APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1979, p. 298-302
0099-2240/79/02-0298/05$02.00/0
Vol. 37, No. 2

Tandem Coagulase/Thermonuclease Agar Method for the Detection of *Staphylococcus aureus*

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Received for publication 4 December 1978

In optimizing previously reported coagulase agar media to obtain a rapid, reliable, and inexpensive coagulase test agar, variations in plasmas, pH, buffer system, fibrinogen, and fibrinolytic inhibitor were investigated. The agar with the following composition was determined best for the demonstration of coagulase production by *Staphylococcus aureus*: 25 ml of 15% bovine fibrinogen (fraction I, type I, citrated, Sigma Chemical Co.), 25 ml of rehydrated rabbit plasma (coagulase plasma ethylenediaminetetraacetic acid, Difco), 10.0 mg of soybean trypsin inhibitor (Schwarz/Mann), and 450 ml of brain heart infusion agar (Difco). In additional studies involving 7 different temperatures and 11 heating times, the thermal destruction of microbial nucleases on plate count agar and coagulase test agar was investigated. Heating the plates for 2.5 h at 65°C destroyed all heat-labile nucleases, but not thermonucleases of *S. aureus*. A tandem agar plate method for the identification of *S. aureus* was developed. Coagulase and thermonuclease activity of 50 colonies can be detected on a single agar plate. Suspect *S. aureus* colonies isolated on various selective media are transferred to coagulase test agar, the plates are incubated at 37°C for 18 h, and the coagulase reaction is recorded. The plates are then heated at 65°C for 2.5 h, overlaid with toluidine blue-metachromatic diffusion agar, and reincubated at 37°C for 3 h, and the thermonuclease reaction is recorded. Studies based on 88 enterotoxigenic *S. aureus* strains and 133 and 48 suspect *S. aureus* strains isolated from fresh salami mixtures on mannitol salt and tellurite-polymyxin-egg yolk agars, respectively, demonstrated 100% agreement between the tandem agar plate method and standard coagulase and thermonuclease tests. Overall, the tandem agar plate method is a rapid and convenient approach contributing to the identification of *S. aureus* from foods.

Staphylococcal food poisoning remains one of the three most common types of food poisonings in the United States. Approximately 25% of all food-borne illness reported in the United States is caused by staphylococcal intoxication (2, 7, 13). Early detection of staphylococcal cells and the enterotoxins produced by them in food or food ingredients is of crucial public health importance.

A rapid, easy, economical, and accurate method for identifying and enumerating *Staphylococcus aureus* has been elusive. Indication of staphylococcal food poisoning potential depends on the isolation and identification of a toxin-producing strain from the food. Proof of staphylococcal food poisoning depends on detection of staphylococcal enterotoxins in the food. Because toxin detection is time consuming, efforts have been made to correlate toxin production to other physiochemical characteristics of the organism (19). Unfortunately, complete correlation of these characteristics with enterotoxigenicity is not possible because the same characteristics may be exhibited by other non-enterotoxigenic *S. aureus* strains (19).

Staphylococcal-type enterotoxins are produced only by *S. aureus* strains. Most strains produce one or more enterotoxins (7). Infrequent production of staphylococcal enterotoxin by other coccal strains has been reported, but on the basis of recent studies it seems that these strains are variants or mutants of *S. aureus* characterized by the absence of coagulase production (12).

Of the numerous physiochemical characteristics of *S. aureus* used for its classification, production of coagulase and thermonuclease (Tnase) is the most reliable practical criterion used in identifying *S. aureus* (4, 11a, 19, 20).

Three methods for detection of coagulase have

been used: (i) tube test, (ii) slide test, and (iii) agar plate test. The tube test is considered the most reliable method for coagulase detection (19, 20). However, subjectivity in judging the test, variability in plasmas, complications due to nonspecific reactions, and incubation time have led to criticism of the test (17, 19, 20). In spite of these shortcomings, the coagulase tube test is used routinely for the identification of *S. aureus* (3, 5, 8, 18). Improvements in agar plate methods for determining coagulase production since its introduction in 1944 have produced media of varying reliability (6, 16, 23).

