APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1980, p. 1080–1085
0099-2240/80/12-1080/06$02.00/0 Vol. 40, No. 6

Single Radial Immunodiffusion Method for Screening Staphylococcal Isolates for Enterotoxin

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A direct system for screening large numbers of staphylococcal isolates for enterotoxin production has been developed. The system employs polyvalent (serotypes A, B, C, D, and E) immunodiffusion assay slides in conjunction with a multiple-culturing system for toxin production. With the combined system, as many as 50 cultures can be screened simultaneously on a single assay slide having a sensitivity of about 0.3 μg/ml. The system should be useful for detecting potential enterotoxin in foods containing a predominance of non-enterotoxigenic strains.

Characteristics such as production of coagulase and thermal stable nuclease, although useful for identifying *Staphylococcus aureus*, do not have 100% correlation with ability to produce enterotoxin. No one characteristic or combination of characteristics is an absolute indicator of enterotoxigenic strains. It has been reported that 92% of enterotoxigenic staphylococci tested produced coagulase, and 93% produced thermal stable nuclease (M. Yeterian, R. W. Bennett, and W. Smith, Abstr. Annu. Meet. Inst. Food Technol. 1977, p. 86). The non-enterotoxigenic staphylococci tested were 84% coagulase positive and 86% positive for thermal stable nuclease.

Therefore, we believe that testing directly for toxin production is the only reliable way for screening isolates. This can be accomplished presently with the microslide gel immunodiffusion test (2) or the optimum sensitivity plate method (7). However, for large numbers of isolates, these methods are time consuming and require a considerable supply of antitoxin.

We have developed and tested a polyvalent single radial immunodiffusion (SRD) method which can be used with a multiple-culturing procedure for screening large numbers of food or other isolates for their ability to produce staphylococcal enterotoxins. The combined system is rapid, inexpensive, and can be used both as a quality control measure during food production and as a regulatory indicator for potential sources of staphylococcal food poisoning. Studies on development of this system are presented in this paper.

(This paper was presented in part at the 79th Annual Meeting of the American Society for Microbiology, May 7, 1979, in Los Angeles, Calif.)

MATERIALS AND METHODS

Cultures. The source of enterotoxigenic staphylococci and estimated values on toxin production for strains used in most experiments are listed in Table 1. Low-producing enterotoxigenic strains (276, 1128, 888, 901, 995, 283, 406, 1004, 1001, 361, 918, 920, 922, and 923) used in one experiment were obtained from M. S. Bergdoll and R. Robbins. Serotypes and estimated toxin concentrations are shown below (see Table 4). Non-enterotoxigenic strains included: D-153, D-158, D-159, D-160, D-165, D-166, D-167, D-192, D-235, D-184, D-87, W-46, and 227, obtained from R. Bennett; 184, 329, 823, 953, and 1017, obtained from M. S. Bergdoll and R. Robbins; and 50 other isolates from routine food samples analyzed at the New York Regional FDA Laboratories, Brooklyn, N.Y. Strains of *Proteus vulgaris*, *Escherichia coli*, *Sarcina lutea*, *Pseudomonas florescens*, *Proteus mirabilis*, *Streptococcus fecalis*, *Micrococcus* spp., and *Bacillus* spp. were from the collection at the FDA Laboratories.

Enterotoxin and antisera. Highly purified enterotoxins (serotypes A, B, C, D, and E) and their corresponding antisera were provided by R. Bennett. Enterotoxin reference standards containing 5, 2.5, 1.25, 0.62, 0.31, and 0.15 μg/ml were purified, and lyophilized toxin was diluted with 0.3% proteose peptone (Difco Laboratories, Detroit, Mich.) in 0.85% NaCl adjusted to pH 7.0.

The sac culture method of Donnelly et al. (3) and the semisolid agar plate method of Casman and Bennett (1) were used to produce enterotoxin. Briefly, in the sac culture method, a dialysis sac containing brain heart infusion broth is placed in an Erlenmeyer flask containing about 20 ml of sterile phosphate-buffered saline and inoculated with 3×10^6 organisms. After shaking at 35°C for 24 h, the phosphate-buffered saline is centrifuged, and the supernatant solution is tested for enterotoxin. In the semisolid agar method, 25 ml of brain heart infusion broth (Difco) containing 0.7% melted agar is poured into plates (100 by 15 mm) and allowed to cool. The plates are inoculated with 0.2 ml of a saline suspension (3×10^6 cells/ml), which is spread carefully over the agar surface with sterile L-shaped glass rods. After 48 h at 35°C, the agar is broken up and centrifuged for 15 min at 15,000 rpm at 4°C, and the supernatant liquid (7 to 10 ml final volume) is tested for enterotoxin.

