Totally Synthetic Medium for Susceptibility Testing

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The results of disk diffusion and plate dilution susceptibility testing of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* on media gelled with agar-agar or with a synthetic hydrogel were compared. Synthetic hydrogel can be combined with a totally defined synthetic amino acid medium to yield a reproducible, totally defined, synthetic solid medium without the antagonistic or booster effects of some components of agar. Such a medium could be used as a reference medium for susceptibility testing.

The results of antimicrobial susceptibility testing performed on solid media are influenced by both the nutrient milieu and the supporting gel. Variability contributed by the nutrient milieu can be eliminated by using a completely synthetic, totally defined culture medium such as SAAM (synthetic amino acid medium). SAAM is isosmolal and is free of inhibitors of antimicrobial agents and macromolecules. It is exceptionally well buffered through the use of a synthetic, nonchelating weak acid-weak base pair (8).

Since its introduction into microbiology in 1881 by a German housewife, agar-agar has been commonly used as the supporting gel (7). As obtained from red seaweeds, agar is a complex mixture of neutral and acidic polysaccharides. Recently, it has been appreciated that variable quantities of lipids, metallic cations, and other unknown substances are also present in agars and may antagonize certain antimicrobial agents (4-6, 10). If a chemically defined, synthetic gelling agent could be substituted for agar, variability in the supporting gel component of solid media might also be avoided.

Synthetic hydrogel (SHG) is a neutral product arising from hydration of cross-linked linear polymers of polyoxethylene (Fig. 1). When SHG is combined with SAAM, a totally defined, reproducible, solid culture medium results.

We carried out susceptibility tests in vitro, using SAAM and Mueller-Hinton (MH) nutrient formulations as liquid media for dilution tests and gelled with agar and SHG for plate dilution and disk diffusion tests. Clinical isolates of *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were examined.

MATERIALS AND METHODS

Bacterial isolates. Recent clinical isolates of *E. coli* (48 strains), *S. aureus* (48 strains), *P. mirabilis*

(30 strains), and *P. aeruginosa* (36 strains) were obtained from the Clinical Microbiology Laboratory of the University of California-Davis, Sacramento Medical Center.

Media. MH broth was purchased from Difco Laboratories, Inc. (Detroit, Mich.). Ionagar no. 2 was purchased from Colab Laboratories (Glenwood, Ill.). SAAM was prepared as previously reported (8). SHG (Neutragel) was obtained from Union Carbide Co. (Tuxedo Junction, N.Y.) as a partially hydrated gel in 100- by 15-mm plastic petri dishes. SAAM or MH broth was added to the plates at twice the final desired concentration to give a plate volume of 20 ml. The plates were stored at 5°C overnight to allow imbibition of the nutrient-bearing liquid. Agar plates were prepared by adding 0.9% Ionagar to SAAM or MH broth (at twice the final desired concentration) to give a plate volume of 20 ml in 100- by 15-mm plastic petri dishes. Plates were used within 24 h of preparation.

Antimicrobial agents. After weighing, standard powders were dissolved in sterile distilled water and filtered through 0.22- μ m-pore-sized filters (Millipore Corp., Bedford, Mass.). Doubling dilutions were prepared in either SAAM or MH broth to reach the final desired concentration. The ranges of concentrations, in micrograms per milliliter, were: penicillin G, 0.25 to 32; penicillin V, 4 to 256; ampicillin, 0.063 to 64; carbenicillin, 0.031 to 512; cephalothin, 0.125 to 16; chloramphenicol, 0.25 to 512; gentamicin, 0.031 to 64; kanamycin, 0.125 to 32; polymyxin B, 0.031 to 8; sulfadiazine, 4 to 512; and tetracycline, 0.063 to 32.

Commercially prepared filter paper disks containing 250 μ g of sulfadiazine, 10 μ g of gentamicin, 30 μ g of kanamycin, 30 μ g of tetracycline, 30 μ g of chloramphenicol, 300 U of polymyxin B, 10 U of penicillin G, 10 U of penicillin V, 10 μ g of ampicillin, 100 μ g of carbenicillin, and 30 μ g of cephalothin were used for agar diffusion testing.

