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Methodology for Recovery of Chemically Treated *Staphylococcus aureus* with Neutralizing Medium

B. P. DEY†* AND F. B. ENGLEY, JR.

Department of Microbiology, School of Medicine, University of Missouri, Columbia, Missouri 65212

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Recovery results of *Staphylococcus aureus* ATCC 6538 treated with phenolics and quaternary ammonium compounds on Dey and Engley (D/E) neutralizing medium at various time intervals were compared by the use of two commonly used media. Two recovery processes were utilized. In one, the chemically treated organisms were plated directly onto an agar medium. In the other, the aliquot was first put in broth and then was plated with agar. By either process, the numbers and the time period for recovery of organism were greater on D/E medium.

To obtain reproducible results in evaluating antimicrobial agents, the need for controlled test conditions was realized and a method was developed some 80 years ago (16). Recognizing the fact that antimicrobial agents are almost invariably required to act in the presence of organic matter, a modified test was proposed whereby the antimicrobial activity of a chemical agent was determined in the presence of organic matter for a given time (4). Another technique was developed (15) which was further modified and adopted as the official method of the Food and Drug Administration (17). This method includes the use of *Staphylococcus aureus* as the test organism, change in phenol dilution, and subculturing against highly inhibitory antimicrobial agents. Another procedure, known as the use dilution test, was also proposed (11) and was subsequently adopted by the Association of Official Analytical Chemists (1). This latter method also eliminates the possibility of carrying over antimicrobial agents by dilution and by the use of an appropriate neutralizer for the agent under test, which is present in the subculture medium for the agent under test.

Other than the methods recommended above, various other in vivo and in vitro procedures have been proposed for evaluating antimicrobial chemicals used for different purposes (5, 7–9, 12, 13, 19; B. P. Dey, F. B. Engley, Jr., and P. E. Rieley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C175, p. 303).

It has been observed that various factors play important roles in the evaluation of antimicrobial agents (18). One such factor is the recovery medium. To determine the actual effect of an antimicrobial agent on test organisms, it is essential that a recovery medium contain appropriate neutralizers to inactivate any agent that is carried over (3, 10).

In the past, Letheem broth (Difco Laboratories) has been used as a recovery medium in the evaluation of quaternary ammonium compounds (14). The only other routine neutralizing medium used over the years consisted of various modifications of thioglycolate broth for neutralizing residual mercurials. However, this medium was originally intended to create an anaerobic condition (2). Another medium, known as Standards Methods Agar, contains lecithin and polysorbate 80 and is utilized by the Association of Official Analytical Chemists and other testing procedures.

At present, there is no single recovery medium which has the ability to inactivate the range of antimicrobial agents that are in current use. According to the need, researchers use different neutralizers in the recovery medium in evaluating different antimicrobial chemicals. Under the circumstances, the value of a medium that would neutralize the action of a wide range of antimicrobial agents can readily be appreciated. Development of one such medium with a broad range of neutralizing capacity for phenolics, quaternary ammonium compounds, halogens, and aldehydes by paper disc assay has been reported (6; B. P. Dey and F. B. Engley, Jr., Bacteriol. Proc., p. 12, 1970; B. P. Dey, M.S. thesis, University of Missouri, Columbia, 1971; Dey et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C175, p. 303). The present paper reports the neutralizing capacity of this medium and presents a technique for enhanced recovery of chemically treated organisms.

† Present address: USDA, FSIS, Science, Division of Pathology and Epidemiology, Epidemiology Branch, BARC-East, Beltsville, MD 20705.