TABLE 1. *Source of enterotoxigenic staphylococcal strains*

Enterotoxin serotype	Strain no.	Estimated toxin production (μg/ml)[a]	Source
A	743	4	R. Bennett[b]
A	246-3A	8	R. Bennett
A	FDA-2	2	R. Meyer[c]
B	243	High	R. Bennett
B	778	High	R. Bennett
B/A/D	485	High/2/3	R. Bennett
B	995	2	R. Robbins[d]
C	834	High	R. Bennett
D	315	3	R. Bennett
A/D	FDA-1	ND[e]	R. Meyer
A/D	878	ND	R. Robbins

[a] Toxin concentrations were determined by R. Robbins using the membrane-over-agar method for production and the optimum sensitivity plate assay (7).

[b] Bureau of Foods, Food and Drug Administration, Washington, D.C.

[c] Food and Drug Administration, N.Y. Regional Laboratory, Brooklyn, N.Y.

[d] Food Research Institute, University of Wisconsin, Madison.

[e] ND, Not determined.

Agar-gel (double strength). Solution A (5.68 g of $NaHPO_4$, 17 g of NaCl, 0.2 g of thimerosal, brought to 1,000 ml with distilled water) was adjusted to pH 7.4 by addition of solution B (5.52 g of NaH_2PO_4, 17 g of NaCl, 0.2 g of thimerosal brought to 1,000 ml with distilled water). Twenty-four grams of Noble agar (Difco) was added to 976 ml of the above mixture. After brief boiling to dissolve the agar, the solution was filtered, hot (>55°C), through Whatman no. 114 paper, dispensed in 20-ml volumes, and stored at 4°C.

SRD assay slides. Standard microslides (75 by 22 mm) were coated by dipping into a beaker of melted and cooled (55 to 75°C) 0.2% agar (Difco, bacteriological grade) and air dried. Appropriate dilutions of antisera were prepared in 0.85% NaCl at 56°C and added in equal volumes to melted double-strength agar-gel at the same temperature. The antiserum-charged agar-gel had a final concentration of 1.2% agar, 1.275% NaCl, 0.1% thimerosal, and 0.2 M sodium phosphate buffer, pH 7.4. Using a warm, wide-tip, 10-ml pipette, 3 ml of charged agar-gel was layered carefully onto the surface of a slide previously coated with 0.2% agar. The pipette was held perpendicular to the slide, and the agar-gel was allowed to flow uniformly over the surface (Fig. 1). If the agar-gel is too viscous due to cooling, the pipette can be guided over the slide to allow for even dispersal. After solidification (1 to 2 min), slides were refrigerated for 10 to 20 min. Two parallel rows of five wells (3-mm diameter) were cut through the agar-gel layer with an immunodiffusion punch set (Gelman Sciences, Inc., Ann Arbor, Mich.), and plugs were removed by aspiration with a Pasteur pipette.

Detection of enterotoxins by the SRD assay. Wells in single radial immunodiffusion (SRD) slides were filled to the top (approximately 10 μl) with test preparations or enterotoxin standards. The slides were incubated for 24 h at 35°C in a moist chamber. Enterotoxin samples, corresponding to the antiserum type in the agar, exhibited a precipitin ring around the well (Fig. 2). Visualization of rings was enhanced by immersing the incubated slide in 4% acetic acid for 10 to 20 min. Slides should be observed immediately, but for future reference they can be preserved by the method of Lumpkins (4).

