JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1985, p. 195–199
0095-1137/85/020195-05$02.00/0

Vol. 21, No. 2

Improvement of Two Toluidine Blue O-Mediated Techniques for DNase Detection

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Received 27 August 1984/Accepted 7 November 1984

Two DNase detection techniques in which the metachromatic dye toluidine blue O (TBO) is used have been improved, and a potential source of difficulty for personnel attempting to use TBO-related methods has been identified. Reducing the concentration of TBO in the Streitfeld plate-flooding method from 0.1 to 0.05% resulted in easier control of staining intensity, less masking of DNase-positive reactions due to overstaining, sharper delineation of zones of DNase activity, and more sensitive detection of weak DNase reactions. Incorporation of 0.005% TBO in DNase agar, rather than the recommended 0.01%, allowed growth and expression of DNase activity by gram-positive as well as gram-negative bacteria. The reduced dye content in the agar also enhanced expression of DNase activity by some organisms and provided sharper delineation of DNase-positive reactions. Because optimum expression of DNase activity depends upon exact TBO concentrations in both the flooding and agar incorporation techniques, strict attention must be paid to the dye content of commercially available TBO dye powders. TBO concentrations must reflect actual dye content; therefore, calculations must include a conversion factor that accounts for the true dye content of the commercial preparation. The conversion factor that we developed is determined by dividing 100 by the percentage of dye in the commercial powder. The grams of commercial dye powder required per 100 ml of dye mixture is calculated by multiplying the percentage of dye required in the dye mixture by the conversion factor.

The metachromatic properties of toluidine blue O (TBO) have been utilized in several methods (2, 5, 6) designed to detect DNase activity in bacteria. These procedures include flooding agar plates containing DNA with a solution of 0.1% TBO (6), incorporation of 0.01% TBO into DNase test agar (5), a colony overlay technique in which colonies on an agar surface are overlaid with molten TBO-DNA agar (2), and microslide techniques for both semisolid and broth cultures (2).

When we tried to use the TBO flooding technique of Streitfeld et al. (6), we encountered overstaining problems that masked some positive DNase reactions. Our observations suggested that 0.1% TBO was too high a concentration to allow proper control of the staining reaction so that overstaining did not mask weaker DNase reactions.

According to Schreier (5), incorporation of TBO into a growth medium provides very sharp delineation of DNase activity but inhibits growth of gram-positive bacteria, thus limiting the technique to gram-negative organisms.

A survey of the dye content of TBO dye powders from various supply houses showed a wide variation in actual dye content. This variation appeared to be a potential cause of difficulty in reproducing TBO methods, in which exact dye content is critical.

The purpose of this study was to determine the optimal concentration of TBO that (i) would allow growth of gram-positive bacteria, as well as accurate detection of DNase production, on DNase test agar containing TBO and (ii) is required to detect DNase activity by flooding DNase test agar with TBO.

MATERIALS AND METHODS

Organisms. The cultures utilized in this study were obtained from the University of North Dakota culture collection. The organisms used were as follows: gram-positive organisms were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Corynebacterium xerosis*, *Bacillus subtilis*, and *Streptococcus pyogenes*; gram-negative cocci were *Branhamella catarrhalis* and *Neisseria sicca*; and gram-negative rods were *Serratia marcescens*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Vibrio alginolyticus*. DNase-negative controls were *C. xerosis*, *S. epidermidis*, *N. sicca*, *E. coli*, and *P. aeruginosa*. Stock cultures were grown for 24 h at 37°C on blood agar prepared by adding 5% defibrinated sheep blood (Colorado Serum Co., Denver, Colo.) to tryptic soy agar (Difco Laboratories, Detroit, Mich.), then refrigerated at 4°C.

Medium for DNase testing. DNase test agar (Difco) was prepared according to the instructions of the manufacturer and used in plates as the basic DNase test medium in these studies. Any additions to the medium are noted.

Inoculum size and inoculation pattern. The usual inoculum size used in this study was the quantity of organisms obtained by touching an inoculating needle to the top of a colony or to growth on a slant. When a heavier inoculum was used, an inoculating loop was utilized to pick up half of a colony about 2 mm in diameter. Five to seven organisms were spot inoculated at regular intervals around the edge of the agar surface, 10 to 15 mm from the periphery. The inoculum was spread over an area about 5 mm in diameter.

Standard DNase test. The standard HCl-DNA precipitation method for detection of DNase production (1) was used as the control for detection of DNase activity and growth of the bacteria on DNase test agar. This test was performed by spot inoculating DNase test plates with the various bacteria, incubating them for 24 to 72 h at 37°C, and detecting DNase activity by flooding the surface of the agar with 1.0 N HCl. The 1.0 N HCl precipitated unhydrolyzed DNA in the medium. Colonies of organisms exhibiting DNase activity were surrounded by a clear zone in a cloudy background.