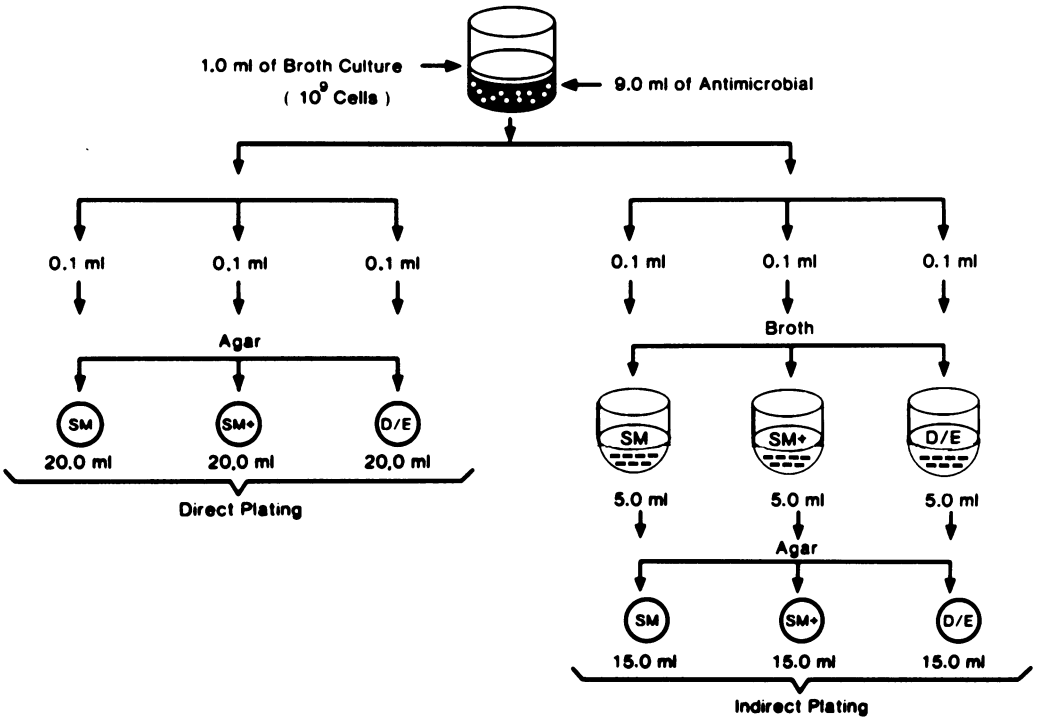

FIG. 1. Scheme for the recovery of chemically treated *S. aureus* ATCC 6538.

MATERIALS AND METHODS

Test organism. *S. aureus* ATCC 6538 was obtained from the Department of Microbiology, School of Medicine, University of Missouri, Columbia, Mo.

Test antimicrobial agents. The following compounds in aqueous solution were used in the study: a 1:50 dilution of phenol (A-91, Fisher Scientific Co., Pittsburgh, Pa.); a 1:128 dilution of phenol "Mikro-Bac" and a quaternary ammonium compound (QUAT) "Mikro-Quat," both obtained from Economics Laboratories, St. Paul, Minn.; a 1:750 dilution of another QUAT "Zephiran" (Sterling Drug, Inc., New York). Except for the 1:50 dilution of phenol, the dilutions used for the commercial disinfectants were suggested by the manufacturers.

Test media. Standard Methods Medium (SM) and Standard Methods Medium with lecithin and polysorbate 80 (SM+ [Difco]) were used as the liquid medium and as the solid medium with the addition of agar (2%). Dey and Engley medium (D/E; currently made by Difco) was tested against SM and SM+ media for recovering chemically treated organisms; it contained (grams per liter) tryptone (Difco), 5.0; yeast extract (Difco), 2.5; dextrose (Difco), 10.0; sodium thioglycolate, 1.0; sodium thiosulfate, 6.0; sodium bisulfite, 2.5; lecithin (soy bean), 7.0; polysorbate (80), 5.0 ml; agar, 20.0.

Procedure for recovery of test organisms. Figure 1 shows a diagrammatic scheme of the experimental method for recovering chemically treated organisms. A 1-ml volume of *S. aureus* ATCC 6538 containing 10^9 cells was added to 9.0 ml of antimicrobial agent of the desired dilution. At intervals of 1, 5, 10, 15, 20, 40, 80, 160, and 320 min, 0.1-ml amounts of the chemical-organism mixture were taken out and plated with SM agar, using the pour plate method. In the same way, 0.1-ml amounts were used for making pour plates with SM+ and D/E agar. This process of organism recovery is described as "direct plating."

Following the same procedure and at the same time, 0.1-ml amounts were added to 5.0 ml of SM, SM+, or D/E neutralizing broth, thoroughly mixed, and left at room temperature for 30 min. After this period, the broth mixture was added to 15.0 ml of appropriate agar, and plates were poured. This process of organism recovery is described as "indirect plating."

The plates from both recovery processes were incubated at 37°C for 48 h, and the colonies were counted.

RESULTS

Recovery of organisms from each antimicrobial agent in each medium at various time intervals was plotted as a curve, an example of which is shown in Fig. 2. A regression analysis on each curve was performed to demonstrate the significant differences between curves due to medium and methodology.

