Detecting the Enterotoxigenicity of *Staphylococcus aureus* Strains

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An optimal sensitivity plate method for examining large numbers of staphyloccocal strains for production of the known enterotoxins (A–E) is presented. Small volumes of relatively concentrated enterotoxin are produced by the semi-solid agar, cellophane-over-agar, or sac culture techniques. Detection of the enterotoxin in the supernatant fluid is accomplished with the optimal sensitivity plate method. In this method small plastic petri dishes (50 mm) were used for a modified Ouchterlonny of high sensitivity.

The staphylococci are important not only in staphylococcus food poisoning but also in many human infections. There is a concern that the enterotoxins which are the cause of food poisoning may also play a role in these infections. It will be necessary to examine large numbers of strains of staphylococci for enterotoxin production that may either be involved or suspected of being involved in staphylococcus food poisoning and in human infections.

Several methods have been proposed and are in use for production and analysis of the enterotoxins. In the analysis of a strain for enterotoxigenicity only a small amount of culture supernatant is needed, thus methods have been provided that give a few milliliters of relatively concentrated enterotoxin. These methods include the sac-culture techniques of Casman and Bennett (3) and Donnelly et al. (5), the semi-solid plate technique of Casman and Bennett (3), and the cellophane-over-solid-agar-plate of Hallander (6). The enterotoxin obtained by these techniques was assayed by the microslide technique (4).

This communication presents a relatively simple method for detection of staphylococcus strains that produce any of the identified enterotoxins (A–E).

**MATERIALS AND METHODS**

**Organisms.** A variety of *Staphylococcus aureus* strains were used for this work, including strains Food Research Institute (FRI)-100 (enterotoxin A), FRI-722 (enterotoxin A), S-6 (enterotoxins A and B), FRI-901 (enterotoxin B), FRI-137 (enterotoxin C₁), FRI-472 (Casman-296; enterotoxin D), and FRI-326 (enterotoxin E). Included in the experiments were strains that produced unidentified enterotoxins as determined by monkey feeding tests.

**MEDIA.** Two media were used in the tests. Medium 1 was the same as that described by Bergdoll et al. (2) which contained 3% N-Z Amine NAK (Humko Sheffield Chemical, Lyndhurst, N.J.) plus 3% protein hydrolysate powder (Mead Johnson International, Evansville, Ind.), and 0.001% of niacin and 0.0005% thiamine per liter of culture medium (3 + 3 medium). The pH was adjusted to 6.8 before autoclaving. Medium 2 was brain heart infusion (BHI) (Difco) as described by Jarvis and Lawrence (7).

**Enterotoxin production: cellophane-over-agar.** The method of Hallander (6), as applied by Jarvis and Lawrence (7), was used. Circles of cellophane were cut from 1/4-inch dialysis tubing (Arthur H. Thomas, Philadelphia) using 9-cm filter paper as a template. The cellophane circles (15 to 20) were placed alternately with filter papers in a 9-cm glass petri plate, moistened with distilled water to eliminate wrinkling, and autoclaved for 20 min at 121 C. The sterile cellophane circles were aseptically transferred to petri plates (100 by 15 mm) containing 15 to 20 ml of medium. One-tenth milliliter of inoculum (5 ml of BHI in a 15 by 125 mm screw-cap test tube inoculated with the appropriate staphylococcus culture and incubated at 37 C for 18 to 20 h) was spread over the cellophane with a bent glass rod. The plates were incubated for 24 h at 37 C after which the growth was harvested from the cellophane with 2.5 ml of 0.01 M Na₂HPO₄. The cell suspension was centrifuged and the supernatant fluid was analyzed for enterotoxin.