Susceptibility testing. Bacteria were grown overnight in 10 ml of SAAM or MH broth to yield about 10^9 organisms/ml. A 10^{-3} dilution of the overnight broth culture was prepared, and the bacteria were inoculated onto plates with a hand-held, 19-pronged, stainless-steel replicator (University of California-Davis, Physical Plant Department) which delivered approximately 0.003 ml/prong. Plates were incubated

FIG. 1. Chemical composition of synthetic hydrogel—cross-linked polymers of polyoxethylene. $\times$ = Very large.

overnight, and the end points for minimal inhibitory concentrations (MICs) were taken as the lowest concentrations preventing growth. Control plates without antimicrobial drugs were also inoculated. For disk diffusion testing, 2 ml of a 10^{-3} dilution of the overnight broth culture (i.e., 10^6 organisms/ml) was flooded onto each plate, the plates were rotated to wet the surface evenly, and the excess solution was sucked off the tilted plate by using a sterile Pasteur pipette. By experience, this gave a consistently confluent lawn of growth. The plates were allowed to stand for about 15 min before the drug-loaded paper disks were placed in a predetermined pattern. After overnight incubation, the diameters of zones of inhibition were measured in millimeters. Four plates were prepared for each organism.

Tube dilution susceptibility testing was performed on 10 isolates of each species tested. Portions (0.5 ml) of a 10^{-4} dilution of the overnight broth culture were added to 0.5 ml of antimicrobial agent (at twice the desired final concentration) to yield a final inoculum of 5×10^4 colony-forming units/ml. After incubation overnight, the lowest concentration preventing visible growth was taken as the MIC. Ten percent of the volume of all tubes without visible growth was transferred to nutrient agar; the lowest concentration yielding no more than five colonies was accepted as indicative of a lethal effect—the minimal lethal concentration.

RESULTS

Tables 1 through 4 show comparisons between (i) mean MICs $\pm$ SEM (standard error of the mean) obtained by broth dilution and plate dilution and (ii) the mean diameters of zones of inhibition $\pm$ SEM on the four solid media. Below the numbers, the letters S, I, or R stand for susceptible, intermediate, or resistant according to the Bauer-Kirby standards and their correlated MICs (12). The gelling component had variable effects on MICs when the same nutrient formulation was used. In general, the plate dilution MICs were higher than those obtained in broth; despite the higher MIC, "susceptible" organisms usually remained in the susceptible classification by either method. In turn, the disk diffusion results—susceptible, intermediate, or resistant—agreed in most instances with the MIC results. Exceptions were seen with sulfadiazine. For example, *S. aureus* tested in SAAM was susceptible to sulfadiazine (mean MIC 16.8 ± 3.47 $\mu\text{g/ml}$); in MH broth the end point was ≥ 32 $\mu\text{g/ml}$, the highest concentration tested

TABLE 1. Results of susceptibility testing of *Staphylococcus aureus* with liquid and solid media^a

Antimicrobial agent	Liquid media, mean MICs ($\mu\text{g/ml}$)		Solid media, mean plate dilution MICs ($\mu\text{g/ml}$)				Mean zone diam (mm)			
	SAAM	MH	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG
Sulfadiazine	16.80 ± 3.47^b S	>32 ?	506.16 ± 5.33 R	196.00 ± 12.05 R	416.00 ± 19.24 R	>256 R	30.23 ± 0.50 S	21.62 ± 0.43 S	15.67 ± 0.61 I	6.00 ± 0.00 R
Chloramphenicol	2.40 ± 0.27 S	2.20 ± 0.20 S	4.08 ± 0.08 S	4.39 ± 0.14 S	4.06 ± 0.08 S	>4 ?	32.08 ± 0.44 S	24.12 ± 0.32 S	26.67 ± 0.22 S	20.08 ± 0.20 S
Tetracycline	0.22 ± 0.04 S	0.18 ± 0.02 S	0.51 ± 0.01 S	0.46 ± 0.01 S	0.48 ± 0.01 S	0.47 ± 0.00 S	31.54 ± 0.27 S	32.19 ± 0.26 S	27.64 ± 0.29 S	26.02 ± 0.21 S
Kanamycin	3.05 ± 1.50 S	0.30 ± 0.03 S	2.33 ± 0.14 S	1.66 ± 0.16 S	1.50 ± 0.17 S	1.60 ± 0.16 S	31.54 ± 0.27 S	28.60 ± 0.16 S	26.14 ± 0.23 S	23.88 ± 0.17 S
Gentamicin	0.09 ± 0.02 S	0.06 ± 0.01 S	0.44 ± 0.03 S	0.16 ± 0.01 S	0.26 ± 0.02 S	0.15 ± 0.02 S	33.44 ± 0.32 S	32.12 ± 0.23 S	26.27 ± 0.23 S	25.69 ± 0.20 S

^a S, Susceptible; I, intermediate; R, resistant (Bauer-Kirby standards).