Production of Tnase has been shown to be a reliable means of identifying *S. aureus* and has been suggested as a confirmatory test when the coagulase tube or agar plate test is questionable (1, 3, 4, 11, 11a, 16, 21). Although other bacteria produce nucleases, production of Tnase is rare and not well documented (22). Several reliable methods are available for Tnase detection (10, 11).

The most common enumeration method for *S. aureus* in foods involves spreading portions of decimal dilutions on a suitable selective medium. A certain percentage of suspect colonies are then picked, grown in broth, and subsequently coagulase tube tested with rabbit plasma. The leftover broth is then heated at 100°C for 15 min and tested for Tnase, usually on toluidine blue-metachromatic diffusion (TB-Mad) agar (11). A more rapid approach (16) reported the use of a coagulase agar plate developed by L. Lotter (Ph.D. thesis, University of California, Davis, 1973) in combination with the nuclease test. This method did not detect all coagulase-producing colonies and did not test for Tnase.

The purpose of this investigation was to develop an improved coagulase plate test agar, incorporate Tnase detection into the method, and develop a rapid, reliable, and inexpensive technique for the detection of both coagulase and Tnase on a single agar plate. The composite method could then be used reliably for the enumeration and identification of *S. aureus.*

MATERIALS AND METHODS

Industrial cultures were obtained during routine microbiological examination of Italian dry salami at various stages of production from two different producers. Suspect *S. aureus* colonies were selected at random from mannitol salt agar (MSA, Difco Laboratories, Detroit, Mich.) and from tellurite-egg yolk-polymyxin agar (TPEY, Difco) plates by using the manufacturer's criteria for identification.

Nuclease-positive cultures were obtained from total plate count agar (PCA, Difco) plates of industrial samples of salami by the TB-Mad overlay procedure of Lachica et al. (11). Several isolates were found to give a positive nuclease reaction. When these isolates were tested further, many were shown to produce a heat-labile nuclease. Heat-stable and heat-labile nuclease isolates were kept as controls and were identified either as *S. aureus* or non-*S. aureus* by additional criteria.

Confirmed enterotoxigenic strains were obtained from the culture collection of one of us (C.G.).

All isolates were characterized as staphylococci or not by the following criteria: cell morphology, pigmentation, catalase reaction, Gram reaction, glucose and mannitol utilization, and production of coagulase and Tnase (3). Later trials on test media were performed on the above cultures with this characterization as a criterion for accuracy. Cultures were stored on PCA slants in screw-capped vials (1.5 by 5.0 cm) at −20°C until needed. Tris(hydroxymethyl)aminomethane (Tris) buffer (Sigma Chemical Co., St. Louis, Mo.) was used where indicated.

Coagulase was detected by two methods: the coagulase tube test reactions were performed with rabbit plasma (coagulase plasma-ethylenediaminetetraacetic acid [EDTA], Difco) by the procedure recommended by the manufacturer (5). Results were determined as no clotting (−) to complete clotting (4+) by the criteria of Turner and Schwartz (22). Coagulase plate tests were performed on agar consisting of 25 ml of 15% bovine fibrinogen (bovine, citrated, fraction I, type I, Sigma Chemical Co.); 10.0 mg of soybean salt-free lyophilized trypsin inhibitor (Schwarz/Mann, Orangeburg, N.Y.) in 25 ml of ethylenediaminetetraacetic acid-treated rabbit plasma (Difco); and 450 ml of brain heart infusion agar (BHI, Difco). The bovine fibrinogin, trypsin inhibitor, and rehydrated rabbit plasma were mixed together and sterilized by filtration through a 1.0-μm Seitz filter. The filtrate-receiving flask was packed in ice during filtration to reduce foaming. The sterile filtrate was gradually heated to 50°C and mixed well with the sterile, cooled BHI agar. The agar was dispensed immediately in 15- to 20-ml amounts into petri dishes and allowed to dry for 24 h at room temperature before being stored in plastic bags under refrigeration. Plates can be stored for at least 6 months without loss of accuracy. Where indicated, pig plasma was included at 14% by the method of Julseth and Dudley (9). The plates were inoculated with pure cultures (up to 50 strains per plate). Coagulase reactions were detected after incubation at 37°C for 18 h by the presence of a precipitation halo around the colonies.