RESULTS

Sensitivity of the SRD assay. The sensitivity of the assay system was determined in separate studies with purified enterotoxins A, B, C, D, and E. Eight dilutions of each antiserum in agar-gel were prepared in the range of 1:50 to 1:400 (i.e., 1:50, 1:75, 1:100, 1:125, etc.). SRD slides were prepared, and separate wells in each slide were charged with one of the six reference standards of purified toxin in the range of 0.15 to 5 μg/ml. After incubation, the slides were examined to determine the lowest antiserum dilution that exhibited the best precipitin reaction over the broadest range of toxin concentration. A second set of antiserum dilutions and SRD slides was prepared in a narrower range of concentrations which bracketed the dilution of antiserum determined above, and the test was repeated before selecting the final working dilution of antitoxin to be employed. Working dilutions were 1:200, 1:400, 1:360, 1:300, and 1:300 for antisera A, B, C, D, and E, respectively (final dilution in agar-gel). The outside diameter of each ring of precipitate that formed on the slides that had been prepared at the selected working dilution was plotted against the toxin concentration (Fig. 3). The lowest concentration of toxin detected in each case was approximately 0.3 μg/ml. Working dilutions must be determined periodically since they vary with age and lot of antiserum.

Effect of incubation time on precipitin ring formation. SRD slides were usually prepared in duplicate and incubated for 24 and 48 h, respectively. Precipitin rings were visible at both times with no detectable difference in diameter or brightness.

Specificity of the assay system. Since highly purified toxin had been employed for production of antiserum, it was not expected that nonspecific antigens would form immune precipitates. However, we tested over 70 nonenterotoxigenic strains. The strains were grown, and extracts were prepared by the semisolid agar method for toxin production. None of the extracts produced visible rings indicating false-positive (i.e., nonspecific) reactions. Among the organisms tested were three potentially troublesome strains (1017, 406, 995) known to produce large amounts of protein A.

Polyvalent assay system. It was found that

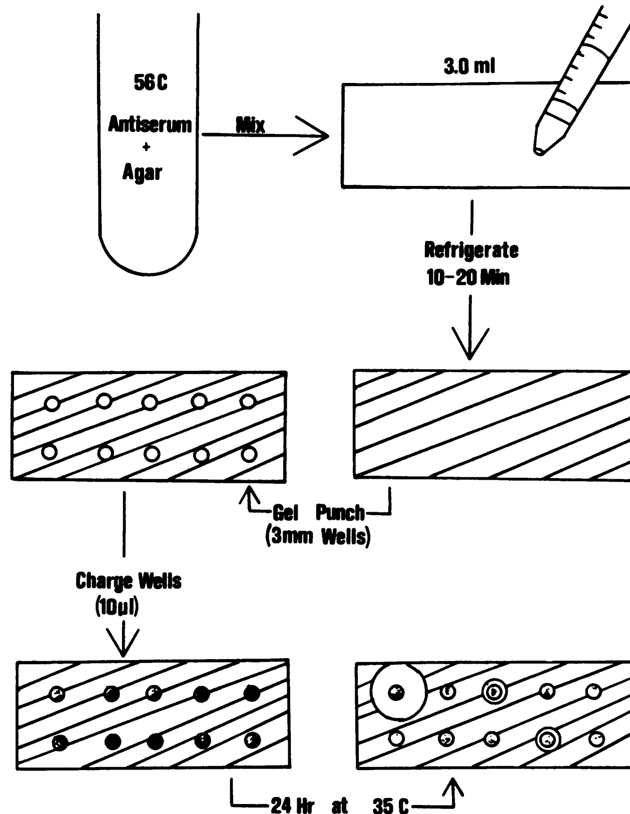

FIG. 1. *Outline of the steps involved in preparation of SRD assay slides.*

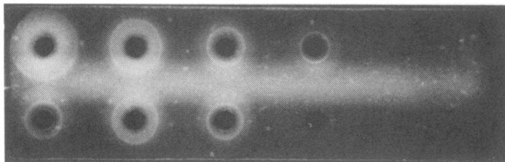

FIG. 2. *Typical monovalent (serotype B) SRD assay slides. Wells were charged as follows: top row (left to right), 5, 2.5, 1.25, 0.62, and 0 µg of purified type B toxin; bottom row (left to right), cultural toxin strain numbers 778, 243, and 485. The last two wells were not charged.*

all five antisera could be incorporated into the same agar-gel of an SRD slide to form a polyvalent detection system. The working dilutions of the antisera were the same as employed in monovalent slides. Separate wells of polyvalent SRD slides were charged with purified toxins (0.6 µg/ml) and also with crude toxins of the five serotypes prepared by the semisolid agar method. In every case, precipitin rings formed in polyvalent sera were equivalent to those formed on control gels containing only the individual antisera. Figure 4 shows a typical polyvalent slide charged with purified toxin. Although the polyvalent system cannot be used to indicate toxin type, a single SRD slide can be used to screen up to 10 preparations for enterotoxin.