^b Mean $\pm$ SEM.

(Table 1). By plate dilution testing, the mean MICs indicated moderate resistance to sulfadiazine regardless of the kind of medium used. However, in disk diffusion tests zone sizes were observed which would be considered susceptible in SAAM with agar or SHG as the gelling agent, intermediate with MH-agar, and resistant with MH-SHG.

Some inconsistency was also seen with sulfadiazine when tested on solid media against *E. coli*—the mean MIC in MH-SHG was 10-fold greater than that in the other plate dilution media. By disk diffusion, the *E. coli* were uniformly susceptible in all media (Table 2).

Ingredients in the gelling compound appeared to affect the results of testing with the aminocyclitols gentamicin and kanamycin. With *E. coli* on media prepared with SAAM, replacing the agar with SHG lowered the mean MIC of gentamicin from 2.38 ± 0.12 $\mu\text{g/ml}$ to 0.31 ± 0.02 $\mu\text{g/ml}$; with media containing MH, the decrease in the mean MIC was less marked, but replacing the agar with SHG lowered the mean MIC from 1.61 ± 0.12 $\mu\text{g/ml}$ to 1.14 ± 0.05 $\mu\text{g/ml}$ (Table 2).

A similar reduction in MIC of gentamicin was noted against *P. aeruginosa* (Table 3). With SAAM, replacing agar with SHG decreased the MIC from 3.76 ± 0.37 $\mu\text{g/ml}$; with MH, the mean MIC with agar was 4.01 ± 0.54 $\mu\text{g/ml}$, and with SHG it was 1.33 ± 0.17 $\mu\text{g/ml}$.

SHG proved to be especially useful for testing *P. mirabilis* since swarming was prevented. The results of testing with broth and solid media correlated quite well for all of the antimicrobial agents except penicillin G (Table 4). In SAAM or MH broth, the 10 test strains were susceptible; on solid media, the mean MICs for 30 isolates ranged from susceptible to intermediate (except on the totally defined SAAM-SHG medium). This distinction, however, is slight when the standard errors are considered. With disk diffusion testing, all 30 isolates were intermediate in their susceptibility to penicillin G.

DISCUSSION

The ideal medium for reference testing should be totally defined, reproducible, free of antagonists or boosters of antimicrobial action, well buffered to maintain pH, and available in both liquid and solid formulations. SAAM meets these requirements, but a totally defined, gelling ingredient for plate testing has been lacking. Many components of agar can influence the results of susceptibility testing. Agar has been shown to both inhibit (6) and augment (9) the effect of chlortetracycline against *S. aureus*. In both of these studies, washing the agar reduced, but did not obliterate, the effect of the agar. The

TABLE 2. Results of susceptibility testing of *Escherichia coli* with liquid and solid media^a

Antimicrobial agent	Liquid media, mean MICs ($\mu\text{g/ml}$)		Solid media, mean plate dilution MICs ($\mu\text{g/ml}$)				Mean zone diam (mm)			
	SAAM	MH	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG
Sulfadiazine	6.40 ± 0.65^b S	5.60 ± 0.65 S	36.33 ± 7.07 S	32.83 ± 7.11 S	28.00 ± 7.04 S	253.33 ± 2.67 R	31.77 ± 0.99 S	25.77 ± 0.75 S	23.71 ± 0.67 S	18.19 ± 0.41 S
Chloramphenicol	1.70 ± 0.15 S	1.80 ± 0.13 S	8.62 ± 0.76 S	7.83 ± 0.69 S	17.25 ± 0.63 I	8.25 ± 0.70 S	31.79 ± 0.57 S	27.46 ± 0.41 S	27.71 ± 0.29 S	23.44 ± 0.34 S
Tetracycline	0.75 ± 0.08 S	0.42 ± 0.04 S	5.04 ± 0.28 I	3.48 ± 0.24 S	2.27 ± 0.27 S	2.50 ± 0.27 S	23.29 ± 0.78 S	22.46 ± 0.72 S	24.46 ± 0.74 S	21.52 ± 0.62 S
Kanamycin	0.85 ± 0.15 S	0.60 ± 0.07 S	0.50 ± 0.06 S	0.23 ± 0.02 S	1.61 ± 0.12 S	1.14 ± 0.05 S	32.81 ± 0.57 S	29.56 ± 0.52 S	23.29 ± 0.37 S	21.25 ± 0.62 S
Gentamicin	0.22 ± 0.04 S	0.10 ± 0.01 S	2.38 ± 0.12 S	0.31 ± 0.02 S	1.61 ± 0.12 S	1.14 ± 0.05 S	31.38 ± 0.33 S	31.38 ± 0.33 S	22.67 ± 0.14 S	24.31 ± 0.17 S
Polymyxin B	0.20 ± 0.02 S	0.15 ± 0.04 S	2.38 ± 0.12 I	0.31 ± 0.02 S	0.55 ± 0.08 S	0.35 ± 0.04 S	17.46 ± 0.17 S	20.60 ± 0.11 S	13.75 ± 0.08 S	16.89 ± 0.06 S