**Enterotoxin production: semi-solid agar.** The method as originally outlined by Casman and Bennett (3) was used in this investigation. In this method one loopful of growth from a BHI slant (18 to 20 h) was suspended in 4 ml of 0.02 M sodium phosphate, pH 7.4, in 0.9% NaCl (phosphate-buffered saline) and 4 drops of the suspension were spread over the agar surface (15 to 20 ml of medium containing 0.7% agar in 10-cm petri plates) with a bent glass rod. The plates were incubated at 37 C for 48 h. The entire contents were centrifuged in 50-ml polycarbonate centrifuge tubes at 39,100 × g for 10 min and the supernatant fluid was analyzed for enterotoxin.
Enterotoxin production: sac culture. For the sac culture method of Donnelly et al. (5), 100 ml of medium was placed inside a sac made from a piece of dialysis tubing approximately 3 cm wide by 65 to 70 cm long (A. H. Thomas, Philadelphia). The tube was placed in a 250-ml Erlenmeyer flask in a U-shape and the flask was stoppered and autoclaved. Two milliliters of the inoculum (prepared in the same manner as for the semi-solid agar method) were added to 18 ml of sterile phosphate-buffered saline and the mixture was transferred into the Erlenmeyer flask. The flasks were incubated at 37 C on a gyratory shaker at 200 rpm for 24 h. The liquid was removed from the flasks and centrifuged, and the supernatant fluid was analyzed for enterotoxin.

Enterotoxin production: shake flasks. The method outlined by Kato et al. (8) was used. In this method 50 ml of medium in 250-ml Erlenmeyer flasks were inoculated with a 1% inoculum (same as for the cellophane-over-agar method) and incubated for 18 to 24 h at 37 C on a gyratory shaker at 230 rpm. The culture was centrifuged and the supernatant fluid was examined for enterotoxin.

Assay of enterotoxin. The enterotoxin was assayed by the single gel diffusion tube method of Bergdoll (8; M. S. Bergdoll, Proc. 14th Res. Conf. Amer. Meat Inst. Found., Circl. no. 70, p. 47, 1962) using standard curves prepared with known amounts of purified or standardized crude enterotoxins.

Detection of enterotoxin. Detection of the enterotoxins in the culture supernatant fluids was accomplished with the optimal sensitivity plate (OSP). Small plastic petri dishes (Falcon Plastic no. 1006) (50 by 12 mm) with tight lids were used. Three milliliters of 1.2% Noble Agar (Difco) PBS with thimerosal (1 to 10,000) were used for each plate. A \( \frac{3}{4} \) -inch thick Plexiglas template (Fig. 1) was positioned over each plate, and then cork borer was used to cut seven wells in the agar. The agar plugs were removed with suction. The antiserum was placed in the center well, the control enterotoxin in the two small wells, and the unknown samples in the four larger outer wells.

For development, the petri dishes were closed and stored in a humidified box at 37 C overnight. A nearly airtight plastic rectangular box, such as is commonly used for refrigerator storage, was used; 5-ml breakers containing water were placed in each corner. At 37 C, 18 h is sufficient for full development as compared to 24 to 30 h at room temperature.

RESULTS AND DISCUSSION

Enterotoxigenic staphylococci. The means for actually determining whether a staphylococcus strain is enterotoxigenic is dependent upon biological methods such as the monkey feeding test. The criterion used at the FRI for labeling strains enterotoxigenic is emesis in two or more of six rhesus monkeys when each is given the equivalent of 1 liter of supernatant fluid from a staphylococcus strain grown in shake flasks (M. S. Bergdoll, Proc. 14th Res. Conf. Amer. Meat Inst. Found., Circl. no. 70, p. 47, 1962). This indicates the production of 10 to 20 \( \mu \)g of enterotoxin per liter or 0.01 to 0.02 \( \mu \)g per ml. The sensitivity of the method described in this paper is sufficient to detect the enterotoxin obtained from strains producing these small amounts. A strain (FRI-988) producing very small amounts of enterotoxin A, the enterotoxin most commonly encountered in food poisoning, serves as a control strain to determine if the methods, as used, are sensitive enough.

Comparison of membranes in the cellophane-over-agar method. Membranes given us by other workers were tested concurrently with the dialysis tubing used at FRI to determine if the type of membranes might be responsible for the poor results reported by some laboratories (9, 11). Table 1 indicates the dialysis tubing (Union Carbide obtainable from A. H. Thomas) is the superior membrane. We chose this film for more uniformity of pore size, molecular weight cut-off of 12,000, lack of interfering surfactants, and ease of availability. It appears these features result in consistent results and one should not leave the choice of cellophane to chance. If this tubing is stored in the refrigerator, good results are obtainable for at least one year.