Multiple-culturing technique. In an extensive screening for enterotoxigenic strains, the utility of the polyvalent SRD slide would be enhanced if several strains could be tested as a pool in a single well. Such a system would allow simultaneous testing of a number of isolates from a single food sample. Although toxin could be produced in separate cultures and pooled for testing, it would be less time consuming if several suspected isolates were grown together in a single culture, which could then be analyzed for enterotoxin. The dialysis sac and semisolid agar methods were tried initially with a mixed inoculum containing cells of a type A strain, a type B strain, and three non-enterotoxigenic strains, suspended together in saline. Neither method for enterotoxin production gave yields with mixed cultures that were readily detected by the monovalent SRD system. Since control cultures of individual toxigenic strains were positive, it became obvious that competition between organisms in mixed inocula prevented production of enterotoxin at detectable levels.

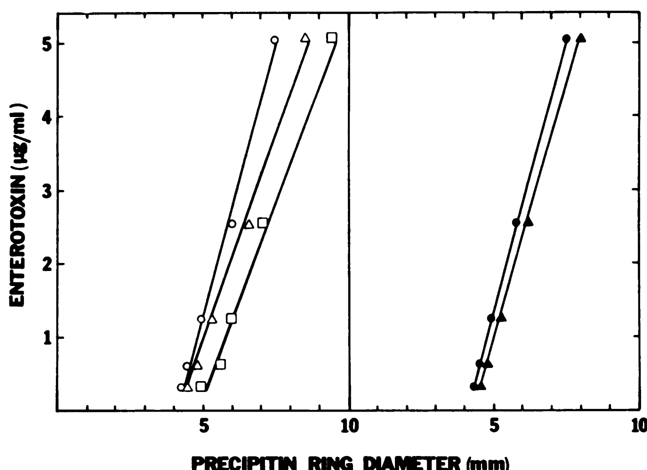

FIG. 3. *Relationship of precipitin ring diameter and staphylococcal enterotoxin concentration assayed by the SRD method. Symbols: ○, serotype C; △, serotype B; □, serotype A; ●, serotype D; ▲, serotype E.*

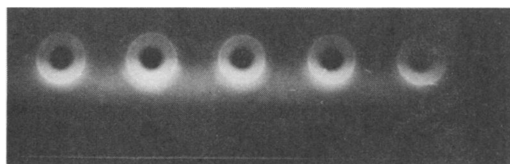

FIG. 4. *Typical polyvalent SRD assay slides. Wells were charged as follows: top row (left to right), 2.5 µg of purified staphylococcal enterotoxins A, B, C, D, and E per ml. All other wells were not charged.*

It was thought that strain competition could be avoided by using a semisolid agar plate, if separate areas of the agar surface were inoculated with individual strains.

We tried this, using a type A strain, a type B strain, and six non-enterotoxigenic strains (i.e., diluent strains) inoculated onto 40 ml of semisolid agar in large plates (150 by 15 mm). A paper template, marked off in eight (or the desired number) of equal-sized sectors, was placed under the bottom of the petri plate. Saline suspensions containing 3×10^6 cells/ml were prepared from heart infusion slants (24 h) of each of the eight strains. One or two drops (0.05 to 0.1 ml) of each suspension was spread over a separate sector of the agar surface with a sterile glass rod. Overlapping of inoculum and cutting into the agar surface were carefully avoided. After incubation (48 h at 35°C), the soft agar was centrifuged, and the supernatant liquid (10 to 15 ml, final volume) was analyzed for enterotoxin. Types A and B toxins were detected by monovalent SRD slides, as well as by double-diffusion microslides used as controls. To test this further, a number of toxigenic and nontoxigenic (diluent) strains in various combinations were grown on soft agar plates inoculated in separate areas as above. Table 2 shows the results obtained with three different type B strains. Each strain was grown in combination with up to 10 non-enterotoxigenic strains. The enterotoxin from the semisolid agar plate was analyzed both by monovalent SRD and by the microslide method as a control. Enterotoxin was detected by the SRD system in cultures of strains 243 and 485 grown together with up to 10 diluent strains. Toxin from strain 778 could be detected in cultures with up to seven diluent strains by SRD and in culture with 10 diluent strains by the microslide method. In all cases, the microslide method confirmed that the enterotoxin was type B.