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TABLE 1. Detection of DNase activity by the TBO flooding technique

Organism	DNase control (1.0 N HCl)	Activity detected with dye concn (%) of[a]:						
		0.5	0.25	0.1	0.075	0.05	0.025	0.01
S. aureus	+	TMD[b]	+	3+	3+	3+	+	+
S. epidermidis	−	TMD	−	−	−	−	−	−
C. xerosis	−	TMD	−	−	−	−	−	−
B. subtilis	2+	TMD	+	+	2+	2+	+	+
S. pyogenes	−	TMD	+	2+	3+	3+	+	+/−
S. marcescens	3+	TMD	−	−	3+	3+	−	+/−
E. coli	−	TMD	−	−	−	−	−	−
B. catarrhalis	−	TMD	−	2+	2+	2+	−	−
P. aeruginosa	−	TMD	−	−	−	−	−	−
N. sicca	−	TMD	−	−	−	−	−	−
V. alginolyticus	+/−	TMD	−	+	+	+	−	−

[a] Zone intensities: 3+, strong; 2+, moderate; +, weak; +/−, questionable; −, no zone.
[b] TMD, Too much dye; concentration of dye masked detection of DNase production.

Detection of DNase activity by TBO flooding. The final concentrations of TBO (MCB Manufacturing Chemists, Inc., Gibbstown, N.J.) used for flooding in this study were 1.0, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, and 0.01%. DNase test plates were spot inoculated with various microorganisms and incubated at 37°C for 24 to 72 h. After significant growth had occurred, the plates were flooded with 5 to 6 ml of a TBO solution. The dye solution was allowed to stand on the agar surface for 3 to 5 min and then decanted. The plates were observed for DNase activity immediately after the dye was decanted and at 5-min intervals for 30 min. Appearance of bright-pink zones surrounding colonies on a royal-blue background indicated DNase activity. No change in the background color was evident for DNase-negative organisms.

DNase test agar containing TBO. TBO (Sigma Chemical Co., St. Louis, Mo.) was added to DNase agar before it was autoclaved. The final concentrations of dye in the agar were 0.5, 0.1, 0.075, 0.05, 0.025, 0.01, 0.0075, 0.005, 0.0025, 0.001, 0.00075, 0.0005, and 0.00025%. The plates were spot inoculated with various organisms, incubated at 37°C for 24 to 72 h, and read for DNase production. DNase activity was manifested as the presence of bright-pink zones surrounding organisms on a royal-blue background. Occasionally DNase activity was manifested as a clear zone surrounding a colony or beneath a colony or both. No change in the background color was evident for DNase-negative organisms.

Standardization of dye content. Commercial dye preparations contain actual dye contents varying at least from 60 to 95%. Therefore, it was necessary to adjust for the dye content in the commercial preparation, as stated on the label, so that dye solutions and media contained the specified percentage of dye rather than the percentage of dye powder supplied by the manufacturer. The conversion factor (CF) used was: CF = 100/percentage of dye in commercial preparation. For determination of the amount of dye powder (in grams) required for 100 ml of water or agar, the final percentage of dye required was multiplied by the conversion factor.

RESULTS

Four strong and two questionable DNase-producing organisms and five non-DNase-producing organisms were tested for DNase activity by the 1.0 N HCl flooding technique, TBO flooding, and TBO incorporation into DNase test agar. The TBO studies were performed with various concentrations of dye to determine the optimal dye concentration for both bacterial growth and detection of DNase activity. The standard HCl-DNA precipitation DNase test served as the control.

DNase detection by flooding DNase test agar with TBO. Observations in our laboratory have suggested that the 0.1% TBO solution recommended by Streitfeld et al. (6) is not the optimal dye concentration for visualizing DNase activity by the TBO flooding technique. Table 1 shows the results of the TBO flooding technique in which various dye concentrations from 0.5 to 0.01% were used. Solutions containing ≥0.5% TBO masked the DNase activity, as the entire plate stained dark blue. TBO dye concentrations of <0.5% allowed varying degrees of DNase detection. The contrast between the blue background and pink zones of DNase activity increased as TBO concentrations increased from 0.01 to 0.1%. When the 0.01% TBO solution was used, DNase activity was difficult to observe because the colors were too faint. DNase activity exhibited by 0.075 and 0.05% TBO were at maximal levels for all DNase-positive organisms and equivalent to each other (Table 1). Also, 0.1% TBO completely masked DNase activity of *S. marcescens* and reduced the observed reaction intensity of *B. subtilis* and *S. pyogenes* (Table 1). A comparison of the TBO method with the standard HCl precipitation method shows that HCl precipitation did not enable the detection of DNase activity by *B. catarrhalis* and was questionable for *V. alginolyticus* and weak for *S. aureus*.