Production of enterotoxin by different methods. The results obtained from a comparison of the cellophane-over-agar, semi-solid agar, sac-culture, and shake-flask methods for production of enterotoxins A-E are given in Table 2. Overall, the sac-culture method of Donnelly et al. (5) was superior. The cellophane-over-agar method was about equal to the sac-culture method for enterotoxins A, D and E; however, it produced much less enterotoxins B and C. Since enterotoxins B and C are usually produced in larger quantity, the amounts produced in this method were sufficient for detection.

| Table 1. Enterotoxin production: cellophane-over-agar method |
|------------------------------------------------------------|
| Staphylococcus strain | Enterotoxin production (\( \mu \)g/ml)* | Shake-flask |
|-----------------------|------------------------------------------|-------------|
|                       | 1            | 2          | 3        |                          |
| FRI-100 (A)*          | 26           | 2          | 4        | 3                       |
|                       | (19-35)*     | (6-3)      | (2-5)    | (2.5-4.0)               |
| FRI-722 (A)           | 190          | 26         | 50       | 46                      |
|                       | (130-230)    | (18-39)    | (38-60)  | (40-46)                 |
| S-6 (B)               | 880          | 150        | 310      | 360                     |
|                       | (800-950)    | (110-180)  | (270-360)| (350-370)               |

* 1. 3/4-inch visking dialysis tubing (Union Carbide Co.); 2. Einmahl Zellgass; 3. cellophane supplied by H. O. Halander.
* Type of enterotoxin in parenthesis.
* Range of enterotoxin concentration of five replicates.
The results obtained using different media also are given in Table 2. Any of the media tested appeared to be satisfactory for all of the production methods. The 3 + 3 medium is the one that has been found to be most useful in the FRI for all strains. Since the ingredients for this medium are not too readily available, probably BHI would be the medium of choice for most users.

Five staphylococcus strains that were low producers of enterotoxin A were grown using the different production methods. The results from these studies are given in Table 3. Positive results were obtained with all methods except in shake flasks, with the sac-culture method being slightly superior to the cellophane-over-agar method. Questionable results had been obtained in other laboratories with these strains; in the present experiments positive identification of enterotoxin production was obtained in all cases by using the combined cellophane-over-agar and OSP methods.

| Table 2. Enterotoxin production by different methods* |
|---------------------------------|
| Enterotoxin | Enterotoxin amount (µg/ml) |
| | Shake-flask | Semi-solid | Sac-culture | Cellophane-over-agar |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| A (FRI-100) | 2.6 | 4 | 7 | 8 | 14 | 21 | 26 | 19-27* | 21 | 32 |
| B (FRI-901) | 26.0 | 11 | 12 | 11 | 100 | 260 | 220 | 15-35 | 1 | 3 |
| C (FRI-137) | 72.0 | 20 | 36 | 0 | 360 | 250 | 400 | 143-185 | 110 | 86 |
| D (FRI-472) | 0.5c | 1-2 | 1 | 1 | 4 | 11 | 5 | 4 | 5 | 4 |
| E (FRI-326) | 4.1 | 10 | 4 | 5 | 16 | 25 | 28 | 16-32 | 25 | 28 |

*The results represent five replicates. Medium 1, 3 + 3; medium 2, BHI(1X); medium 3, BHI(2X).

*The higher values were obtained with new cellophane from A. H. Thomas; the lower values from cellophane approximately 1-year-old.

*Values for enterotoxin D were estimated from the OSP plate.

| Table 3. Enterotoxin A production by Staphylococcus strains producing small amounts: comparison of production methods |
|---------------------------------|
| Staphylococcus strain | Production method* |
| | Shake-flash | Semi-solid agar | Sac-culture | Cellophane-over-agar |
| | BHI(1X) | BHI(1X) | BHI(2X) | BHI(1X) | 3 + 3 medium |
| G-1507 | - | + (S)c | - | + | + |
| G-1508 | - | + | - | + | + |
| L-17 | - | + (HS)d | - | + (HS) | + |
| L-19 | - | + (HS) | - | + | + |
| T-F68 | - | + (HS) | - | + | + |

*All results were without concentration of supernatant fluid.

*Negative.