Nuclease-producing colonies were detected by the TB-Mad method of Lachica et al. (11). Tnase was determined by two methods: the microslide well test of Lachica et al. (11), and a TB-Mad agar modification of Lachica (10). For the TB-Mad modification, cultures were inoculated onto solid agar plates (PCA or coagulase plate test agar), incubated (18 h at 37°C), heated (2.5 h at 65°C) to denature heat-labile nuclease, cooled, and overlaid with TB-Mad agar and read in the usual manner.

RESULTS AND DISCUSSION

Detection of coagulase by the agar plate method. In optimizing an earlier coagulase plate

test agar reported by Lotter (Ph.D. thesis), variation in plasma, pH, buffer system, fibrinogen, and fibrinolytic inhibitor was investigated. Each ingredient was varied while all other parameters were held constant. The strength and accuracy of the reaction on control strains were used to determine the ideal formulation.

We investigated the sensitivities of pig plasma, rabbit plasma, and pig-rabbit plasma systems in detecting the coagulase reaction on solid agar plates. Rabbit plasma is the accepted plasma for coagulase testing, whereas pig plasma has been shown by some investigators to increase the sensitivity of the test (9, 14). Preliminary experiments revealed that pig plasma alone was unsatisfactory because of inadequate and undependable reactions. All other tests were run with rabbit plasma or pig-rabbit plasma systems. Precipitation halos were scored: no halo (−), detectable precipitation (+1), dense halo (+2), or opaque zone (+3). Agar with 5% rabbit plasma was selected as the superior system for further use because of its 100% accuracy in detecting coagulase production. In addition, the precipitation reactions were more visible, as indicated by the mean score of 2.96 as compared to the mean score of 2.65 for the rabbit-pig plasma system. The latter system failed to detect coagulase production by *S. aureus* enterotoxigenic strain S-6.

Personal communication with L. Lotter of Bundesanstadt für Fleischforschung, Kulmbach, Germany, indicated that Tris buffer might aid in obtaining a strong coagulase reaction on the coagulase agar plate test. To detect the effects of the addition of Tris buffer to the phosphate buffer already in the medium, growth of *S. aureus* enterotoxigenic strain 196-E was studied in various concentrations of Tris buffer at different pH values. Growth was followed photometrically on a Coleman spectrophotometer, model 6/20. Calculated generation times are given in Table 1. Although BHI broth with 0.005 to 0.05 M Tris buffer at nearly all pH values improved the growth of the test strain over the BHI broth alone, there was no visible enhancement of growth or coagulase reaction when Tris buffer was incorporated into the coagulase agar test plates. The inclusion of Tris buffer into the medium was discontinued.

Depending on strain, fibrinolysis of the precipitation halos on coagulase agar plates occurred at different times. The inconvenience of reading plate reactions at several times to eliminate the possibility of false negatives was avoided by adding soybean trypsin inhibitor to the medium. Increasing amounts of inhibitor were added to different batches of medium until no further fibrinolysis was noted and no inhibition of growth or coagulase reaction could be observed. The optimum concentration of soybean trypsin inhibitor was found to be 0.002% (wt/vol).