Effect of TBO on bacterial growth. The data in Table 2 show the effects of 13 different TBO concentrations on the growth of 11 bacterial species. Three gram-negative rods, *P. aeruginosa*, *E. coli*, and *S. marcescens*, showed very little or no inhibition of growth at any concentration of TBO from 0.5 to 0.00025%. A fourth gram-negative rod, *V. alginolyticus*, was inhibited completely when dye concentrations exceeded 0.025% but showed no inhibition in the presence of <0.025% TBO. The gram-negative cocci were inhibited to the greatest extent. *B. catarrhalis* showed complete inhibition of growth when the concentration of TBO exceeded 0.0075% unless a heavier inoculum was used. When a heavier inoculum was used, barely detectable growth occurred at 0.01% TBO, and significantly better growth occurred at all lower TBO concentrations. *N. sicca* was able to grow at TBO concentrations of up to 0.01% with the usual inoculum level. TBO concentrations exceeding 0.05% completely inhibited the growth of all gram-positive bacteria except *S. aureus*. All gram-positive bacteria grew at 0.01%,

TABLE 2. Growth on TBO-containing DNase plates

Organism	Growth with final dye concn (%) of[a]:													
	0.5	0.1	0.075	0.05	0.025	0.01	0.0075	0.005	0.0025	0.001	0.00075	0.0005	0.00025	0.0
S. aureus	NG	NG	2+	2+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+
S. epidermidis	NG	NG	NG	+	2+	2+	2+	3+	3+	3+	3+	3+	4+	3+
C. xerosis	NG	NG	NG	+	+	+	2+	3+	3+	2+	3+	4+	4+	4+
B. subtilis	NG	NG	NG	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
S. pyogenes	NG	NG	NG	+/−	2+	4+	3+	3+	3+	4+	4+	4+	4+	3+
S. marcescens	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
E. coli	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
B. catarrhalis	NG	NG	NG	NG	NG	NG	+/−[b]	+/−[b]	+/−[b]	+/−[b]	+[b]	+[b]	3+	2+
P. aeruginosa	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
N. sicca	NG	NG	NG	NG	NG	3+	2+	2+	3+	2+	2+	3+	3+	2+
V. alginolyticus	NG	NG	NG	NG	+	4+	4+	4+	4+	4+	4+	4+	4+	4+

[a] Amount of growth: NG, no growth; 4+, heavy; 3+, moderate; 2+, light; +, poor but detectable; +/−, questionable.
[b] These results were obtained with the usual inoculum size used in this study. When a heavier inoculum (one-half of a 2-mm colony) was used, growth of *B. catarrhalis* ranged from + to 2+ for dye concentrations of ≤0.0075% in 48 h.

but growth of *C. xerosis* and *S. epidermidis* was light at best. At 0.005% TBO, all gram-positive bacteria grew well.

DNase detection by incorporation of TBO into DNase test agar. The concentration of TBO incorporated into the DNase test agar affected the ability to detect DNase activity (Table 3). TBO concentrations of ≥0.025% masked the pink zones indicative of DNase production. Although the strongly DNase-positive organisms *S. marcescens*, *S. pyogenes*, and *S. aureus* grew significantly on DNase agar plates containing 0.025% TBO (Table 2), no discernible pink zones indicating DNase activity were evident. The production of DNase by *S. aureus* was partially masked on plates containing 0.01% TBO, even though good growth occurred. Pink zones of DNase activity were most obvious and exhibited most contrast with the blue background at TBO concentrations of 0.0075, 0.005, and 0.0025%. As the dye content decreased to <0.0025%, the blue and pink faded and the contrast between the background and the DNase zones decreased markedly. Although *B. catarrhalis* exhibited only slight growth in this concentration range of TBO with the usual inoculum size, zones of DNase activity were seen easily when a heavier inoculum was utilized.

Colorless, rather than pink, zones appeared around and beneath the colonies of both *B. subtilis* and *V. alginolyticus*. Since no precipitate formed in these zones when 1.0 N HCl was added to them, the colorless zones indicated DNase activity for these two organisms. No zones indicative of DNase production were detected for *S. epidermidis*, *C. xerosis*, *E. coli*, *P. aeruginosa*, or *N. sicca*.