Table 1 shows the data based on the value for the log count (Y) versus the log time (X) based on the log (counts) greater than zero. Use of the log (count) versus time (T) plot and the log (count) versus time (T) plot including one zero value (i.e., the first time at which the log count is

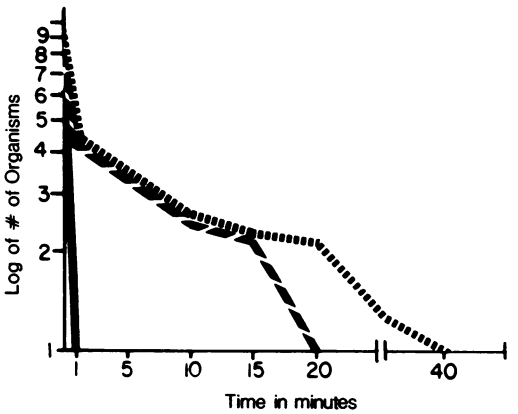

FIG. 2. Recovery of *S. aureus* ATCC 6538 by direct plating from 1:128 phenol on different agar (SM ■, SM+ ▨, D/E ▥) at various time intervals.

zero in a test series) were also examined before settling on the model (20):

$$\log (\text{count}) = A + B \log(T)$$

Here, A and B denote the intercept and the slope of the curve, respectively, and are shown for each trial. The intercept corresponds to the log (count) at T = 1 since log(1) = 0. The slope values indicate the nature of the recovery curve. The least significant difference between two B values was approximately 0.755.

The differences in intercept (A) associated with SM+ versus D/E were generally minor; however, they varied greatly among antimicrobial agents. The rate of decline in log (count) with log (time) was considerably slower for D/E than for SM+, since the slope (B) value was lower in all cases. Because the intercepts were similar but the decline was slower, the recovery counts for D/E were higher than the count for SM+. The differences between slopes of D/E and SM+ were larger with Zephiran and Mikro-Quat. The difference between 8, the initial log count, and the intercept measures the initial mortality in the first minute. The effect of method at t-1 was minor for Mikro-Bac, but the indirect method yielded higher counts for the other three antimicrobial agents.

Because the organisms in most trials were recovered for the first four timings (1, 5, 10, and 15 min) on SM+ and D/E medium, an analysis of variance was done to determine the effect of medium and methodology on the organism recovery. Two significant interactions on the recovery of organisms were observed (Table 2). The first was between the antimicrobial agents and the media; the second was between the antimicrobial agents and the method for recovery. Antimicrobial agents remaining the same, the differential effects on the recovery were due to media and the method for recovery. The smallest differences were associated with phenol and Mikro-Bac and were significantly larger with Zephiran and Mikro-Quat. The change in the recovery rate on SM+ medium was not influenced by the change in phenol but was influenced by the method for recovery. Recovery on SM+ medium changed due to the change in QUAT and the change in the method. However, the rate of recovery on D/E medium was influenced by the change in phenol only.

The recovery time of organisms varied with the medium and the method for recovery (Table 3). By either method, the recovery times of chemically treated organisms were comparatively higher in D/E medium than in the other two media.

DISCUSSION

The bactericidal activity of an antimicrobial agent is determined by its capacity to kill a certain number of microorganisms in a given period of time. Beyond this time, media with neutralizers are used to inactivate the aftereffect of the agent to recover remaining viable organisms. Without a specific neutralizer present in

TABLE 1. Mathematical evaluation of recovery curves of chemically treated *S. aureus* ATCC 6538 on different media by two methods

Antimicrobial agent	Recovery method	Medium	*n*	Intercept (A)	Slope (B)
Phenol	Direct[a]	SM	—[b]	—	—
		SM+	4	4.727	−2.786
		D/E	5	5.661	−2.589
	Indirect	SM	2	2.903	−0.292
		SM+	5	5.736	−2.329
		D/E	6	5.905	−2.094
Mikro-Bac	Direct	SM	—	—	—
		SM+	5	7.267	−4.595
		D/E	6	7.735	−4.299
	Indirect	SM	4	6.967	−4.808
		SM+	5	7.628	−4.254
		D/E	7	7.505	−3.522
Zephiran	Direct	SM	—	—	—
		SM+	4	5.603	−3.039
		D/E	7	5.600	−2.141
	Indirect	SM	—	—	—
		SM+	5	6.052	−3.074
		D/E	8	6.495	−1.808
Mikro-Quat	Direct	SM	—	—	—
		SM+	4	4.023	−2.427
		D/E	5	4.950	−1.896
	Indirect	SM	—	—	—
		SM+	4	7.628	−5.323
		D/E	8	7.627	−3.236

[a] Data used for Fig. 2. Least significant difference, 0.755 at 5% level.