^{a, b} See Table 1.

TABLE 3. Results of susceptibility testing of *Pseudomonas aeruginosa* with liquid and solid media^a

Antimicrobial	Liquid media, mean MICs ($\mu\text{g/ml}$)				Solid media, mean plate dilution MICs ($\mu\text{g/ml}$)				Mean zone diam (mm)			
	SAAM	MH	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	MH-SHG
Polymyxin B	0.32 $\pm$ 0.04 ^b S	0.18 $\pm$ 0.03 S	4.00 $\pm$ 0.00 R	0.81 $\pm$ 0.14 S	1.50 $\pm$ 0.15 S	0.63 $\pm$ 0.10 S	18.69 $\pm$ 0.25 S	21.50 $\pm$ 0.23 S	16.25 $\pm$ 0.35 S	18.47 $\pm$ 0.18 S	18.69 $\pm$ 0.25 S	18.47 $\pm$ 0.18 S
Gentamicin	0.61 $\pm$ 0.16 S	0.41 $\pm$ 0.18 S	3.76 $\pm$ 0.37 S	1.31 $\pm$ 0.16 S	4.01 $\pm$ 0.54 S	1.33 $\pm$ 0.17 S	24.17 $\pm$ 0.59 S	25.28 $\pm$ 0.60 S	22.42 $\pm$ 0.50 S	19.81 $\pm$ 0.33 S	24.17 $\pm$ 0.59 S	19.81 $\pm$ 0.33 S
Carbenicillin	116.40 $\pm$ 101.02 I-S	142.80 $\pm$ 100.8 I-S	63.67 $\pm$ 10.70 S	46.78 $\pm$ 9.60 S	72.11 $\pm$ 11.60 S	67.67 $\pm$ 8.97 S	19.53 $\pm$ 0.86 S	18.17 $\pm$ 0.72 S	15.89 $\pm$ 0.80 I	14.22 $\pm$ 0.63 I	19.53 $\pm$ 0.86 S	14.22 $\pm$ 0.63 I
Chloramphenicol	61.20 $\pm$ 24.31 R	41.60 $\pm$ 9.60 R	227.56 $\pm$ 24.25 R	231.11 $\pm$ 20.60 R	245.33 $\pm$ 19.67 R	259.56 $\pm$ 17.84 R	7.67 $\pm$ 0.54 R	7.69 $\pm$ 0.38 R	7.06 $\pm$ 0.46 R	7.94 $\pm$ 0.44 R	7.67 $\pm$ 0.54 R	7.94 $\pm$ 0.44 R
Tetracycline	62.00 $\pm$ 50.03 I-R	45.60 $\pm$ 24.02 R	103.11 $\pm$ 6.88 R	51.11 $\pm$ 6.83 R	49.00 $\pm$ 7.88 R	46.67 $\pm$ 7.19 R	10.47 $\pm$ 0.31 R	11.97 $\pm$ 0.36 R	12.08 $\pm$ 0.60 R	9.11 $\pm$ 0.46 R	10.47 $\pm$ 0.31 R	9.11 $\pm$ 0.46 R

^{a,b} See Table 1.TABLE 4. Results of susceptibility testing of *Proteus mirabilis* with liquid and solid media^a