TABLE 1. *Effect of type of buffer, molarity, and pH on the generation time of S. aureus enterotoxigenic strain 196-E grown in BHI broth for 12 at 37°C*

Addition to BHI-phosphate buffer (concn, M)	Generation time (min) in medium at pH				
	7.0	7.2	7.4	7.6	7.8
Tris (0.0005)	38.6	40.5	45.0	45.0	54.1
Tris (0.005)	36.1	36.9	39.8	43.0	51.0
Tris (0.05)	31.9	34.3	43.6	47.5	54.1
Tris (0.5)	113.6	66.0	150.7	223.6	1155.0
BHI-phosphate buffer alone	40.5	44.4	45.3	49.5	53.7

The concentration of fibrinogen affected the intensity of the precipitation halo on the coagulase agar test plates. Of the several concentrations tested, 0.75% (wt/vol) fibrinogen in the agar medium was found to be optimal for halo formation. No false positive reactions were observed.

As a result of the above studies, an optimum coagulase plate test agar was developed (see above). To verify the reliability of this medium, a total of 261 organisms including enterotoxigenic strains and industrial isolates from MSA and TPEY agar plates were tested on the coagulase agar. The data are summarized in Table 2. Of the 261 organisms tested, 109 (41.8%) were coagulase tube test positive. All of the 109 organisms (100%) were detected accurately by the coagulase agar plate method.

The time needed for the traditional tube method of identifying coagulase-positive staphylococci after primary isolation on selective and differential media is 24 to 30 h (18). By comparison, the coagulase agar plate test requires 18 h. In addition to saving time and supplies, the coagulase agar plates can be used later for the Tnase test.

Detection of Tnase by the modified overlay technique. Tnase detection was made by a modification of the overlay procedure of Lachica et al. (10, 11). By subjecting known heat-labile cultures and known heat-stable cultures grown on PCA plates to various time-temperature treatments, a convenient time-temperature optimum for accurately differentiating heat-stable from heat-labile nuclease-producing strains was found. Heat-stable nuclease produced by 15 *S. aureus* strains was not affected by heating at 45, 50, 55, or 60°C for up to 6 h. At 65, 70, and 75°C the ratio of heat-stable nuclease strains to total number of strains tested was reduced from 1.0 to 0.93 in 6.0, 4.0, and 4.5 h, respectively. The results for heat-labile nuclease-producing strains are shown in Fig. 1. It was found that 65°C for

TABLE 2. *Comparison of five methods used for the demonstration of coagulase and Tnase production by suspect or known S. aureus strains from three sources*

Source of test strains	Total no. of strains tested	No. of strains identified by:				
		Tnase microslide	Tnase overlay on PCA	Tnase overlay on coagulase plate test agar (TAP)[a]	Coagulase tube test	Coagulase agar plate test (TAP)
Suspect *S. aureus* isolated from MSA	133	4	4	4	5	5
Suspect *S. aureus* isolated from TPEY	48	28	28	28	24	24
Known enterotoxigenic *S. aureus* strains	80	80	80	80	80	80

[a] TAP, Tandem agar plate.

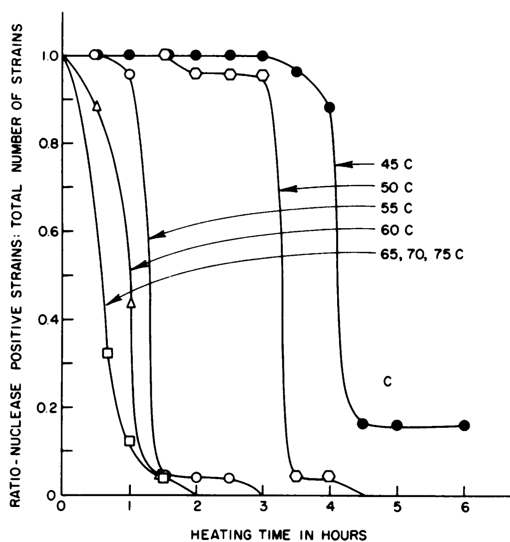

FIG. 1. *Effect of heating time and temperature on the destruction of nucleases produced by non-S. aureus on plate count agar as determined by the TB-Mad overlay method. A group of 25 gram-positive and gram-negative rods and cocci producing heat-labile nuclease (15 min at 100°C) as determined by the Tnase microslide test (11) were tested.*

