FA and gel diffusion techniques were treated with 2 drops of 1:5 unconjugated antiserum, incubated for 30 min at 37°C, washed once with buffer for 10 min, air dried, and stained with various dilutions of the conjugate (1:5-1:160). Smears so treated were negative for fluorescent precipitates or clouds, but there were cells showing varying fluorescence in the walls. It is believed that surface antigens of these cells reacted with anti-staphylococcal antibodies normally existing in the conjugate after specific precipitates were washed off during the first washing.

Smears made from foods positive for large amounts of enterotoxin were stained with normal conjugated antiserum obtained from a young rabbit never used before for any immunization. The smears were negative for enterotoxin, but individual cells were stained green. It was difficult to demonstrate the presence of enterotoxin in smears made from nonsterile meat products or their slurries previously inoculated with type B strains when the toxin was present only in small amounts. In this case, it was difficult to identify fluorescent particles as being specific precipitates rather than autofluorescent meat material. As the concentration of toxin around the cells increased, the morphology of the specific precipitates became characteristic, permitting identification. As with culture media (Genigeorgis and Sadler, 1966) the demonstration of enterotoxin B by FAT in foods is based only on the presence of morphologically specific fluorescent precipitates around the bacterial cell and not on the presence of fluorescent cells alone. Efforts to prepare a conjugated antienterotoxin B serum which will specifically stain the enterotoxin-producing cells even in the absence of enough toxin to form precipitates, were unsuccessful. Such a conjugated antiserum will, of course, be ideal since precipitates can be washed off while an antigen attached on the cell cannot.
REFERENCES


Ms. rec’d 12/18/65.

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