DISCUSSION

The metachromatic dye TBO exhibits either its true orthochromatic or its metachromatic staining spectrum when complexed with other substances (3, 4). TBO exhibits a royal-blue coloration in the presence of unhydrolyzed DNA. In the presence of hydrolyzed DNA, TBO forms complexes with mononucleotides or oligonucleotides which shift the color spectrum to bright pink, which is the metachromatic staining pattern of TBO. Therefore, DNase-producing organisms which hydrolyze the DNA incorporated into a medium cause the color of the agar to shift from royal blue to bright pink.

Attempts to use the TBO flooding method of Streitfeld et al. (6) to detect DNase activity of *S. aureus* were less than satisfactory because the plates easily overstained and the pink DNase reaction zones often were partially or fully obscured by the intense blue caused by overstaining. Although use of 0.1% TBO, as recommended by Streitfeld et al., produced a deep-royal-blue background with dark-pink zones, indicating DNase activity, for most DNase-positive organisms tested, DNase production by *S. marcescens* was

TABLE 3. Detection of DNase activity with TBO-containing DNase plates

Organism	Control (1.0 N HCl)	Activity detected with final dye concn (%) of[a]:									
		0.05	0.025	0.01	0.0075	0.005	0.0025	0.001	0.00075	0.0005	0.00025
S. aureus	+	−	−	+	3+	3+	3+	3+	+	−	−
S. epidermidis	−	−	−	−	−	−	−	−	−	−	−
C. xerosis	−	−	−	−	−	−	−	−	−	−	−
B. subtilis	2+	−	−	2+[b]	2+[b]	2+[b]	+[b]	+[b]	−	+/−	−
S. pyogenes	−	−	+/−	4+	4+	4+	4+	4+	4+	+	−
S. marcescens	3+	−	+/−	4+	4+	4+	4+	4+	2+	+	+/−
E. coli	−	−	−	−	−	−	−	−	−	−	−
B. catarrhalis	−	−	−	+	2+[c]	2+[c]	+[c]	+[c]	−	−	−
P. aeruginosa	−	−	−	−	−	−	−	−	−	−	−
N. sicca	−	−	−	−	−	−	−	−	−	−	−
V. alginolyticus	+/−	−	−	+[b]	+[b]	+[b]	+[b]	−	−	−	−

[a] Zone intensities: 4+, very strong; 3+, strong; 2+, moderate; +, weak; +/−, questionable; −, no zone.
[b] Colorless around and under colony. Addition of 1.0 N HCl to the colorless areas did not result in precipitation of unhydrolyzed DNA.
[c] Distinct, well-defined, pink zones were best exhibited by *B. catarrhalis* when a heavy inoculum (one-half of a 2-mm colony) was used. The heavy inoculum was used to obtain these data.

completely masked by overstaining, and apparent DNase activity by *B. subtilis* was reduced by at least 50%. The results of the present study show that the 0.1% TBO solution recommended by Streitfeld et al. (6) is not the optimal dye concentration for determining DNase activity when the flooding technique is used. When the concentration of TBO solution used was 0.075 or 0.05%, five of the six DNase-producing microorganisms exhibited brilliant, well-defined, pink zones indicative of DNase activity. The sixth organism, *V. alginolyticus*, was weakly DNase positive, but produced distinct, well-defined, pink zones in this medium.

Two factors in addition to dye concentration affected the results of the TBO flooding procedure: the length of time the dye was in contact with the agar and the amount of time between the decanting of the dye solution and the reading of the DNase activity. There was a direct relationship between the length of time the dye was on the agar surface and the darkness of the blue background. The longer the dye remained on the agar, the darker the background became. As a result of this relationship, it was important to decant the solutions with higher concentrations of TBO (0.25 to 0.075%) after short periods of time, otherwise the background color masked the pink zones of DNase activity. Lower dye concentrations (e.g., 0.05 and 0.025%) were less sensitive to the time of contact and background color; therefore, these solutions could be kept on the agar surface for longer periods without masking DNase reactions. The color development that occurred during the 15 to 20 min after the dye solution was decanted did not increase zone diameters but did markedly increase the contrast between the royal-blue background and the bright-pink zones of DNase activity. The color, intensities, and relationships between background and DNase zones were stable for at least 2 h after development; in fact, all reactions were still distinct after refrigeration for 24 h.