2.5 h denatured all heat-labile nucleases produced by bacteria other than *S. aureus*, but left Tnase intact. This time-temperature treatment was selected because it allowed a margin of at least 5°C and 30 min beyond the treatment which would leave heat-labile nucleases functional and temperature increases up to 75°C did not inactivate Tnase. The treatment is easily performed in most standard laboratory incubators in a reasonable time, and the plates may be read at up to 5 h at 65°C without complete destruction of Tnase activity. A total of 261 cultures were tested for Tnase by the modified agar overlay procedure (Table 2). When results were compared with the microslide well assay for Tnase, 112 of 112 (100%) positive cultures were detected. Because the production of Tnase is rare among bacteria other than staphylococci, an estimate of *S. aureus*, and thus potentially enterotoxigenic staphylococci, can be obtained by testing for the production of Tnase (12).

Perhaps the greatest advantage of the coagulase agar plate test and the Tnase agar overlay method for detecting *S. aureus* is that they can be combined on a single agar plate. Suspect *S. aureus* colonies from recovery media are transferred onto coagulase agar test plates which are incubated for 18 h at 37°C. Coagulase is detected by the presence of a fibrin halo. The plates are then heated for 2.5 h at 65°C, cooled, overlaid with TB-Mad agar, and incubated (3 h at 37°C), and Tnase is detected by the presence of a pink halo. Thus, the tandem agar plate method detects coagulase and Tnase on a single agar plate.

The accuracy of the tandem agar plate method to detect coagulase and Tnase activity was tested by comparing results obtained by this method to those obtained by the coagulase tube test and the microslide well test for Tnase.

Table 2 shows the number of coagulase tube test-positive and Tnase microslide test-positive cultures detected by the tandem agar plate method: all 109 tube test- and all 112 Tnase microslide-positive strains were detected accurately by the coagulase agar plate and the Tnase overlay technique. Then when the tandem agar plate method was employed, coagulase and Tnase activities of all suspect and known *S. aureus* strains were demonstrated correctly.

Table 3 gives the Tnase and coagulase reactions of all the organisms tested. Of the 133 presumptive *S. aureus* strains isolated on MSA, only 4% produced coagulase and Tnase and one strain produced only coagulase. Of the 48 presumptive *S. aureus* strains isolated in TPEY agar, only 24 (50%) produced coagulase and Tnase, and four (8.3%) strains produced only Tnase. All 261 test strains were characterized

TABLE 3. *Comparison of two methods used for the demonstration of coagulase and Tnase production by suspect or known S. aureus obtained from three sources*

Detection method	Source of bacterial cultures	No. of strains			
		Tnase-positive, coagulase-positive	Tnase-negative, coagulase-positive	Tnase-positive, coagulase-negative	Tnase-negative, coagulase-negative
Tnase microslide and coagulase tube test	MSA	4	1	0	128
	TPEY	24	0	4	20
	Ent[a]	80	0	0	0
TAP[b]	MSA	4	1	0	128
	TPEY	24	0	4	20
	Ent	80	0	0	0

[a] Ent, Known enterotoxigenic *S. aureus*.
[b] TAP, Tandem agar plate.

correctly with regard to coagulase and Tnase activities with the tandem agar plate method, and thus the method is considered a significant contribution to the identification of *S. aureus* from foods.

ACKNOWLEDGMENTS

This work was partially supported by a grant-in-aid from Gallo Salami, Inc., San Francisco, Calif., and Galileo-Capri Salami, Inc., San Lorenzo, Calif.

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