TABLE 2. *Detection of enterotoxin B in semisolid agar inoculated with various combinations of toxigenic and nontoxigenic staphylococci*

No. of nontoxigenic strains	Enterotoxin detected[a]					
	243		778		485	
	SRD	DD	SRD	DD	SRD	DD
0	+	+	+	+	+	+
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	−	+	+	+
9	+	+	−	+	+	+
10	+	+	−	+	+	+

[a] Three type B enterotoxigenic strains (243, 778, 485) were tested in mixed cultures with from 0 to 10 nontoxigenic strains by monovalent SRD assay slides and by double-diffusion (DD) microslides; +, toxin detected; −, no toxin detected.

To determine whether an antigen excess situation would occur if all the organisms on the plate produced the same enterotoxin type, the entire plate was inoculated with 10 samples of the same type B strain. The precipitin reactions on SRD slides and on double-diffusion slides indicated that enterotoxin was not in excess.

Experiments similar to those in Table 2 were performed with serotypes A, C, and D enterotoxigenic strains. Table 3 summarizes the results of these experiments, including those with type B described above. Enterotoxin was detected by the SRD system with all enterotoxin types tested at a dilution of at least 5 and, in several cases, with up to 10 non-enterotoxigenic organisms. In all cases, the microslide system detected toxin in the presence of all 10 diluent strains. It was concluded that, for routine use, no more than five strains should be grown together on semisolid agar plates when screening for toxin by SRD.

Occasionally, staphylococci are encountered which produce very low levels of enterotoxin compared to the strains employed in the studies above. A number of these low-producing strains were obtained from M. S. Bergdoll and were analyzed by the multiple-culture system to determine whether or not they could be detected when grown with four nontoxigenic organisms.

With some of these cultures, information was supplied as to the amount of toxin produced. In others, we determined approximate concentrations using the microslide procedure after individual growth on separate plates of semisolid agar. The results are shown in Table 4. All but three strains (276, 923, and 283) produced toxin sufficient for detection by both poly- and monovalent SRD assays when cultured individually or in multiple culture with four nontoxigenic strains. One isolate, 361, produced enough type C enterotoxin to be detected by polyvalent SRD after growth in multiple culture, but not enough enterotoxin D to be detected with the monovalent SRD. In all cases, toxin was detected by the microslide system.

TABLE 3. *Detection of enterotoxin in semisolid agar inoculated with various combinations of toxigenic and nontoxigenic staphylococci*

Toxigenic strain no.	Enterotoxin (serotype)	No. of nontoxigenic strains[a]
743	A	10
246-3A	A	10
FDA-1	A/D	7/8
FDA-2	A	10
243	B	10
778	B	7
485	B	10
834	C	5
315	D	7

[a] Each enterotoxigenic strain was grown in semisolid agar culture with from 0 to 10 nontoxigenic strains and assayed by SRD. Numbers indicate the highest number of diluent nontoxigenic strains that resulted in a preparation having detectable enterotoxin levels. Enterotoxin was confirmed by the microslide assay.

TABLE 4. *Detection of enterotoxin in semisolid agar inoculated with low enterotoxin-producing strains of staphylococci either individually or in combination with four nontoxigenic strains*

Strain no.	Enterotoxin serotype	Estimated concn (μg/ml)	Individual culture		Multiple culture	
			Mono SRD	Poly SRD	Mono SRD	Poly SRD
276	A	0.3[a]	+	+	+	+
1128	A	0.3[a]	–	–	–	–
888	A	0.3[a]	+	+	+	+
922	A	0.3[a]	+	+	+	+
923	A	0.3[a]	–	–	–	–
901	B	11.0[b]	+	+	+	+
995	B	2.0[b]	+	+	+	+
283	A/B	2.0[b]/0.3[a]	–/–	–	–/–	–
406	C	7.0[b]	+	+	+	+
1004	C	3.0[b]	+	+	+	+
1001	C	1.0[b]	+	+	+	+
361	C/D		+/+	+	+/–	+
918	E		+	+	+	+
920	E		+	+	+	+

[a] Values for estimated concentrations were determined in our laboratory by the microslide procedure after 48 h of growth on semisolid agar plates.