On the basis of zone clarity, color contrast, and effects of time, we concluded that 0.05% TBO was the optimal concentration of dye used for flooding the DNase plates. This dye concentration yielded excellent results regardless of whether the dye was left on the agar surface for 3 or 5 min. In fact, contact times of up to 10 min usually yielded readable plates. Also, use of the 0.05% TBO solution resulted in definite pink DNase zones and good color contrast immediately upon decanting as well as after 15 to 20 minutes. Although the 0.075% TBO solution showed equivalent end results for the detection of DNase activity, this solution was more sensitive to time, often exhibiting overstaining when left on for 5 min, and usually did not show sharp zones of DNase activity immediately after the dye solution was decanted. The TBO flooding technique produced much more clearly delineated zones of DNase activity than did the DNA precipitation DNase test, in which 1.0 N HCl was used.

Schreier (5) detected DNase activity by growing bacteria on DNase test agar containing 0.01% TBO but stated that the growth of gram-positive bacteria was inhibited. In our study, 0.01% TBO in DNase test agar did not completely inhibit the growth of gram-positive organisms and was not the optimal concentration for visualizing DNase activity. When DNase test agar contained 0.01% TBO, it was difficult to detect the production of DNase by *S. aureus* and *B. catarrhalis*. In fact, growth and DNase production by *B. catarrhalis* were inhibited completely by 0.01% TBO unless a very heavy inoculum was used, and even then growth and DNase activity were faint. *B. catarrhalis* was the only microorganism whose growth was inhibited substantially by TBO concentrations in the range of 0.005 to 0.0005%; however, light to moderate growth and DNase activity were observed at these concentrations when a heavy inoculum (one-half of a 2-mm colony) was utilized. It is likely that organisms other than *B. catarrhalis* are sensitive to 0.005% TBO; however, use of a heavy inoculum may allow enough growth to ensure a valid test for DNase activity.

The report of Schreier (5) that gram-positive bacteria are inhibited on TBO-DNase agar is a single statement at the end of the discussion; there is no data supporting the statement from which one could see which organisms were tested or draw conclusions regarding degrees of inhibition. It is unlikely that the inhibition noted by Schreier was due to TBO concentrations in excess of 0.01% because, in our study, higher TBO concentrations almost totally masked DNase reactions of all organisms tested (Table 3), including *S. marcescens*, the main object of Schreier's study. Schreier's instructions (5) for making TBO-DNA agar instruct one to weigh out 100 mg of toluidine blue O (Allied Chemical Corp., New York) but make no reference to the dye content of the dye powder. Since in our hands an actual TBO content of 0.01% produced a very dark blue agar, it is probable that the actual dye content of the preparation used by Schreier was significantly less than 100%. Therefore, it seems likely that some component in the dye formulation other than TBO was responsible for the inhibition observed by Schreier.

When TBO concentrations of 0.0075 to 0.001% were utilized, pink zones indicative of DNase activity were very evident. The best contrast between the royal-blue background and the bright-pink zones of DNase activity was exhibited on plates containing 0.005% TBO.

On the basis of color contrast, zone clarity, and growth inhibition, we concluded that the optimal TBO concentration for incorporation into DNase test agar was 0.005%. This concentration of dye enabled all bacteria tested to grow and form distinct pink zones of DNase activity exhibiting excellent contrast with the royal-blue background.

Although 0.005% TBO was chosen as the optimal dye concentration for incorporation into DNase test agar, good results also were obtained when 0.0025 or 0.0075% TBO was incorporated into the agar medium. Incorporation of TBO into DNase test agar exhibited a much clearer delineation of DNase production than that yielded by the HCl-DNA precipitation DNase test.

In summary, two techniques involving use of the metachromatic dye TBO to detect DNase activity by bacteria have been improved. It has been shown that a TBO solution of 0.05% is superior to 0.1% TBO as a flooding agent to detect DNase activity of colonies grown on DNase agar and that incorporation of 0.005% TBO into DNase agar is superior to 0.01% TBO, since 0.005% TBO allows growth and DNase production by all bacteria tested and provides better delineation of DNase activity. Both modified TBO-DNase tests are greatly superior to the hydrochloride-DNA precipitation technique.

A caution regarding calculations for TBO concentrations is necessary because dye preparations sold by different chemical supply houses have vastly different dye contents (range of at least 60 to 94% actual dye content). The calculations we used accounted for such differences. Some of the difficulties we encountered early in these studies suggested to us that some authors using TBO must have based their calculations on the weight of the dye preparation rather than the actual dye content. Our attempts to reproduce some of the work resulted in dye concentrations much in excess of that actually desired. Such a result would have occurred only if the original authors had based their calcu-

lations on weight of dye preparation rather than actual dye content, as we had done. The problem is further compounded by the fact that no author mentioned the percentage of dye content of the TBO powder used, making it impossible to know what actual dye content had been used. If other investigators or laboratorians are to be capable of using data or methods involving TBO, it is of utmost importance that calculations be based on the actual dye content rather than on the weight of the dye preparation.

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