*Positive after treatment with H₂PO₄.

*Hook after treatment with H₂PO₄.

*Hook.
A range of 1 to 4 μg/ml in the unknowns give easily observable lines, roughly halfway between the toxin and the serum wells. Overall, the OSP will detect the presence of enterotoxin at concentrations from 0.5 μg/ml to upwards of 64 μg/ml.

At 0.5 μg/ml a positive hook (Fig. 2, 1XA-U1), that is, a curving around of the standard line, is usually observed. Flooding the plate with 0.1 M H₃PO₄ will make a line more visible. It is well to confirm a hook by concentration of the sample and re-running it (Fig. 2, 5XA-U1). The results obtained with a high concentration of enterotoxin in the unknown are shown in Fig. 2, 1XC, and Fig. 3. In the first (Fig. 2, 1XC-U4), the line appears to be split into two lines and in the latter only a short standard line may be visible until after the plate is treated with 0.1 M H₃PO₄ (Fig. 3). Note that the standard line is not only short, but appears cut off in the direction of U2. Sometimes an unknown appears to be positive after concentration, as indicated in Fig. 2, 5XB-U2. The hook shown could be due to the high concentration of enterotoxin B in U1; therefore, U2 should be re-run separately.

Hundreds of cultures have been checked for enterotoxigenicity by the cellophane-over-agar-OSP combination in the FRI. There has been no evidence that any false positives have been obtained or that any producers of known enterotoxins were missed. Strains that were determined to be questionable producers of enterotoxin A were found to be enterotoxigenic at the FRI (Table 3). Eight strains that Toshack and Thorsteinson (10) examined in their laboratories by the soft agar plate method for enterotoxin production and the microslide for analysis and found to produce enterotoxin D were found to be D producers at the FRI. Eight strains which they found to be negative for enterotoxins A–D were found to be negative for A–E at the FRI. All eight of the latter were enterotoxigenic by the monkey-feeding test (1) at a level that would have been easily detectable by the OSP method if they did produce known enterotoxins; these results confirm the hypothesis that the strains produce as yet unidentified enterotoxin.

Fig. 1. Plexiglas template for OSP. Drawing not to scale.

Fig. 2. Enterotoxin analysis in OSP. A, B, C indicates enterotoxins A, B, C, respectively (4 μg/ml). aA, aB, aC indicates anti-enterotoxins A, B, C, respectively. Unknown U1 contains approximately 0.5 μg of A and 6 μg of B per ml; unknown U2 contains no enterotoxins A, B, C; unknown U3 contains approximately 2 μg of A per ml; unknown U4 contains approximately 16 μg of C per ml. IX indicates unconcentrated unknowns; 5X indicates 5 times concentrated unknowns.

Fig. 3. Enterotoxin analysis in OSP. Unknown sample U2 contains >64 μg of enterotoxin per ml, left plate is untreated; right plate is after treatment with 0.1 M H₃PO₄.
The sensitivity of the OSP method is not equivalent to the microslide technique, but with concentrated supernatant from the cellophane-over-agar production method the sensitivity is adequate for detection of enterotoxigenic strains. The requirements for purification of the test samples are less for the OSP than for the microslide, and the volume of sample is similar. The amount of antiserum per assay is about the same for the two methods. The major advantage of the OSP method is the ease in handling and the lower technical ability requirements of the operator. It is easier to prepare and to set up for enterotoxin analysis. Also, it is easier to observe the development of the precipitate lines as this can be done at any stage in the development and can be delayed for a longer period of time if necessary. Lines of identity, spurs, and crossing of lines are more readily visible in the OSP method.

The results show that the OSP plate is sufficiently sensitive for use with the cellophane-over-agar and sac-culture-production methods, but questionable with the semi-solid agar, and inadequate with shake flasks for detection of low enterotoxin producers. If one chooses to utilize the latter production methods, then it is necessary to do the enterotoxin analysis with the microslide or to concentrate the culture supernatant fluid for use with OSP.

The results show the sac-culture-production method to be superior for all-around enterotoxin production, but because of the simpler operation of the cellophane-over-agar method it is recommended as the one of choice. When questionable results are obtained with this method, the sac-culture method could be used as a secondary method for confirmation.

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