[b] Estimated concentrations were supplied by M. S. Bergdoll. Values were obtained from preparations made in semisolid agar, shake flask, or membrane-over-agar methods assayed by the optimum sensitivity plate method (7).

Interference by non-staphylococcal organisms. An experiment was performed to determine whether the growth of nonstaphylococcal organisms in the multiple-culturing system would affect enterotoxin production and detection. Combinations of enterotoxigenic strains and non-staphylococcal strains were inoculated on separate areas of semisolid plates as listed in Table 5. After incubation, preparations from these plates were tested for enterotoxin by SRD and by the microslide system (Table 5), and in every case enterotoxin was detected.

DISCUSSION

We have shown that SRD, first described by Mancini (5) for quantitation of immunoglobulins, can be used for the detection of crude staphylococcal enterotoxin. The sensitivity of the method (0.3 μg/ml) is comparable to the optimum sensitivity plate method of Robbins (7) and allowed detection of enterotoxin from all nine of the first group of organisms tested and

TABLE 5. *Detection of enterotoxin in semisolid agar inoculated with various combinations of enterotoxigenic staphylococci with non-staphylococcal strains*

Enterotoxigenic strain no. and serotype	Non-staphylococcal strains	Enterotoxins detected by:	
		SRD	DD
246-3A, A	*P. vulgaris* *P. florescens* *Micrococcus* sp.	Yes	Yes
243, B	*P. vulgaris* *E. coli* *S. lutea*	Yes	Yes
834, C	*E. coli* *S. fecalis* *Bacillus* sp.	Yes	Yes

from 11 of the 14 low-producing strains. The method can detect individual toxin serotypes or can be used as a polyvalent system by incorporating all five type antisera. Up to 10 determinations can be made on a single assay slide.

The presence of staphylococci in foods has sanitary significance indicative of poor manufacturing practice or improper handling, but the potential public health problem is greater if the strains present are capable of producing enterotoxin. Detection of enterotoxigenic staphylococci after an outbreak of food poisoning is not a difficult task since they are usually present in high numbers, unless, after growth and toxin production, the food is processed (e.g., heated) sufficiently to kill cells. However, from a preventive point of view, it may be difficult to screen routine food samples for strains which have the potential for subsequent growth and toxin production.

A screening program may require the examination of many staphylococcal isolates from a single sample. This point was illustrated by the results of an analysis of butter samples implicated in a food poisoning outbreak (personal communication, M. S. Bergdoll). A mixture of enterotoxigenic and non-enterotoxigenic staphylococci was found with the latter predominating; only one out of 16 staphylococcal isolates produced enterotoxin. In a study by Noleto and Bergdoll (6), milk was inoculated with enterotoxigenic and non-enterotoxigenic staphylococci, and the latter were capable of outgrowing the former. Enterotoxin was detected in the milk, although the enterotoxigenic organisms were difficult to isolate because of the predominance of the other staphylococci. Thus, enterotoxigenic strains can be outnumbered and remain undetected in foods, if only a few isolates are tested.

The multiple-culturing system in combination with the SRD assay described here is an effective way of screening large numbers of isolates. With the exception of three low-producing strains, all cultures examined were capable of producing detectable levels of enterotoxin when grown on semisolid agar with at least four other nontoxigenic strains. Reducing the number of strains tested in the multiple-culturing system to lower than five would not be effective in detecting the three strains that were missed since they were not detected in individual culture by the SRD procedure (Table 4).

Using five strains for each semisolid agar plate, one polyvalent SRD assay slide could be used to screen up to 50 cultures for enterotoxin. As suggested by Noleto and Bergdoll (6), it may be necessary to examine such high numbers in food-poisoning outbreaks when toxigenic strains coexist with non-enterotoxigenic staphylococci. If required, cultures producing positive reactions in a single well can be examined individually for ability to produce toxin and for toxin typing.

ACKNOWLEDGMENTS

We are grateful to James D. Macmillan, Department of Microbiology and Biochemistry, Cook College, Rutgers University, for his invaluable assistance in the preparation of this manuscript. We thank M. S. Bergdoll and R. Robbins, Food Research Institute, University of Wisconsin, Madison, for sending us cultures of staphylococci used in this study. Special thanks and appreciation are given to R. W. Bennett, Food and Drug Administration, Washington, D.C., for his generous gifts of purified enterotoxin, antisera, and staphylococcal cultures, and for his invaluable advice and cooperation throughout this study.

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