[b] —, No recovery was made.

TABLE 2. Effect of medium and methodology on the recovery rate (mean log count) of chemically treated *S. aureus* ATCC 6538

Antimicrobial agent	Recovery (mean log count)[a]					
	Medium			Method		
	SM+	D/E	Difference	Direct	Indirect	Difference
Phenol	3.7310	4.0690	0.3380	3.5360	4.2640	0.7280
Mikro-Bac	4.1565	4.6820	0.5255	4.1885	4.6500	0.4615
Zephiran	3.7020	4.7535	1.0515	3.8009	4.6545	0.8536
Mikro-Quat	3.0400	4.5370	1.4970	3.0050	4.5720	1.5670

[a] Each mean (least significant difference, 0.5576) is based on counts at four times (1, 5, 10, and 15 min). Least significant difference, 0.7886 at 5% level.

the recovery medium, the carried-over antimicrobial agent in the medium may inhibit growth of viable organisms (12). As the SM medium contains no neutralizer for phenol or QUAT, the antimicrobial agent carried over by a direct plating procedure inhibited the growth of viable organisms. Thus, no recovery of organisms at any time period was made on this medium. Organisms exposed to the same antimicrobial agents for longer periods of time were recovered on both SM+ and D/E media. This indicates that both media were able to neutralize the carried-over phenols or the QUATs, allowing the viable organisms to grow. Comparatively, the number of recoveries of phenol-treated organisms at any time was greater on D/E medium than on SM+ medium. Also, the organisms were recovered on D/E medium for a longer period of time. This may be due to the increased concentration of lecithin in D/E medium, which is known to have phenol-neutralizing capacity (6; Dey and Engley, Bacteriol. Proc., p. 12, 1970; Dey, M.S. thesis). The higher concentration of lecithin in D/E medium could be the reason for the greater number and longer period of recoveries for the QUAT-treated organisms on D/E medium than on SM+ medium.

The use of a broth recovery medium before final plating minimizes the effect of carried-over antimicrobial agent, and the rate and duration of recovery of chemically exposed microorganisms increase. For this reason, organisms exposed to phenolics could be recovered on SM medium which contained no specific neutralizer for phenol (Tables 1 and 3). However, by this procedure (i.e., indirect plating), QUAT-treated organisms cannot be recovered on SM medium. Nonrecovery of such organisms on SM medium indicates that dilution does not play a role in counteracting QUATs, as these are known to inhibit growth of organisms at very low concentrations. Greater recovery of QUAT-treated organisms on SM+ and D/E media by this procedure, as opposed to direct plating, was due to the stepwise reduction in the strength of carried-over antimicrobial agents (Table 2). It was achieved first by dilution and then by the neutralizer present in the broth and in the final agar medium. Comparatively better rates and duration of recovery of QUAT-exposed organisms in D/E medium than in SM+ medium demonstrates the advantage of higher concentration of lecithin in D/E medium in neutralizing the residual QUAT more thoroughly. Therefore, to neutralize the action of residual phenol or QUAT in the recovery process, it is essential that an adequate amount of a suitable neutralizer be present in the recovery medium. Based on the data, it appears that the recovery of phenol or QUAT-treated organisms depends on the neutralizing capacity

TABLE 3. Endpoint recovery time on different media and by two different methods for chemically treated *S. aureus* ATCC 6538

Antimicrobial agent	Recovery method	Medium	Recovery time (min)
Phenol	Direct	SM	0
		SM+	15
		D/E	20
	Indirect	SM	5
		SM+	20
		D/E	40
Mikro-Bac	Direct	SM	0
		SM+	20
		D/E	40
	Indirect	SM	15
		SM+	20
		D/E	80
Zephiran	Direct	SM	0
		SM+	15
		D/E	80
	Indirect	SM	0
		SM+	20
		D/E	160
Mikro-Quat	Direct	SM	0
		SM+	15
		D/E	20
	Indirect	SM	0
		SM+	15
		D/E	160

of the recovery medium and also on the technique for recovery. Among the three media tested, D/E medium produced better recovery of organisms exposed to either antimicrobial agent. Even with a change in recovery process, better recovery results were obtained on D/E medium. The media show promise in evaluating differences between two antimicrobial agents containing similar active ingredients.

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