Antimicrobial	Liquid media, mean MICs ($\mu\text{g/ml}$)				Solid media, mean plate dilution MICs ($\mu\text{g/ml}$)				Mean zone diam (mm)			
	SAAM	MH	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	SAAM-SHG	MH-agar	MH-SHG	SAAM-agar	MH-SHG
Penicillin G	1.55 $\pm$ 0.73 S	1.40 $\pm$ 0.74 S	8.84 $\pm$ 1.70 S-I	5.87 $\pm$ 1.77 S	7.23 $\pm$ 1.72 S-I	7.92 $\pm$ 2.20 S-I	18.43 $\pm$ 0.57 I	17.62 $\pm$ 0.45 I	19.92 $\pm$ 0.56 I	16.92 $\pm$ 0.33 I	18.43 $\pm$ 0.57 I	16.92 $\pm$ 0.33 I
Ampicillin	2.70 $\pm$ 0.73 S	2.55 $\pm$ 0.78 S	8.47 $\pm$ 1.00 S-I	4.68 $\pm$ 0.59 S	5.21 $\pm$ 0.62 S	7.32 $\pm$ 0.76 S	25.14 $\pm$ 0.73 S	22.62 $\pm$ 0.62 S	25.63 $\pm$ 0.60 S	20.18 $\pm$ 0.56 S	25.14 $\pm$ 0.73 S	20.18 $\pm$ 0.56 S
Carbenicillin	0.52 $\pm$ 0.06 S	0.40 $\pm$ 0.08 S	1.82 $\pm$ 0.09 S	1.08 $\pm$ 0.10 S	0.76 $\pm$ 0.10 S	1.07 $\pm$ 0.10 S	31.24 $\pm$ 0.53 S	27.70 $\pm$ 0.42 S	31.24 $\pm$ 0.53 S	27.70 $\pm$ 0.42 S	31.24 $\pm$ 0.53 S	27.70 $\pm$ 0.42 S
Penicillin V	36.80 $\pm$ 4.80 R	24.80 $\pm$ 5.12 R	118.74 $\pm$ 9.27 R	62.32 $\pm$ 6.71 R	91.79 $\pm$ 6.84 R	64.82 $\pm$ 8.05 R	6.00 $\pm$ 0.00 R	6.00 $\pm$ 0.00 R	6.00 $\pm$ 0.00 R	6.00 $\pm$ 0.00 R	6.00 $\pm$ 0.00 R	6.00 $\pm$ 0.00 R
Cephalothin	1.55 $\pm$ 0.34 S	1.70 $\pm$ 0.33 S	7.68 $\pm$ 0.84 S	3.32 $\pm$ 0.20 S	11.10 $\pm$ 0.84 S	5.05 $\pm$ 0.41 S	27.43 $\pm$ 0.38 S	23.81 $\pm$ 0.31 S	25.63 $\pm$ 0.36 S	21.42 $\pm$ 0.36 S	27.43 $\pm$ 0.38 S	21.42 $\pm$ 0.36 S

^{a,b} See Table 1.

activities of polymyxin B, neomycin, kanamycin, and streptomycin are also adversely affected by agar (6).

Cations in the test medium have been shown to have marked effects on the results of testing *P. aeruginosa* with gentamicin (5, 9, 14). Cations also exert effects on tetracycline, presumably by chelation (13, 16). This was inferred from the correlation of content of Ca^{2+} and Mg^{2+} in MH agars from different manufacturers with significant differences in disk diffusion susceptibility test results (1, 3).

Agar also contains substances that can stimulate growth of certain microorganisms. These include biotin, thiamine, and other unknowns (15); Mg^{2+} can also stimulate growth of certain microorganisms (2). Inhibition of the growth of pathogenic *Neisseria* spp. is attributable to unsaturated fatty acids present in agar (11).

SHG avoids the uncertainties associated with agar. It consists solely of linear polymers of polyoxethylene formed into a matrix by cross-linking. SHG is biologically inert, transparent (facilitating examination of colonial morphology), and strong (it is nearly impossible to gouge with an inoculating loop). It permits diffusion of antimicrobial agents from paper disks and inhibits swarming of *Proteus* spp. SHG can be used with a totally defined medium such as SAAM to provide a reproducible, totally defined, completely synthetic medium. This combination could provide a useful reference medium for susceptibility testing.

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